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Colin R. MacKenzie  
Birgit Henrich *Editors*

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# METHODS IN MOLECULAR BIOLOGY™

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# **Diagnosis of Sexually Transmitted Diseases**

## **Methods and Protocols**

Edited by

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## Preface

Sexually transmitted infections (STI) continue to be a major cause of morbidity and mortality in the twenty-first century, both in developed industrial countries and in the developing world. The WHO estimates that there are 448 million new curable infections per year, many of which will result in infertility, perinatal morbidity, and death (<http://who.int>). Human immunodeficiency virus infections and the ensuing opportunistic infections are a major drain on the human and financial resources of many countries in the developing world, and even with the availability of effective treatment the epidemic is not yet contained. Many STI are inapparent or asymptomatic, which makes their control very difficult and places the emphasis on screening for infections among this group. In wealthy industrialized countries most people have ready and regular access to health services, such as during school, at the work place, or during pregnancy, and therefore screening for asymptomatic STI poses no major problem other than in the disadvantaged. The vast majority of the population in the developing world has no regular access to health care, and services are often days away from the home. Screening in this group is difficult and there is a need for simple reliable cheap diagnostic methods that can be performed at the point of care, on the same day and by staff with limited training. To add to the already considerable burden of STI, an increase in the resistance of many of the treatable organisms causing infection has been observed recently, and thus a need for rapid detection of resistance is desirable.

A number of organisms can cause STI, the most common of which are *Treponema pallidum*, *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, *Haemophilus ducreyi*, *Mycoplasma genitalium* and *M. hominis*, *Ureaplasma urealyticum*, *U. parvum*, *Klebsiella granulomatis*, *Candida*, viruses such as HIV, HSV, HBV, HCV, CMV, HPV, parasites such as *Trichomonas vaginalis*, scabies. Testing for this wide range of pathogens has not been useful due to technological limitations in regions where STI prevalence is highest, and therefore a syndromic approach has been propagated by the WHO (<http://who.int>). This syndromic approach to therapy classifies STI into seven syndrome complexes; urethral discharge, genital ulcer disease, inguinal swelling, scrotal swelling, vaginal discharge, lower abdominal pain, and neonatal eye infections. Application of this approach has probably led to an overtreatment as many patients with symptoms do not have an STI. Therefore newer better applicable diagnostic tools are urgently required and the term “ASSURED” tests was introduced to describe the ideal test: Affordable, Sensitive, Specific, User-friendly, Rapid and robust, Equipment-free, Delivered.

Molecular biological methods are very attractive for the diagnosis of STI since a well-defined range of pathogens is responsible for the infection. In particular, the screening of asymptomatic patients requires an affordable and reliable (sensitive and specific) test. In addition new advances in molecular biology methods and techniques will undoubtedly lead to simple robust and affordable tests. We only have to look at the advances in PCR technology to see how far we have come in a decade. Tests developed and applied in developing countries will more than likely lead to ASSURED tests in developing countries.

This book strives to cover the full range of molecular testing for STI. Special attention was given to including a range of methods from the simple and inexpensive to complex

sophisticated methods, thus hoping to provide scientists in many different situations with the information they seek. Aspects of DNA extraction from small-volume samples or difficult tissues, simple, nested, or multiplex PCR, use of duplex primers or other modifications of primers and PCR conditions, sequence analysis for genotyping, denaturing gel analysis, microarrays using liquid beads or microspheres, and silicon nanoparticle-enhanced microcantilever detection of DNA are dealt with in individual chapters. Due to the increasing concern for antibiotic resistance in modern medicine and the appearance of resistant neisseria, the rapid and simple testing for resistance genes is an important factor for molecular testing, and chapters addressing this and the expression of virulence and host factors have also been included. Due to the high prevalence of STI and the asymptomatic nature of many diseases, self-collection of specimens has become more common, and the issue concerning self-collected specimens is dealt with in Chapter 27. Where appropriate, the authors have provided a set of guidelines to aid the reader in the process of establishing a method from scratch. We have also included chapters on the ethical issues of sexual abuse and molecular testing as well as those issues pertaining to STI-testing and close the book with a review about the “transparent patient.”

Laboratory diagnostic tests must meet strict standards in order to be approved for use in the diagnosis of disease in patients. In many instances the application determines the standards to be met; for example, a test in a low-prevalence region requires a higher specificity than would be required in a high-prevalence population. Redesigning molecular tests to suit point-of-care testing will be a challenge for the future; however, the market is huge and the need for inexpensive tests in middle-income regions such as China, India, South Africa, and South America is urgent considering the expanding burden of HIV and related diseases in these regions. Molecular testing will undergo the same evolution as other methods; the greater the usage the better the evaluation resulting in the truly robust methods becoming established and refined and the less useful tests falling by the wayside. We hope this book will be a useful adjunct to the literature in order to help this process.

The twenty-first century will certainly witness an exciting expansion in the use of molecular methodologies for the diagnosis of a wide range of human (and animal) diseases, and this will be particularly relevant for the diagnosis of STI. We are confident that the current volume in the series of “Molecular Methods” will be a valuable addition to the reference literature for the scientist looking to establish this kind of test in their own lab.

*Düsseldorf, Germany*

*Colin R. MacKenzie  
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# Chapter 1

## Overview of Molecular Biological Methods for the Detection of Pathogens Causing Sexually Transmitted Infections

Fernando Vazquez, Luis Otero, Santiago Melón, and María de Oña

### Abstract

We review here different state-of-the-art molecular methods currently used in the diagnosis of sexually transmitted infections.

**Key words:** Sexually transmitted infections, Diagnosis, Molecular biology techniques

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### 1. Introduction

The use of molecular biology techniques (MBTs) in the diagnosis of sexually transmitted infections (STIs) and genital infections has produced significant advances in the knowledge of these diseases for several reasons: (a) the use of noninvasive samples (urine) or self-taken samples, (b) the use of samples with low-load DNA or RNA even from not viable microorganisms or not detected by other ways such as *Mycoplasma genitalium* or Human papilloma virus (HPV), (c) the development of more sensitive and specific procedures for mass screening studies and for the diagnosis of asymptomatic patients, (d) the improvement of the diagnosis, due to enhanced sensitivity and specificity (i.e., *Chlamydia trachomatis* infection), (e) to know the implication of certain microorganisms in different syndromes such as urethritis (i.e., *Ureplasma urealyticum* vs. *U. parvum*) or in bacterial vaginosis, (f) the typing of microorganisms (i.e., *Treponema pallidum*, *C. trachomatis*, HPV), and (g) the determination of susceptibility to antimicrobial drugs (Herpes Simplex virus or Human Immunodeficiency virus). Nevertheless, MBTs are not free from the problem of false negative or positive results, sometimes have a higher cost than conventional methods, have the additional problem of environmental contamination with DNA, may be inhibited, and

require strict quality controls. Furthermore, data on genomic antimicrobial resistance are limited (1, 2).

For diagnostic laboratory tests, the “gold standard” test should be used whenever possible. In the population being tested, any nucleic acid amplification tests (NAATs) for bacterial STIs such as chlamydia and gonorrhoea should give a positive predictive value (PPV) of over 90 % (3).

In low prevalence populations it may be necessary to use a supplementary or confirmatory test to achieve an acceptable PPV (4). In general, it is important that those laboratories working with NAATs have standards in place, and they must be appropriately accredited with a nationally approved accreditation scheme and also comply with international standards for medical laboratory accreditation, ISO 15189, and standards such as the molecular methods of the Joint Commission International (5). Provided all conditions are met, NAATs (mostly based on the amplification and hybridization methods) are more sensitive than culture in extra-genital samples, as it has been recently reported (6).

Recently the tendency has been to use new molecular diagnostic methods such as multiplex PCRs (mPCRs) and multiplex PCR-based reverse line blot or microarray (mPCR/RLB) for a syndromic approach both in women (7) and men (8), or real-time PCR methods (RT-PCR). Fast and low-cost molecular methods are also necessary in resource-poor or point-of-care settings. In this way, methods such as the loop-mediated isothermal amplification (LAMP) are being used to diagnose different STIs (9–11) although the methodological problems of this technique must be solved.

*Gonococcal infection.* Gonorrhoea is primarily an uncomplicated infection of the lower genital tract and is believed to be symptomatic in most men (90–95 %) and asymptomatic in approximately 50 % of women, but it can lead to complicated infection, sequelae of infertility, and ectopic pregnancy and can facilitate the acquisition and transmission of HIV specially in men who have sex with men (3). Ever since the advent of molecular methods, the diagnosis of *N. gonorrhoeae* has been troubled by false negative and false positive results compared with culture, which is the current reference method. Commensal *Neisseria* spp. and *Neisseria meningitidis* are genetically closely related to *N. gonorrhoeae* and may be the cause of positive results when using molecular tests having too-low specificity. Screening assays for *N. gonorrhoeae* exhibit low positive predictive values which are exacerbated in low-prevalence populations. The use of NAATs in gonococcal disease leads to a presumptive diagnosis (recommendation grade C) and confirmation by culture in low prevalence areas is required. In rectal and oropharyngeal samples, NAATs are more sensitive than culture (grade C) although they are not recommended because the presence of commensal *Neisseria* in these anatomical areas, which reduces the specificity. In women urine samples are valid (grade C). NAATs

are recommended for urine and noninvasive samples (evidence level II, grade B); however, sensitivity is lower than in endocervical samples (level III). Vaginal or tampon samples can also be used as specimens (level III) (12).

There are two types of MBT diagnostic methods of interest: (a) hybridization methods such as the commercially available GenProbe PACE II (Gen-Probe, San Diego, USA) and the Digene Hybrid Capture II assay (Digene Corp, Gaithersburg, USA). Both use an oligonucleotide-specific probe; however, both have a lower sensitivity and specificity than the culture and (b) amplification methods both in-house and commercial.

NAATs could improve gonococcal control in men who have sex with men (MSM), since gonococcal culture of rectal and oropharyngeal samples has a sensitivity of only 50 %. In recent years, there is abundant literature showing that NAATs have a higher sensitivity and specificity than bacterial culture for the screening of *N. gonorrhoeae* in asymptomatic men and women; for example an RT-PCR using a quadruplex assay (LDQA) targeting the cryptic plasmid and MOMP genes of *C. trachomatis*, the *porA* pseudogene of *N. gonorrhoeae* and a synthetic internal control (13) or automated assay (14). Bachman et al. (15) found over 60 % and 80 % of gonococcal and chlamydial infections respectively, among men who have sex with men, whereas over 20 % of chlamydial infections in women went undetected if the rectal site was not tested with NAAT.

Different genotyping methods have been described and remain a challenging problem (16): Restriction Fragment Length Polymorphism (RFLP), *opa* (based on the family of 11 *opa* genes), analysis of internal fragments of two hypervariable genes *porB* (transferrin-binding protein B subunit gene) and *tbpB*. The two former methods require acrylamide or agarose gel electrophoresis, while the latter two are used together to type *N. gonorrhoeae* (multiantigen sequence typing multiantigen sequences [NG-MAST]) and are easy to compare in different laboratories. The concordance of genotypes in sexual patterns is higher with NG-MAST than with *opa* typing. NG-MAST and Multilocus Sequence Typing (MLST) appeared more efficient than the classical serotyping scheme. MLST seems to be suited to large-scale epidemiological purposes, while the NG-MAST, as well as *porB*-typing, is more appropriate for local outbreak investigation (17). It is possible to identify mixed infections and to contribute significantly to studies of host immunity, gonococcal epidemiology, and pathogenesis (18) using *porB* variable-region (VR) typing in direct clinical specimens. In recent years, MBTs (RT-PCR) have been developed to detect resistance to penicillin and quinolones: hybridization probe (LightCycler platform) (19, 20) and RT-PCR with Taq-Man probes (quinolones only) (21).

*Chlamydial infection.* *Chlamydia trachomatis* infection is the most common bacterial sexually transmitted infection (STI) in the

United States, and may lead to an insidious and often chronic, unrecognized disease. Several important sequelae can result from *C. trachomatis* infection in women; the most serious of these include pelvic inflammatory disease (PID), ectopic pregnancy, and infertility. Although the technology for diagnosing *C. trachomatis* continues to be a rapidly developing field, the standard of care for all cases, including medicolegal cases, is a nucleic acid amplification technique (NAAT) (22). In clinical evaluations, NAAT methods have been shown to be more sensitive than culture and other non-culture methods, and have become the preferred method for diagnosing *C. trachomatis* infections in clinical laboratories. In women, a cervical swab or vulvovaginal swab are the specimens of choice. If a speculum examination is not possible then a first-catch urine sample can be used. In men a urethral swab is the specimen of choice; however, first voided urine sample is reported to be as good. In both men and women none of the NAATs have FDA approval for rectal, pharyngeal, and conjunctival specimens. However, in the absence of culture or direct fluorescence antibody tests, NAATs may be used. Under US law, laboratories may offer NAAT testing for diagnosis of extragenital chlamydia after internal validation of the method by a verification study performed by a second laboratory (23). There are reports that have found that available commercial NAATs are more sensitive for the detection of rectal chlamydial infection than culture (6). For medicolegal cases, a NAAT should be taken from all the sites where penetration has occurred. The BASHH guideline (22) recommends NAATs rather than cultures due to the low sensitivity of the latter (60–80 %) and the lack of availability of culture in many centers. A reactive NAAT result must be confirmed using a different NAAT that targets a different sequence.

NAATs specificity lies between 95 and 98 %, and its sensitivity between 88 and 90 %. NAATs are recognized as a reference method to diagnose *C. trachomatis* infections (24). An inhibitory control should be used for each specimen (25), as substances may be present in biological fluids which can inhibit NAATs. In addition for population screening programs or symptomatic patient diagnosis NAATs are more sensitive than culture and are more sensitive and specific than enzyme immunoassays (EIAs). Commercially available NAATs include Becton Dickinson BDProbe Tec<sup>®</sup>, Gen-Probe AmpCT, Aptima<sup>®</sup>, and Roche Amplicor<sup>®</sup>; both can detect *N. gonorrhoeae* in the same sample. The Abbott RealTime CT new formulation assay (m2000 real-time PCR) consists of a duplex PCR which targets different parts of the cryptic plasmid in *C. trachomatis*. The version 2 of the Roche Cobas TaqMan CT assay comprises a duplex PCR targeting a sequence in the cryptic plasmid and another in the *omp1* gene, and the Gen-Probe Aptima Combo 2 assay (AC2) targets the *C. trachomatis* 23S rRNA molecule.

A new variant of *C. trachomatis* (nvCT) was identified in Sweden in 2006, and is characterized by a 377 bp deletion in

ORF-1 of the multicopy cryptic plasmid, which includes the target region of two commercial NAATs (Roche and Abbott) and several in-house NAATs. The Strand Displacement Assay (SDA, Beckton Dickinson) can detect the nvCT variant because it uses a different DNA target sequence in the cryptic plasmid. The newly redesigned Roche and Abbott commercial assays include, in addition to the sequence affected by deletion, new targets in the cryptic plasmid or in the chromosomal *ompA* gene, allowing for the detection of both the wild-type and new variant *C. trachomatis* strains with the same accuracy. The new variant has not spread extensively and only sporadic cases have been reported outside the Nordic countries, suggesting that the cryptic plasmid may play a role in the infectivity of the pathogen.

*C. trachomatis* comprises three serogroups and 19 serovars; the knowledge of circulating *C. trachomatis* serovars can be beneficial for the success of sexual network surveillance and monitoring treatment. Typically, *C. trachomatis* serovars are predicted by direct *omp1* gene PCR-RFLP analysis or by nucleotide sequencing. Genotyping procedures can be labor-intensive, are not readily available, and may lack the capacity to identify multiple serovars.

*Lymphogranuloma venereum*. Lymphogranuloma venereum (LGV) is caused by the more invasive L serovars (L1, L2a, L2b and L3) of *C. trachomatis*. Since the recent outbreaks of LGV proctitis in Europe and North America among MSM it is recommended that all MSM with a positive NAAT from rectal and urethral/urine samples and/or isolate reporting rectal symptoms and/or who are in contact with someone with LGV, should send a sample to check for LGV. The first step is the detection of *C. trachomatis* using a nucleic acid amplification test (NAAT); routinely available NAATs for *C. trachomatis* will detect all serovars including LGV serovars and are licensed for genital specimens. There are no licensed NAATs for the detection of *C. trachomatis* in rectal specimens but data are available supporting the validity of these tests (level III, grade B) (26). Confirmation of the presence of LGV-specific DNA can then be obtained using RT-PCR (level IV, grade C) (27–29). Alternatively, genotyping can be performed by amplifying the *omp1* gene followed by restriction endonuclease digestion to identify specific serovars (30). An additional RFLP method is based on the digestion of the CrP gene, which differentiates between L1, L2a, L2b, L3 (31) (level III, grade B) (26). All LGV positive samples must be genotyped to determine the LGV serovar (30) (level III, grade B) (26). Typing for epidemiological purposes using DNA sequencing of the *omp1* gene should only be performed at a reference laboratory.

*Genital mycoplasma*. *Ureaplasma* spp. and *Mycoplasma hominis* can be isolated from the lower genital tract in many sexually active asymptomatic adults. Nevertheless, there is evidence that these species play etiologic roles in some genital tract diseases of both men

and women. *Ureaplasma urealyticum* is a cause of urethritis in men. *M. hominis* has been associated with some cases of PID and is involved in the alterations of the vaginal microbiota observed in bacterial vaginosis. *M. hominis* and *Ureaplasma* spp. can be isolated easily and rapidly from clinical specimens by culture in specific media.

*Ureaplasma urealyticum* has now been designated as two distinct species: *Ureaplasma parvum* (formerly known as “biovar 1”) reported to be nonpathogenic in men, and *U. urealyticum* (formerly known as “biovar 2”) associated with urethritis. Differences are based on whole-cell DNA homology, genome size, serotype grouping, sequence homology and divergence among urease and other selected genes (32). Since serotyping to differentiate *U. parvum* and *U. urealyticum* presents difficulties, molecular methods to differentiate the two species have been described against different targets: specific sequences in the urease gene (33) and 16S rDNA gene amplification and sequencing (34).

It has been shown that *M. genitalium* is a cause of disease in both males (nongonococcal urethritis) and females (cervicitis, urethritis, and PID). The diagnosis of *Mycoplasma genitalium* is difficult. The culture is very slow (weeks or months) and has low sensitivity due to the low organism load. Thus, MBTs are the only diagnostic tests that may provide relevant clinical information. Appropriate specimens are urethral exudate, cervical exudate, and first-catch urine for both men and women. PCR is the main method developed using different target sequences: major rRNA gene (16S) (35), the adhesin MgPa (36), and *gap* gene encoding glyceraldehyde 3-phosphate dehydrogenase (37). Methods capable of detecting two genes simultaneously (16S rRNA and adhesin MgPa), and methodologies such as TMA (Gen-Probe investigational use) which target ribosomal RNA-like features (38) have also been described.

*Syphilis*. Direct detection methods such as dark-field microscopy and direct immunofluorescence are relatively insensitive for *Treponema pallidum* diagnosis (38). Polymerase chain reaction (PCR) provides a sensitive and specific assay to directly detect the presence of pathogenic *T. pallidum* in swabs and biopsy specimens from genital and mucosal ulcers, placental specimens, and cerebrospinal fluid. Oral or other lesions can be used, but contamination (and possible cross-reaction) with commensal treponemes is likely. PCR is available only in reference laboratories, so usually it is not a replacement for dark-field microscopy in the clinic setting.

In certain scenarios PCR may be helpful for diagnosis by revealing *T. pallidum* in tissue samples, vitreous fluid and CSF. For example, syphilitic gummata may be identified by PCR when diagnosing congenital syphilis of exudates from suspicious lesions or body fluids (e.g., nasal discharge). A PCR assay targeting the *polA* gene (39), the 47-kDa integral membrane lipoprotein gene (40), the *bmp* gene (41), and the 366 bp region of the 16S rRNA (42)

has been published. Other areas of interest in the molecular biology of *T. pallidum* are RFLP typing of *tpr* and *arp* genes (43) and the detection of antimicrobial resistance by RT-PCR (44).

*Donovanosis*. Donovanosis or granuloma inguinale is caused by infection with *Klebsiella granulomatis* (*Donovania granulomatis* or *Calymmatobacterium granulomatis*), which was renamed following comparative DNA sequencing studies (45). Alternative phylogenetic analyses have argued in favor of retaining the previous species name, *Calymmatobacterium* (46). PCR diagnostic is not currently available except in Australia, where it was only used in a small eradication program (47) (level IIa, grade B) (48).

*Chancroid*. Infection due to *Haemophilus ducreyi* is infrequent in developed countries. Since culture is difficult and has a low sensitivity (75 %), molecular methods are recommended (49–51) and these can achieve a sensitivity of 95 % (level IIb, grade B) (52). These methods are generally designed with other microorganisms in a multiplex test (53, 54). An in-house multiplex PCR (*H. ducreyi*, *T. pallidum*, and HSV types 1 and 2) detected between 1 and 10 microorganisms, although inhibition problems were observed (54). RT-PCR technology can provide a sensitive, fast and reproducible diagnosis of genital ulcer etiology in a resource-limited setting (55). Also, *H. ducreyi* genetic material detected by PCR in biopsies of esophageal lesions in three HIV-1-infected patients has been reported (56). For epidemiological studies, both Random Amplification of Polymorphic DNA (RAPD) and Pulsed Field Gel Electrophoresis (PFGE) techniques can be used to characterize the diversity of *H. ducreyi* strains; however, RAPD is more rapid and accessible in resource-poor countries, such as those in Africa, than other MBTs that require expensive equipment (57).

*Genital Herpes*. HSVs are host-specific and ubiquitous; direct contact with a lesion with active viral replication is necessary for transmission. Infection persists for life, and relapses may be frequent. HSV-1 is mostly associated with orolabial herpes, whereas HSV-2 manifests more commonly in the genital area; however, both viruses may cause both infections. Genital infection due to HSV-2 presents the highest relapse rate. Although clinical presentations such as mucocutaneous forms are very typical and their diagnosis is typically clinical, because of the relative frequency of atypical lesions, the fact they may appear without associated external lesions (urethritis) and the importance of genotyping the virus, it is recommended to always confirm the diagnosis in the laboratory, where a prognosis (quantification and/or susceptibility assays) may be established and the antiviral treatment monitored. HSV are relatively easy to diagnose in a laboratory setting using a wide range of samples (cutaneous lesions, exudates, CSF, tears, etc.). Obtaining a quality sample determines which types of tests may be carried out. In the case of HSV, genome detection has not been as relevant

as with other viruses, since they replicate abundantly at the infection site and may be retrieved easily with culture techniques or identifiable via antigen detection. However, in samples with low viral load molecular methods are more interesting and relevant.

Even though hybridization genome detection techniques are commercially available, such as Hybrid Capture II (Digene, USA), most of them are based on genome amplification. These detection techniques amplify conserved viral genomic fragments from proteins such as G- or D-glycoprotein, polymerase or thymidine-kinase. The first protocols were designed on low viral load samples, and consequently they were very sensitive and specific (e.g., “nested PCR”) (58). Compared with culture, the diagnostic sensitivity increased by up to 40 %, and could be considered as a new reference standard (i.e., “gold standard”) (59, 60). In addition, they allow for the detection of subclinical infections (61). Nevertheless, the cost of such methods is high, especially if commercially available techniques are employed. Nowadays, classic PCR techniques have been replaced with real-time PCRs, which reduce the time needed to obtain results and the complexity of the procedure (avoiding agarose gels and plaque hybridizations), and allow for the detection of up to 50 copies/ml (62). Protocols which avoid genomic material extraction have been recently developed (63). These RT-PCR techniques can be used to distinguish between HSV-1 and HSV-2. Multi-PCR protocols have also been designed that may identify different STI-related microorganisms (54).

As with other viral infections, due to the close relationship between genotypic changes and the resulting phenotype, NAAT techniques are extremely useful to determine and characterize the infecting strains and to study the response to treatment. In the case of HSV, antivirals are effective, and their use is uncomplicated, although some studies demonstrate resistance to antiviral substances to be as high as 14 % (64). Special precautions must be taken with immunosuppressed patients, such as HIV-positive patients or those with solid organ transplantation, receiving prophylaxis in which mutations in the polymerase or thymidine-kinase genes (UL-23 and UL-30, respectively) may develop leading to antiviral resistance (65). To determine the changes in those genes, genome amplification tests are carried out in the two segments of the aforementioned genes, followed by sequencing (66).

*Candidiasis.* Although vulvovaginal candidiasis and bacterial vaginosis usually are not transmitted sexually, they are included in this chapter because they are often diagnosed in women being evaluated for STIs and are included in the STD guidelines (67).

Since *Candida* is a commensal microbiota in the genital tract, its detection would only be meaningful in cases of vulvovaginitis. MBTs are poorly developed, and their main use is in cases that require more rapid results than those offered by culture. A commercial method is available, the Affirm VP III test (Becton

Dickinson, Franklin Lakes, NJ, USA) that is used for the detection and identification of *Candida* species, *Gardnerella vaginalis*, and *Trichomonas vaginalis* from vaginal fluid specimens. It is based on nucleic acid hybridization and uses two distinct single-stranded probes for each organism, a capture probe and a color development probe. The Affirm VPII has proven to be more sensitive than direct smear to detect *Candida* spp. (68).

*Trichomoniasis*. While the majority of the STI have increased in the last years vulvovaginal and urethral trichomoniasis are decreasing and, in Australia, this decrease from 1947 to 2005 is related to the nitroimidazoles introduction and the Papanicolaou screening (69).

Traditional *Trichomonas vaginalis* detection methods (wet preparation and culture) underestimate the *T. vaginalis* prevalence when compared with MBTs. For the diagnosis of *T. vaginalis* there are two types of MBT tests: (a) The Affirm VP III test (Becton Dickinson, Franklin Lakes, NJ, USA), which was one of the first MBT for *T. vaginalis*; it requires 45 min and offers sensitivity and specificity values of 83 and 100 %, respectively; and (b) PCR in-house methods (70–75) (level III, grade B) (76). The sensitivity of culture compared with PCR has been estimated to be 70 % because a prompt transport and laboratory processing of the specimen is essential before the organism lyses or loses motility, which reduces the sensitivity of the diagnostic procedure (73). RT-PCR Roche LightCycler (Roche Diagnostics) improves the accuracy of traditional diagnostic tests, removes the postamplification step and its sensitivity and specificity values are 90.1 and 100 %, respectively (77, 78). These commercial methods are not certified by the FDA in the USA. The anatomical site from which the sample is collected is very relevant. In women, posterior fornix or self-collected vaginal swabs, or first catch urine (FCU) specimens are valid (level III, grade B) (76). In men, FCU samples are recommended, but urethral swab, FCU samples and semen should be required for optimal sensitivity. Furthermore, recent studies have indicated that the viability of the organism has a profound effect on all testing modalities because *T. vaginalis* possesses highly active endonucleases that could be responsible for the degradation of DNA, resulting in false negative test results. When urine specimens are collected, the sensitivity is affected by a loss of viability as early as 1 h after specimen collection (79). For the *T. vaginalis* phylogenetic analysis, techniques such as RAPD are used.

*Bacterial vaginosis*. BV increases the risk of acquiring other sexually transmitted infections, including HIV (80). Perhaps the most enlightening work on BV organisms was provided by Fredricks et al. (81) using NAAT for bacterial 16S rDNA. Using similar techniques, Ferris et al. (82, 83) concluded that each case of BV has its own unique set of anaerobic species. The pros and cons of the molecular methods in BV can be seen in ref. 84 and are beyond the scope of this chapter. The conclusions of all the studies on bacterial

vaginosis (summarized in ref. 84) are the following: (a) subjects without BV have vaginal bacterial biotas dominated by lactobacilli; (b) the composition of bacterial taxa in subjects with BV is heterogeneous; (c) this may be a polymicrobial syndrome; (d) currently, we do not appreciate the role of the “long tail” of less prevalent bacteria; (e) the importance of using a combination of approaches to attain a complete picture of vaginal bacterial diversity; (f) a better approach would be to use quantitative PCR that offers real-time results; (g) the use of Gram stain analysis to evaluate BV status is advisable. Finally, there is new evidence suggesting that BV may be a biofilm condition in some women, which may contribute to poor treatment responses and high relapse rates (85).

*HIV.* Although genome detection techniques may be used to diagnose the infection, they are normally used to monitor it. More than two decades ago, these techniques were used to determine the viral genome integration in the genome of CD4 lymphocytes (86). They allowed the identification of mutations in the viral genome in those patients with a poor response to zidovudine (87). Subsequently Mellors et al. (88) demonstrated that the viral load determined by viral genome quantification was a good prognostic marker of disease. Nowadays, viral load measurement techniques are based on signal amplification (branched-DNA, VERSANT HIV-1 RNA 3.0 ASSAY, Siemens, Germany) or on the amplification of a target sequence of interest such as the NASBA (Nucleic Acid Sequence Based Amplification, NUCLISENS HIV-1 RNA QT, bioMerieux), via RNA isothermal amplification, and the most common, RT-PCR methodology (AMPLICOR HIV-1 MONITOR V 1.5, Roche Diagnostics, Switzerland; The Abbott RealTime HIV-1 assay, Abbot, USA). Currently, totally automated protocols are commercially available (COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test, v2.0, Roche, USA). Viral load determination has also been applied to genital tract samples to determine the probability of viral transmission (89, 90) or to determine the influence of HIV in the transmission of other STIs (91).

Genome detection techniques are also interesting for the genotyping and characterization of viral fragments (genes “pol” and “env”) associated with a good antiviral response. For example, TRUGENE® HIV-1 Genotyping Assay (Siemens, Germany) and ViroSeq HIV-1 Genotyping System (Abbot, USA) evaluate the changes associated with RT inhibitor-based and proteinase-based antiviral resistance by genome amplification of a fragment of the “pol” gene. In order to do so, international guidelines are used (92, 93). They can be found online at some public institution sites, such as Stanford University (<http://hivdb.stanford.edu/pages/alg/HIVdb.html>), the Max Planck Institute (<http://www.geno-2pheno.org/>) or the Los Alamos National Laboratories (<http://www.hiv.lanl.gov/content/sequence/RESDB/>). These guidelines also include information on antivirals against viral integrase or HIV

coreceptors which may be determined with amplification and sequencing techniques.

MBTs have also been used to carry out phenotype studies (94), which are the basis of virtual phenotype sensitivity studies: the method provides an accurate prediction of phenotypic susceptibility from any qualified genotype sequence and eliminates the need to work-up samples for conventional phenotype testing. (<http://www.vicolab.com/hiv-resistance-products/virconet2>). Finally, studies have been developed which apply proviral genome detection in order to look for all viral subtypes and avoid infections during transfusions (95). NAATs have proven very valuable for HIV study, monitoring and handling. Their great clinical and social repercussion has allowed for the rapid development of the technology and their use in clinical diagnostic laboratories like genome detection, genome quantification, or genotypic characterization; furthermore, their use has spread to other Microbiology areas.

*HPV*. HPV is arguably the most common sexually transmitted agent worldwide, either in its clinical (condylomata) or subclinical forms. HPV is recognized as a causal and necessary factor for cervical cancer (96) and other types of cancer, such as penis, anal, or oral cancer (97). The fact that infection by HPV provokes long-term symptomatology demands a close follow-up (screening) of those individuals susceptible to infection in order to avoid related problems.

HPV serves as paradigm for the use of NAATs for diagnosis and typing due to the difficulty in culturing the virus and in the development of indirect diagnostic techniques. Genome amplification protocols (PCR) with degenerate primers targeted towards the L1 gene fragment (MY09/MY11) allow for the detection of a wide range of viral subtypes, which are then identified with specific probes (98, 99). Other consensual primers (PGMY or GP5+/GP6+) aimed at the same target enhance diagnostic sensitivity (100, 101). The use of these protocols leads to the identification of the low and high cancer risk genotypes (102).

Monitoring can be performed on several types of samples: vaginal, urethral, paraurethral, anal or pharyngeal exudates, biopsies, and endocervical exudates. At this time, several kits are commercially available which allow for the detection of the virus or the detection and typing of the most relevant HPV genotypes: Amplicor HPV test and Linear array HPV Genotyping test (Roche Diagnostics, Switzerland), Innolipa HPV Genotyping Extra (Innogenetics, Belgium), Biopat kit (Biotools, Spain), or Clart Papillomavirus 2 (Genómica, Spain). The latter uses microarray technology to increase the number of hybridizations in a reduced space. Besides genome amplification, direct hybridization protocols on the sample (hybrid capture) approved by the FDA for diagnosing HPV in women (Hybrid Capture 2, Digene, USA) are also used.

These protocols identify high- and low-risk genotypes without specifying the infecting genotype. These techniques have replaced other methods, such as “in situ” hybridization (103, 104).

Amplification protocols have also undergone great advancements with the application of real-time PCR, which reduces reaction times (e.g., HPV RealTime test, Abbot, USA; GenoID, Hungary). In fact, it is now possible to automate the whole process (Cobas® 4800 HPV Test with 16/18S Genotyping, Roche Diagnostics, Switzerland). The sensitivity of such methods made cytological methods (Papanicolau), which are less sensitive and specific, redundant. This high degree of sensitivity allows the period between control visits of women to be extended to 5–6 years (105, 106). Despite the promising outcomes, vaccination does not exempt patients from periodic control visits, because the effects of the vaccine at 15–20 years and the role other genotypes with oncogenic capacity not included in the vaccine may play are still unknown.

Furthermore, there is still a large population of women older than 20 years old, where the vaccination will be not effective.

In conclusion, the search for safer, more precise markers which may allow for a better control of the infection is ongoing (107). These markers include genome quantification via real-time PCR (108), viral integration into the human genome via E2-E1/E6-E7 genes ratio (109), or the search of viral variants by sequencing, pyrosequencing, or allelic discrimination techniques (110, 111).

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## 2. Other STIs

1. Scabies, known colloquially as the 7-year itch, is a contagious skin infection caused the mite *Sarcoptes scabiei*. Molecular methods are used in two situations: (a) cases where only few mites are present or in subtle or atypical skin manifestations (112, 113) and (b) to survey the resistance to acaricides (114).
2. Molluscum contagiosum (MCV). Over the last years the incidence of molluscum contagiosum has been increasing, mainly as a sexually transmitted infection (115) and it is particularly rampant as a result of concurrent human immunodeficiency virus (HIV) infection. Molecular methods have been used for epidemiological and diagnostic purposes. A restriction endonuclease analysis of the molluscum contagiosum genome identifies four types of MCV: MCV-I, II, III, and IV (116) and Thompson et al. (117) described that HIV-1 positive patients had a significantly higher incidence of MCV-2 infections than those not infected with HIV. However, Saral et al. (118) found that MCV-I was the only dominant infecting strain. In this study, a specific PCR reaction amplifying the 393 bp and 575 bp-long regions of the MCV genome was used in the

detection. Subsequent subtyping was performed by digestion of the amplified 575 bp product with restriction endonuclease enzyme *Bam*HI and visualization on agarose gel electrophoresis. A rapid method for identifying patients infected with MCV via swab sampling has been described using two dual-labeled probe RT-PCR assays in conjunction with pyrosequencing for confirmation of PCR products and discrimination between MCV-1 and MCV-2 (119).

In summary, the use of these methods (in Table 1 we present a summary of this technology) is the choice of the laboratories according their possibilities, and they have produced significant advances in the diagnostic and epidemiological studies of these diseases. In the future, low-cost molecular methods, specially in resource-poor countries, and point-of-care tests, automatization and resistance patterns, are necessary to know, in real-time, the trends and enable us to use them both for the prevention strategies and serious sequelae and dynamics of STI.

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### 3. Appendix

#### 3.1. Levels of Evidence

Ia, evidence obtained from meta-analysis of randomized controlled trials

- Ib, evidence obtained from at least one randomized controlled trial
- IIa, evidence obtained from at least one well-designed controlled study without randomization
- IIb, evidence obtained from at least one other type of well-designed quasi-experimental study
- III, evidence obtained from well-designed nonexperimental descriptive studies
- IV, evidence obtained from expert committee reports or opinions and/or clinical experience of respected authorities

#### 3.2. Grading of Recommendation

- A. Evidence at level Ia or Ib (Body of evidence can be trusted to guide practice)
- B. Evidence at level IIa, IIb, or III (Body of evidence can be trusted to guide practice in most situations)
- C. Evidence at level IV (Body of evidence provides some support for recommendation(s) but care should be taken in its application)
- D. (Body of evidence is weak and recommendation must be applied with caution)

**Table 1**  
**Characteristics of NAAT methods**

	Process	Price by sample	Specific robots	Pros	Cons
<i>Preparations of samples</i>					
Manual	Middle	1€	No	Cost	Laborious
Automatic	Little	5–10€	Yes	Easy	Cost
<i>Amplification</i>					
“in-house” PCR	Middle	1–2€	No	Cost, versatility	Standardization
Multiplex PCR	Middle	2–4€	No	Cost, versatility	Standardization
<i>Identification</i>					
Manual gel	High	0.3€	No	Cost	Laborious
Automatic gel	Little	1.5€	Yes	Easy	Cost
Hybridization	High	3–6€	No	Cost, versatility	Laborious
<i>Amplification + identification</i>					
Real-Time PCR	Little	2€	Yes	Easy	Standardization
<i>Characterization</i>					
Hybridization	High	6–9€	No	Mix population	Laborious
RFLP	Middle	2€	No	Easy	Interpretation
Sequencing	Middle	6–9€	Yes	Acute identification	Laborious
<i>Commercial methods</i>					
Based on Hybridization	Middle	30–50€	Yes	Standardized	Cost
Gen-Probe					
HCI assay					
Affirm VP					
Branched-DNA					
Linear array					
Based on Amplification	Middle	30–60€	Yes	Standardized	Cost
LDQA					
BD-Probe					
Cobas-Amplicolor					
LigtCycler					
Nuclisens					
Based on Sequencing	Middle	180–200€	Yes	Standardized	Cost
NG-MAST					
MLST					
Trugene					

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## Guidelines for the Qualitative Detection of Viral Genomes in Dried Blood Spots

Davide Gibellini, Elisa De Crignis, and Maria Carla Re

### Abstract

Dried blood spots (DBSs) are a useful alternative to blood sampling especially in children or for screening high-risk populations in developing countries. DBS blood collection can be employed in the diagnosis of viral infections by PCR or RT-PCR and also in viral genome sequencing. In addition, the advent of multiplex PCR approaches has led to further diagnostic and methodological improvements allowing simultaneous detection of two or more different viral genomes in the same sample and amplification reaction. This chapter describes general guidelines for the qualitative viral genome amplification and detection in DBS providing an example application of a qualitative real-time SYBR Green-based multiplex RT-PCR assay targeting two major viral pathogens, HIV-1 and HCV.

**Key words:** Real-time RT-PCR, Dried blood spots, SYBR Green, HIV, HCV

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### 1. Introduction

PCR direct detection of viral genomes in clinical samples has dramatically enhanced the performance of clinical virology laboratories leading to major improvements in patient management and the correct choice of therapy (1–3). PCR has evolved into a more useful procedure represented by real-time PCR that has further increased PCR applications in viral infection diagnosis offering several advantages over classic PCR. Real-time PCR shows shorter analysis time, lower contamination risk, and the possibility to perform reliable and easy quantitative detection of multiple targets using different fluorophores. Software has been developed to manage assay results and many real-time PCR dedicated instruments are available (4–6). Classic and real-time PCR can detect either one target (“monoplex” PCR) or multiple targets (“multiplex” PCR) in a single tube for the concomitant detection of two or more

viruses in clinical samples. The multiplex PCR assays save time and money by identifying mixed infections (5, 7–10). Although some commercial kits have appeared in recent years, specific multiplex PCR settings are complex because they require a careful optimization of specific PCR amplification reagents. Both monoplex and multiplex platforms can be applied on different samples and then even on DBS sampling where the blood sample is directly placed on filter paper and dried facilitating blood collection and storage even for molecular analysis (11, 12). This sampling alternative is very useful in specific patient groups such as children from whom it is relatively difficult to obtain large blood volumes. DBS is also suited to developing countries where health system infrastructure may be wanting (13, 14). This chapter describes general guidelines for DBS preparation and amplification of viral targets providing an illustrative application of a SYBR Green-based real-time multiplex assay for HIV-1/HCV detection where this procedure has been successfully applied in practice (15).

### **1.1. General Guidelines for DBS Preparation and Viral Nucleic Acid Amplification**

DBS assessment and subsequent qualitative determination of viral genomes can be summarized in three major points: preparation of DBS, nucleic acid extraction/purification, and specific viral target amplification and detection (Table 1).

#### **1.1.1. Clinical Sample Preparation**

##### **Blood Collection**

Blood is collected from patients by venipuncture in an EDTA-containing vacutainer tube (at least 1 mL of blood is recommended) or with a sterile lancet either from the heel or finger tip. Finger tip and heel blood collections are especially indicated for pediatric patients from whom it is often difficult to obtain optimal volumes of blood. The operator must collect and handle the blood sample taking into account that all human blood samples could potentially be infectious for blood-borne pathogens and hence standard “universal” precautions should be taken before, during, and after the blood collection ([http://www.cdc.gov/ncidod/dhqp/bp\\_universal\\_precautions.html](http://www.cdc.gov/ncidod/dhqp/bp_universal_precautions.html)) and also when processing blood derived samples such as DBS.

##### **Dried Blood Spot Assessment**

The blood is placed on a solid support, represented by filter paper, just after the blood collection at room temperature or, at least, within 6 h to achieve reliable results. Specific filter papers (i.e., S&S n°903, Whatman BFC 180) can be used to retain the blood on their surface with similar performances without any interference with the following steps of nucleic acid extraction and amplification.

The blood must be spotted carefully onto a paper support without excessive sample spreading: the optimal blood volume is 50 µL and several blood spots (i.e., six or more) are recommended for each patient in order to obtain sample replicates useful for eventual re-testing or for recording. When the blood is collected with a sterile lancet either from the heel or from finger tip, it is not

**Table 1**  
**Flowchart of qualitative real-time PCR/RT-PCR in DBS**

DBS assessment	A. Sample collection	<ol style="list-style-type: none"> <li>1. Collect blood sample by venopuncture or with a sterile lancet from heel or finger tip</li> <li>2. Place blood sample (50 <math>\mu</math>L) on a paper support minimizing blood spreading. It is recommended to prepare at least six blood spots</li> </ol>
	B. Drying procedure	<ol style="list-style-type: none"> <li>3. Dry blood spots at room temperature for 3 h in a safety cabinet or, alternatively, at 37 °C for 1 h in a bio-containment box placed in a drying oven</li> </ol>
	C. Storage	<ol style="list-style-type: none"> <li>4. Refrigerate DBS at +4 C or alternatively freeze (at -20 °C or less) in an individual envelope if the extraction and amplification assay is not immediately performed.</li> </ol>
Nucleic acid extraction and purification	D. Excision	<ol style="list-style-type: none"> <li>5. Excise three punches from DBS</li> <li>6. Clean puncher between the samples to avoid carry over contamination</li> </ol>
	E. Extraction and purification kit	<ol style="list-style-type: none"> <li>7. Select nucleic acid extraction method. The choice of procedure is related to the kind of nucleic acid target involved (DNA/RNA). We strongly recommend the use of DBS validated commercial kits</li> </ol>
Amplification and detection of molecular target(s)	F. Real-time PCR/RT-PCR technique	<ol style="list-style-type: none"> <li>8. Assess real time PCR/RT-PCR technique. The choice of technique is related both to nucleic acid target and amplification format. All critical parameters must be titrated to obtain better sensitivity and specificity performance (see also Subheading Monoplex and Multiplex Procedure)</li> <li>9. Analyze qualitative data using the software dedicated to real time apparatus only when all controls are fully satisfied</li> </ol>
	G. Controls	<ol style="list-style-type: none"> <li>10. Run each sample, positive and negative controls in duplicate. Check the extraction performance analyzing the presence of specific cellular mRNA or single copy gene DNA. Amplification run is validated when all controls are properly identified</li> </ol>

possible to establish the exact blood volume spotted but, in this case too, the operator should try to minimize blood spreading. Following this stage, blood spots can be dried at room temperature for 3 h in a safety cabinet or, alternatively, at 37 °C for 1 h in a biocontainment box placed in a drying oven with the paper in a horizontal position. Drying time, at room temperature, may be longer if the humidity is high and then is essential to check visually that the sample is fully dried to avoid the failure of assay.

**Storage of Dried Blood Spots**

A major advantage of blood sampling like DBS is that the samples can be handled and/or shipped easily because they can be stored for a long time before processing. The storage of DBS is a crucial point

in DBS management and can be done with different procedures. Both DNA and RNA are well conserved for long periods when DBS samples are refrigerated (+4 °C) in an individual envelope such as a hybridization bag or in a zip-closure bag with desiccant packs or frozen (−20 °C or less) showing a reliable recovery of RNA or DNA for amplification after at least 1 year of storage (12, 16, 17).

DBS exposure to higher temperatures such as room temperature or 37 °C seems to be variably associated with a progressive degradation of RNA, whereas DNA remains more stable in these conditions. Several studies have demonstrated that RNA stability progressively declines at 37 °C and after two months RNA loss is very consistent (18). Room temperature storage in a dedicated envelope with desiccant packs does not consistently affect RNA stability for at least 3 months (19), even though other studies show a good RNA stability for at least 1 year (16, 20). These conditions allow the possibility of DBS shipping to reference laboratories from rural areas where the cold chain of transport is not attainable.

### *1.1.2. Extraction and Purification of Nucleic Acids from DBS*

#### *Preliminary Steps*

To extract the nucleic acids from a paper support, the sample must be excised from the support in paper disks using a paper puncher. To avoid carryover contaminations, between the different DBS samples, the puncher must be thoroughly cleaned with 70 % ethanol followed by PBS wash and by repeated punching of clean filter paper between consecutive samples. This approach is sufficient to rule out carry over contamination even though other alternative procedures have recently been described (21). The carryover contamination may, however, be monitored by placing reference negative samples between DBS achieved from patients.

#### *Nucleic Acid Extraction*

The choice of nucleic acid extraction and purification procedure is obviously related to the viral nucleic acid target and several methods are able to extract DNA, RNA or both. Although it is possible to use in-house extraction techniques (22–24), it is strongly recommended to employ commercial DNA and/or RNA extraction kits (i.e., Qiagen, Siemens, Roche, Invitrogen, etc.) that generally assure a higher recovery and purity of nucleic acids without the presence of amplification inhibitors. Importantly, these manufacturers have assessed effective nucleic acid extraction protocols specifically applied to DBS. These commercial kits generally allow a reliable recovery of nucleic acids greater than 75–80 %. We encourage the use of these commercial kits because in-house extraction procedures may exhibit some problems of reliability and nucleic acid recovery effectiveness.

### *1.1.3. Amplification and Detection of Nucleic Acids Purified from DBS*

Correct DBS preparation and optimal nucleic acid extraction and purification procedures are essential for the subsequent amplification and detection steps that can be performed by classical or real-time PCR/RT-PCR. The real-time approach is more functional for the

detection of viral genomes due to its flexibility and technical characteristics. Real-time PCR or RT-PCR will disclose one (monoplex) or more (multiplex) specific viral genomes in the same run using different chemistries (i.e., SYBR Green, Taqman, etc.).

#### Monoplex and Multiplex Procedure

As shown in several reports, a single viral target can be detected in DBS using or slightly adapting qualitative or quantitative current classical or real-time protocols already validated on nucleic acids purified from blood or other body fluids without significant changes in the mixture composition or amplification cycles. Although real-time qualitative amplification monoplex assays are widely used in pathogen diagnostics to qualitatively discriminate specific nucleic acid targets, it is essential to determine assay performance such as the low-end sensitivity and dynamic range of amplification. To estimate the sensitivity limits, the protocol needs to assay blood samples with a known scalar number of viral genomes. The lowest viral concentration detected in over 95 % of replicates indicates the sensitivity limit. In parallel, as previously indicated (25), it is important to detect the dynamic range of amplification analyzing the calibration curve determined by amplification of reference sample dilutions. The range must cover at least three orders of magnitude (25). The assessment of RT-PCR or PCR multiplex procedures (especially real-time multiplex) is more complex because the multiplex protocols suitable for adaption to DBS using the same format are not yet as widely available as for monoplex PCR. For example, a well-known multiplex format is the Taqman approach where oligo probes (specific for each pathogen and labeled with distinct fluorescent dyes with separate excitation and emission wavelengths) discriminate the different pathogens after appropriate optimization of the real-time instrument, fluorescent calibration and scanning. On the other hand, even fluorochrome dyes binding non sequence-specific dsDNA such as SYBR Green may be used in the multiplex platforms. This approach is less complex and expensive than Taqman because it needs a lower number of oligos for the amplification reaction but distinct viral amplicon melting temperature values must be obtained for accurate identification of the specific target (4, 26–28). Nevertheless, a reasonable starting point for the assessment of multiplex real-time RT-PCR or PCR procedures may be to attempt to combine two or more effective target-specific monoplex techniques with the same format. This approach can be useful but unfortunately the addition of extra primers and probes may result in a loss of performance with reduced sensitivity and/or formation of unspecific amplification products. To overcome this drawback, an in-depth analysis of the target region is required to determine primers/probes compatible with the multiplex assay. Several oligo primer design software programs are available to disclose the more promising oligos for specific multiplex platform (e.g., Oligo, Primerplex). The amplification effectiveness

of selected primers/probes must be challenged in an amplification mixture where a careful optimization of the concentrations of the oligos and key PCR reagents, such as the magnesium chloride (tested generally between 1.5 and 8 mM), DNA polymerase (1–3 U/reaction), dNTPs and reverse transcriptase (only for multiplex RT-PCR), is essential to achieve reliable and sensitive results. These parameters can be evaluated by a chessboard procedure where scalar concentrations of these molecules are challenged among them on known reference samples positive for the target viruses to identify the optimal amplification mixture. In addition, it is noteworthy that some companies have assessed pre-formed master mix solutions that can be directly used in several multiplex contexts or appropriately modified (7). The sensitivity limit and dynamic range of amplification can be determined using a reference sample positive for the viruses of interest with a procedure similar to those indicated in the monoplex approach.

Since multiplex formats detect mixed viral infections, it is conceivable that the two (or more) viral targets, present in the DBS at very different concentrations, can suffer a relevant competition where the less represented target may be undetectable. To overcome this drawback, a chessboard procedure challenging scalar opposite dilution of two or more viral genomes in reference samples can reveal the conditions affecting the amplification of less represented viral target. This analysis is pivotal because it is possible that, in a mixed infection, the burden of a specific virus may be generally higher than in another. This problem can be solved or at least minimized by decreasing the primer concentration of preferentially amplified target. When the monoplex or multiplex qualitative real-time assays are set on reference samples, it is important to verify the assay specificity on a large group of positive and negative samples. Moreover, qualitative real-time assays must include positive and negative controls in each run. Positive controls with high and low viral concentrations are recommended. In particular, the low positive control should be added at a concentration near the lower limit of detection to monitor the sensitivity performance of amplification. Positive controls can be prepared for example, by pooling negative blood sample with reference positive plasma. On the other hand, negative controls are represented by a negative blood sample. To check nucleic acid extraction in each sample, cellular genes are amplified in the same or parallel amplification tube: GAPDH,  $\beta$ -actin can be chosen for RT-PCR, whereas for PCR it is important to select single copy genes such as HLA or  $\beta$ -globin. Viral detection analysis must be performed with the dedicated software of real-time instrument and the manufacturer' instructions must be followed to optimize analysis performance.

The next section provides an illustrative example of qualitative real-time multiplex amplification procedures represented by simultaneous amplification of HIV-1/HCV genomes in DBS using a SYBR Green-based qualitative real-time RT-PCR.

## 2. Materials

All reagents should be stored at room temperature (unless otherwise indicated) and all solutions must be nuclease-free. All waste disposal regulations should be followed when disposing of waste materials.

### **2.1. Dried Blood Spot Preparation**

1. EDTA-containing vacutainer blood collection tubes.
2. Whatman No. 3 filter paper (Whatman, Maidstone, UK) (see Note 1 and Subheading 1.1.1).
3. Micropipettes and pipette tips with aerosol barrier.
4. Drying oven instrument.

### **2.2. Nucleic Acid Extraction**

1. Single-hole paper punch (3 mm diameter).
2. Water baths or heating blocks (56, 70, and 85 °C).
3. Microcentrifuge (Eppendorf 5415D microcentrifuge, Eppendorf, Hamburg, Germany).
4. Micropipettes and pipette tips with aerosol barrier.
5. Vortexer.
6. Microcentrifuge tubes (1.5 and 2 mL).
7. QIAmp Mini spin column (Qiagen, Hilden, Germany) (see Notes 2 and 3).
8. ATL, AL, AW1, and AW2 buffers (Qiagen) (see Note 2).
9. Proteinase K solution (600 mAU/mL; Qiagen). Store at 2–8 °C (see Note 2).
10. Ethanol (100 and 70 %).
11. Sterile PBS (phosphate buffered saline) buffer.
12. Deionized, double-distilled, nuclease (RNAase and DNAase)-free water.

### **2.3. SYBR Green Multiplex Real-Time RT-PCR Amplification**

1. Light Cycler instrument (Roche, Mannheim, Germany).
2. Capillaries (20 µL; Roche).
3. 2× Quantitect SYBR Green PCR master mix (Qiagen). Store at –20 °C (see Note 4).
4. Reverse transcriptase (RT) mix (Qiagen). Store at –20 °C (see Note 4).
5. Taq polymerase (Invitrogen, Carlsbad, USA). Store at –20 °C.
6. dNTPs mix (10 mM). Store at –20 °C.
7. MgCl<sub>2</sub> (25 mM).
8. Oligonucleotide primers. Store at –20 °C. For sequences see Table 2.
9. Deionized double-distilled water.

**Table 2**  
**Oligonucleotide sequences**

Oligos	Sequences	Amplicon length
HIV-1 gag (29) K03455	5'-TGCTATGTCAGTTCCCCTTGGTTCTCT-3' (SK431; 1473–1499) 5'-AGTTGGAGGACATCAAGCAGCCATGCAAAT-3' (SK462; 1358–1387)	142 bp
HCV 5'NCR (30) AF009606	5'-GTCTAGCCATGGCGTTAGTATGAG-3' (HCVP1; 77–100) 5'-ACCCTATCAGGCAGTACCACAAG-3' (HCVP2; 280–302)	226 bp
$\beta$ -Actin NM_001101.3	5'-GACAGGATGCAGAAGGAGATCACT-3' (ACT1; 1015–1038) 5'-TGATCCACATCTGCTGGAAGGT-3' (ACT2; 1135–1156)	142 bp

### 3. Methods

Blood specimens must be considered to be infectious and manipulated under strict biosafety precautions. Essential precautions include the availability of adequate hand washing facilities, work practice controls, proper use of protective equipment such as gloves, laboratory coats, the routine decontamination of work areas, and the proper disposal of waste material (see also Subheading 1.1). The following protocol is based on specific steps allowing the amplification of specific targets of HCV and HIV-1 genomes from dried blood spots.

#### 3.1. Preparation of Dried Blood Spots

1. Collect at least 1 mL of venous blood by venipuncture in EDTA-containing vacutainer tube (see Subheading 1.1.1).
2. Transport the sample tube to the virology laboratory at room temperature (see Subheading 1.1.1).
3. Carefully apply 50  $\mu$ L aliquots of whole blood to Whatman No. 3 filter paper using a micropipettor and sterile tips with aerosol barrier (see Subheading 1.1.1).
4. Let the blood spotted filter papers dry at 37 °C for 1 h in a biocontainment box placed in a drying oven (see Subheading 1.1.1).

#### 3.2. Nuclear Acid Extraction and Purification from Dried Blood Spots

1. Use a single-hole paper punch to excise three filter punches where DBS is visible (each punch about 3 mm in diameter; see Subheading 1.1.2).
2. Place the three punched-out circles into a microcentrifuge tube (1.5 mL) and add 180  $\mu$ L of ATL buffer (see Note 2).
3. Incubate at 85 °C for 10 min in a water bath. Briefly centrifuge to remove the drops from inside the lid.

4. Add 20  $\mu\text{L}$  of proteinase K solution to the sample and mix by vortexing.
5. Incubate at 56  $^{\circ}\text{C}$  for 1 h in a water bath. Briefly centrifuge to remove the drops from inside the lid.
6. Add 200  $\mu\text{L}$  of AL buffer to the sample. Mix thoroughly by vortexing.
7. Incubate at 70  $^{\circ}\text{C}$  for 10 min in a water bath. Briefly centrifuge to remove the drops from inside the lid.
8. Add 200  $\mu\text{L}$  of 100 % ethanol to the sample, and mix thoroughly by vortexing. Briefly centrifuge to remove the drops from inside the lid.
9. Carefully apply the mixture to the QIAamp Mini spin column (placed in a 2 mL sterile collection tube) without wetting the rim (see Note 3).
10. Centrifuge at  $6,000 \times g$  for 1 min. Place the QIAamp Mini spin column in a clean sterile 2 mL collection tube and discard the tube containing the filtrate.
11. Add 500  $\mu\text{L}$  of AW1 buffer in the QIAamp Mini spin column without wetting the rim.
12. Centrifuge at  $6,000 \times g$  for 1 min and place the QIAamp Mini spin column in a clean sterile 2 mL collection tube and discard the collection tube containing the filtrate.
13. Add 500  $\mu\text{L}$  of AW2 buffer to the QIAamp Mini spin column without wetting the rim. Close the cap and centrifuge at  $16,000 \times g$  for 3 min.
14. Place the QIAamp Mini spin column in a new clean and sterile 2 mL collection tube and discard the collection tube containing the filtrate.
15. Centrifuge at  $16,000 \times g$  for 1 min.
16. Place the QIAamp Mini spin column in a clean and sterile 1.5 mL microcentrifuge tube and discard the collection tube containing the filtrate.
17. Carefully add 30  $\mu\text{L}$  of deionized, double-distilled, RNAase and DNAase-free water to the center of the filter and incubate at room temperature for 2 min, and then centrifuge at  $6,000 \times g$  for 1 min.
18. The eluate can be processed immediately or stored at  $-70^{\circ}\text{C}$  until use.

### **3.3. SYBR Green-Based Real-Time RT-PCR Amplification and Detection**

All materials and equipment must be rigorously clean and sterile. A separate set of micropipettes and microcentrifuge tubes must be used exclusively for this procedure.

**Table 3**  
**Amplification reaction mixture**

Reagents for each sample	Volume
2× Quantitect SYBR Green PCR Master Mix	10 µL
10 mM dNTPs	0.4 µL
Taq polymerase (5 U/µL)	0.4 µL
25 mM MgCl <sub>2</sub>	0.2 µL
HIV-1 oligonucleotide mix (25 µM each) (see Note 6)	0.32 µL
HCV oligonucleotide mix (25 µM each) (see Note 6)	0.16 µL
RT Mix	0.25 µL
Deionized, double-distilled, nuclease-free water	0.27 µL
Total	12 µL

**Table 4**  
**SYBR Green real-time multiplex RT-PCR cycling conditions**

Steps	Cycles	Temperature and times
Retrotranscription	1	50 °C for 20 min
Taq activation	1	95 °C for 15 min
Amplification	45	94 °C for 5 s 60 °C for 15 s 72 °C for 30 s 78 °C for 3 s (see Note 8)
Melting	1	from 60 to 95 °C with an increase of 0.4 °C/s
Cooling	1	25 °C for 2 min

1. Add the following reagents (Table 3) for each sample in a nuclease-free microcentrifuge tube (see Notes 4 and 5):
2. Add 8 µL of purified sample and transfer the whole PCR amplification mixture to the Light Cycler capillary and perform the amplification reaction in the Light Cycler instrument (see Note 7) following the procedure shown in Table 4.
3. Data analysis is performed by LightCycler 5.3.2 software (see Note 7). The melting peaks are detectable at 81.6 °C and 86.5 °C for HIV-1 and HCV respectively. The controls are summarized in Table 5.

**Table 5**  
**SYBR Green real-time multiplex RT-PCR controls**

<i>Negative controls</i>	Two HIV-1/HCV negative blood samples in duplicate (see Note 9 and Subheading 1.1.3)
<i>Positive controls</i>	HIV-1/HCV high positive blood sample in duplicate (see Note 9 and Subheading 1.1.3) HIV-1/HCV low positive blood sample in duplicate (see Note 9 and Subheading 1.1.3)
<i>Nucleic acid extraction control</i>	Amplification of $\beta$ -actin mRNA in each tested sample in a parallel separate run (see Note 10 and Subheading 1.1.3)

## 4. Notes

1. We place the dried blood spots on Whatman No. 3 filter paper but other filter papers such as S&S n°903 (Schelchter and Schuell, Dassel, Germany) or Whatman BFC 180 (Whatman) are useful alternative.
2. The ATL, AL, AW1, AW2 buffers, QIAamp mini columns, and proteinase K are supplied in the QIAamp DNA mini kit (Qiagen, cat n°51304). Before using for the first time AW1 and AW2 buffers (supplied in a concentrated solution) must be diluted with 100 % ethanol (44 mL of AW1 plus 25 mL of 100 % ethanol and 43 mL of AW1 plus 30 mL of 100 % ethanol) and are stable for 1 year at room temperature. Proteinase K solution is stable for up to 2 months when stored at 2–8 °C. Storage at –20 °C prolongs the stability of protease up to 6 months but repeated freezing and thawing of protease K must be avoided. For this reason, we recommend the storage of suitable aliquots of proteinase K at –20 °C. AL and AW1 buffers contain guanidine hydrochloride and it is thus essential to not add bleach or acidic solutions directly to the sample-preparation waste because it can form highly reactive compounds. For more information, please consult the material safety data sheets of AL, ATL, AW1, AW2, and proteinase K solutions online (<http://www.qiagen.com/support/msds.aspx>).
3. We use as nucleic acid extraction and purification procedure (fully described in Subheading 3.2, steps 2–18) the same protocol with minor adjustments indicated for dried blood spots in the QIAamp DNA mini kit. The QIAamp mini spin columns effectively bind both RNA and DNA nucleic acids (31), potentially allowing the extraction of any viral genome by DBS. Hence, these columns can represent a good tool to purify any

virus to develop future real-time multiplex PCR or RT-PCR for the diagnosis of mixed viral infections. In our model, HIV-1 is advantageously detected because the presence of proviral DNA and viral RNA genome can be determined in the same amplification reaction.

4. Both Quantitect SYBR Green PCR Master Mix and RT mix are available in the Quantitect SYBR Green RT-PCR kit (Qiagen cat n°204243). These solutions are stable at  $-20^{\circ}\text{C}$  for at least 1 year. The material safety data sheets are available online (<http://www.qiagen.com/support/msds.aspx>).
5. The RT-PCR mixture of this multiplex assay was optimized with a chessboard procedure by which the concentrations of magnesium chloride, Taq, dNTPs, and HIV-1 and HCV oligos were adjusted.
6. The oligonucleotide sequences recognize the major HIV-1 subtypes and HCV genotypes as checked by BLAST program (<http://blast.ncbi.nlm.nih.gov/>).
7. For the use and maintenance of LightCycler and dedicated software, it is essential to follow the instructions in the instrument manual. The technique may be applied to other commercial real-time PCR dedicated instruments: in this case, it is sufficient to scale-up the contents of all reagents appropriately when other real-time instruments with higher volumes of amplification reaction mixture are employed.
8. The single fluorescence detection is performed at  $78^{\circ}\text{C}$  to rule out any possible interference of rare unspecific products such as the formation of primer-dimers.
9. Both negative and positive controls are treated as the samples obtained from patients and are analyzed in duplicate. Negative control samples are represented by HIV-1/HCV negative blood samples from healthy donors. Positive control samples can be obtained mixing HIV-1/HCV positive plasma with negative healthy donor blood sample and spotted onto the filter. The positive reference plasma can be supplied by specific sources (e.g., NIBSC, London, UK, Siemens, Munich, Germany). Alternatively, HIV-1/HCV positive blood samples can be used when the quantitation of viral genomes has been previously performed by gold standard techniques such as (b-DNA, quantitative real-time RT-PCR, etc.). The positive controls are represented by high and low positive controls (4). The high positive control is represented by DBS with HIV-1/HCV at concentration of  $1 \times 10^5$  HIV-1 copies/mL and  $5 \times 10^5$  HCV copies/mL, whereas a low positive control is indicated by an HIV-1/HCV positive sample with  $1 \times 10^3$  HIV-1 copies/mL and  $5 \times 10^3$  HCV copies/mL. The analysis of scalar HIV-1/HCV reference blood samples showed

that, in our hands, this SYBR-green based real-time assay consistently detects  $4 \times 10^2$  copies/mL and  $2.5 \times 10^3$  copies/mL of blood for HIV-1 and HCV respectively (15).

10.  $\beta$ -Actin mRNA was selected as the target to determine the effectiveness of extraction and purification of nucleic acids (32). The oligonucleotide primers for  $\beta$ -actin are described in Table 2.2. The amplification mixture solution and the amplification conditions are the same as those shown in Tables 2.3 and 2.4 except for the volumes of oligos and water used in the mixture solution described in Table 2.3: 0.25  $\mu$ L of  $\beta$ -actin primer mix (25  $\mu$ M each) and 0.5  $\mu$ L of water.

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## Guidelines for the Quantification of HIV and HCV in Small Volume Whole Blood Samples

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### Abstract

The quantification of Hepatitis C Virus (HCV) and Human Immunodeficiency Virus (HIV) in whole blood provides several advantages over the quantification in plasma samples. The application of small samples of capillary blood allows for application in point-of-care diagnostic testing methods.

Here we describe two protocols of extracting viral RNA from small samples of whole blood by hybridization to biotinylated LNA-modified 2'-O-Methyl-RNA or to biotinylated DNA, indirect capturing to streptavidin-coated beads, and subsequent quantification by one-step non-nested qRT-PCR. Further, we provide some general guidelines on extraction and quantification of HIV and HCV in small volume whole blood samples.

**Key words:** Hepatitis C virus, Human immunodeficiency virus, Viral load, Whole blood, Capture assay, Point-of-care testing, qRT-PCR, Guidelines

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### 1. Introduction

Worldwide approximately 130–170 million people are infected with the hepatitis C virus (HCV) (1) and more than 30 million people with the human immunodeficiency virus (HIV) (2) with the highest prevalence in developing and transitional economy countries. The determination of the viral load in plasma is the current standard of care for patients with these chronic viral infections and provides important information on activity and prognosis of infection (3), response to antiviral treatment (4, 5), and therapy adherence (6). There is an urgent need for simple, rapid, and affordable point-of-care viral load assays in settings with limited infrastructure. These assays will require small volumes of whole blood (WB) instead of large volumes of plasma.

Furthermore, quantification of viral concentration from WB may provide some advantages over plasma: Although the liver is the main

site of HCV replication, HCV RNA has been detected in circulating extrahepatic compartments such as in peripheral blood mononuclear cells (7, 8), in cryoprecipitates (9), attached to erythrocytes (10), and to platelets (11). Similarly, HIV-1 RNA has not only been found in circulating CD4<sup>+</sup> T cells (12), but also in CD4<sup>-</sup>/CD8<sup>-</sup> T cells (13), in monocytes (14), and associated with erythrocytes (15) and platelets (16). Thus, quantification of viral load in plasma or serum samples may underestimate the total circulating viral load.

Whereas we could recently demonstrate a very good correlation of the HCV viral load in 10  $\mu$ L WB and in 1 mL plasma in patients with chronic hepatitis C ( $R^2 = 0.919$ ) without a substantial benefit for WB analysis (17), HIV-1 RNA is detectable in WB even when the plasma viral load is below the limit of detection. In fact, in almost 60 % of 10  $\mu$ L WB samples from patients with undetectable HIV viral loads in 1 mL plasma, HIV was detectable (18). These findings demonstrate that quantification of viral loads from small-volume WB samples can serve as a valid and sensitive marker for HIV and HCV therapy monitoring and provide the prerequisite to develop a point-of-care diagnostic assay.

Here we describe two related methods to efficiently extract viral RNA from WB by means of a sequence-specific oligonucleotide hybridization assay. We developed an indirect HCV RNA capture assay using a 25-mer 2'-O-methyl-RNA that is modified by locked nucleic acids (LNA) which significantly increases the RNA extraction yield compared to silica-based total nucleic acid extraction (17) and compared to 25-mer DNA oligonucleotides using the same protocol in WB. 2'-O-methyl-RNA forms nuclease-resistant stable complexes with RNA (19) whereas LNA modification allows for an exceptionally high binding affinity to complementary RNA (20). For HIV-1 RNA hybridization we report a different protocol using three 60-mer DNA oligonucleotides to ensure strong hybridization to HIV-1 RNA. Both protocols use different streptavidin-coated matrices for the binding of the biotinylated capture oligonucleotides. The 2.8  $\mu$ m superparamagnetic polystyrene beads demonstrate a good dispersion and specific binding in chaotropic salts, but require a magnetic device for processing, whereas dried pellets of 34  $\mu$ m beads of highly cross linked agarose (sepharose) can be easily stored at ambient temperatures, dissolved, and recollected by filtration. To simplify the process of reverse transcription of extracted viral RNA, amplification, and detection we describe optimized one-step non-nested RT-PCR protocols for the quantification of HCV and HIV-1 viral load. However, to estimate extraction efficiency and amplification quality we strongly recommend the use of an internal standard RNA during extraction and amplification.

Applying small volume whole blood samples instead of large-volume plasma samples for viral load quantification of HIV or HCV requires a comprehensive assay validation including determination of analytical sensitivity, precision, linearity range, and demonstration of matrix equivalence to plasma (see Table 1).

**Table 1**  
**Guideline checklist and general considerations for the quantitation of viral RNA in small volume whole blood samples**

Item to check	Options/quality control parameters	Importance <sup>a</sup>	Notes
<i>Specimen selection</i> Source of clinical specimens	Capillary whole blood <i>or</i> venous EDTA whole blood	O	In settings with limited infrastructure venous blood draw, transport of fresh samples, and generation of plasma are difficult and sometimes impossible. The use of freshly obtained capillary blood allows use in simple, rapid, and affordable point-of-care viral load assays
Sample volume	10–100 µL	P	Sensitivity and precision decrease with lower sample volume. On the other hand, finger-prick sample volumes greater than 100 µL are not feasible
<i>Nucleic acid extraction</i>			
Method of nucleic acid extraction	Sequence-specific RNA extraction (vs. total nucleic acid extraction)	P	Because of a high cellular human nucleic acid concentration in whole blood, a sequence-specific extraction may provide advantages over total nucleic extraction as we have demonstrated for HCV (17)
Method of RNA hybridization	Indirect hybridization <i>or</i> Direct hybridization	O	In our hands, indirect capturing with biotin-labeled antisense probes and subsequent binding of the hybrids to a streptavidin-coated bead matrix improved viral RNA extraction compared with direct capturing using immobilized antisense probes
Selection of antisense oligonucleotide for specific extraction	LNA-modified 2'-O-Methyl-RNA <i>or</i> LNA-modified DNA <i>or</i> DNA	O	We evaluated different combinations of one to four sequence-specific oligonucleotides as hybridization probes complementary to conserved viral RNA regions. Shorter LNA-modified oligonucleotides (20-mer to 30-mer) possess superior viral RNA binding than DNA oligonucleotides with the same length and identical nucleotide sequences. If you use DNA, rather try longer antisense oligonucleotides (50-mer to 80-mer)

(continued)

**Table 1**  
**(continued)**

<b>Item to check</b>	<b>Options/quality control parameters</b>	<b>Importance<sup>a</sup></b>	<b>Notes</b>
Internal positive control	Artificial standard RNA as internal extraction and amplification control	E	The Internal Standard RNA must contain the capture binding sites and primer binding sites identical to that used for isolation and amplification of the respective analyte and a unique binding site for a labeled TaqMan probe (best with equal GC content). Because during amplification of both, the analyte and the Internal Standard RNA, the same primers are incorporated in the PCR-product, high amounts of Internal Standard RNA may impair the quantification of the target viral RNA. Therefore, we recommend to carefully adjust the initial amount of the competitive Internal Standard RNA applied. We used the standard at an initial concentration that results in a CT of 30–32 for lowest interference. For details on the HCV Internal Standard RNA see ref. (17)
Negative control	Viral free whole blood sample	P	We recommend the processing of a virus free whole blood sample as negative control for extraction and amplification within every run
<i>qRT-PCR</i>			
<i>qRT-PCR</i> type	One-step <i>qRT-PCR</i>	P	We recommend a one-step non-nested <i>qRT-PCR</i> protocol for reasons of simplicity, robustness, and rapidness
<i>qRT-PCR</i> validation	$R^2$ , $E_{T_{PCR}}$	E	Analyze a serial dilution of the target RNA for over at least four logs in replicates. Determine the coefficient of determination ( $R^2$ ) and the slope of the standard curve. Calculate the <i>qRT-PCR</i> efficacy $E_{T_{PCR}}$ with the following formula: $E_{T_{PCR}} = 10 \times (-1/\text{slope}_{T_{PCR}}) - 1$ . $R^2$ should be $>0.99$ ; $E$ between 1.8 and 2.2, and, thus, the slope of the standard curve between $-3.58$ and $-3.10$
PCR optimization	Temperature and times of denaturation and annealing/extension	P	Test target RNA in <i>qRT-PCR</i> with denaturation temperatures between 92 °C and 95 °C and denaturation times between 1 and 15 s in 4- to 5-s increments. To determine optimal annealing/extension temperature combine times between 20 and 50 s with temperatures between 60 °C and 66 °C in 2 °C increments. Select the conditions that repeatedly result in the lowest CT values and sufficient <i>qPCR</i> quality parameters

<i>Assay validation</i>	$R^2$ , $E$	$E$
Calibration curve		Since HCV and HIV whole blood standards do not exist, use plasma samples that are calibrated against the WHO international standard HCV-RNA 96/790 (21) or against the HIV-1 first international standard (NIBSC Code 97/656) (22), respectively, in replicates over at least four logs. Generate a calibration curve by plotting mean nominal log viral RNA concentrations and corresponding CT values (see Fig. 1). Evaluate linearity with the coefficient of determination ( $R^2$ ), the slope of the linear regression, and the assay efficacy $E_{\text{assay}} = 10 \times (-1/\text{slope}) - 1$ . When the internal standard RNA is coamplified, $E_{\text{assay}}$ should be $>1.6$ and $R^2 > 0.99$
Linearity	Polynomial method (EP6-A)	Determine linearity by the absolute difference of observed and nominal log-transformed viral titers in plasma with the polynomial method according to the NCCLS document EP6-A (24). The linear range should cover at least four logs
Analytical sensitivity	95% limit of detection (LOD)	Use Probit analysis for tested log-transformed concentration levels to statistically determine the 95% and 50% LOD. Aim for a 95% LOD of $<10$ IU/sample for HCV and $<50$ copies/sample for HIV-1
Precision	%CV	Determine intra-assay and inter-assay coefficients of variation (%CV) for different viral loads in replicates with the proposed formula for $\log_{10}$ -normal distributed values: $\%CV = 100\% \times (10^{SD \times SD_{\ln(10)} - 1})^{1/2}$ (SD: standard deviation). The SD should be $<0.2$ log; the respective %CV should be $<48\%$
Accuracy	Mean log difference	Determine the mean log differences of observed and detected plasma viral loads for different genotypes by using a genotype reference panel
Matrix equivalence	$R^2$ , regression slope, and intercept	Spike viral RNA-positive samples into pairs of plasma and whole blood from viral RNA-negative donors. Determine the mean log difference of measurement results in plasma and in WB and the coefficient of determination ( $R^2$ ). In linear regression, $R^2$ should be $>0.99$ , the slope should not differ from 1.0 (0.9–1.1), and the intercept should be $-0.1$ to 0.1 log
Diagnostic specificity	False positive rate	Determine the false-positive rate in whole blood from noninfected sero-negative individuals. The diagnostic specificity should be 100%

\* $E$  essential,  $P$  preferable,  $O$  optional

## 2. Materials

Prepare all solutions using ultrapure nuclease-free water and analytical grade reagents. Prepare and store all reagents at room temperature unless indicated otherwise. All work with potentially infectious material such as blood or plasma samples from patients infected with HCV and/or HIV has to be performed in Biosafety level 2+ facilities (use of a biosafety level 2 facility with containment equipment and practices of biosafety level 3). Diligently follow all waste disposal regulations when disposing of waste materials.

### 2.1. Specific HCV RNA Extraction

1. HCV Lysis/hybridization buffer: 4.3 M bicine buffered guanidine hydrochloride, 20 mM EDTA, 10 % (v/v) Triton X-100, 0.2 % (w/v) saponins from quillaja bark, pH 8.5. For a 20 mL solution use 10.75 mL of an 8 M guanidine hydrochloride solution buffered with 0.05 M bicine, 200 mg saponins from quillaja bark (Sigma, Saint Louis, MO, USA), 2 mL of Triton X-100, and 0.8 mL of a 0.5 M EDTA Stock solution (pH = 8.0), and adjust with water to 20 mL.
2. HCV Bead buffer: 10 mM Tris-HCl, 0.05 % (v/v) Tween-20, pH 7.5. For a 20 mL solution solve 100  $\mu$ L of a 10 % polysorbate 20 (Tween-20) solution and 200  $\mu$ L of a 1 M Tris-HCl stock solution (pH = 7.4) in 20 mL water, and adjust pH (see Note 1).
3. HCV 10 $\times$  Washing buffer: 1.5 M ammonium chloride, 10 mM EDTA, 100 mM Tris-HCl. For 10 mL use 3 mL of a 5 M ammonium chloride stock solution, 1 mL of a 1 M Tris-HCl stock solution (pH = 7.4), 0.2 mL of a 0.5 EDTA stock solution (pH = 8.0), and 5.8 mL water.
4. HCV Washing buffer I: 150 mM ammonium chloride, 1 mM EDTA, 10 mM Tris-HCl, 0.1 % (w/v) SDS, pH 8.0. For 20 mL use 2 mL of the 10 $\times$  washing buffer, 100  $\mu$ L of a 20 % (w/v) SDS solution (Ambion, Austin, TX, USA), 17.9 mL water, and adjust pH to 8.0 (see Note 2).
5. HCV Washing buffer II: 150 mM ammonium chloride, 1 mM EDTA, 10 mM Tris-HCl, 0.05 % (w/v) Tween-20, pH 8.0. For 20 mL use 2 mL of the 10 $\times$  washing buffer, 100  $\mu$ L of a 10 % (v/v) polysorbate 20 (Tween-20) solution, 17.9 mL water, adjust pH to 8.0 (see Note 3).
6. 2.8  $\mu$ m streptavidin coated superparamagnetic polystyrene beads, 10 mg/mL. Dynabeads M-270 Streptavidin (Invitrogen, Carlsbad, CA, USA) stored at 4 °C (see Note 4).
7. Biotinylated LNA-modified 2'-O-methyl-RNA antisense capture probe, 2.5  $\mu$ M: suggested sequence: AaCgCcAtGgCtAgAc GcUtUcUgC-biotin (capital letters refer to 2'-O-methyl-RNA,

lower case letters to LNA), nucleotide positions 92–68 according to the H77 reference strain of HCV (AF009606). Dilute a thawed and well mixed 25  $\mu\text{M}$  stock solution of the biotinylated LNA-modified 2'-*O*-methyl-RNA antisense capture probe (RiboTask, Odense, Denmark) 1:10 with water and store at 4 °C (see Notes 5 and 6).

8. Internal Standard RNA: approximately 100–500 copies/sample (see Table 1, Internal positive control).
9. Magnetic separation stand (e.g., CD 3002 or MagnaSphere, Promega, Madison, WI, USA).
10. 1.5 mL Safe-Lock micro test tubes.

## **2.2. Amplification and Detection of HCV RNA**

1. RNA UltraSense One-Step Quantitative RT-PCR System (Invitrogen, Carlsbad, CA, USA).
2. Smart Cycler II System (Cepheid, Sunnyvale, CA, USA).
3. HCV Primers, 600 nM each, suggested sequences: sense primer GTGGTCTGCGGAACCGGTGA, nucleotide positions 143–162 according to the H77 reference strain of HCV (AF009606); antisense primer CGCAAGCACCTATCAGG CAGT, position 309–288. Dilute the 100  $\mu\text{M}$  DNA primer stock solutions (Metabion International AG, Martinsried, Germany) 1:10 with water for a working solution of 10  $\mu\text{M}$  and store at 4 °C (see Note 6).
4. FAM-labeled HCV TaqMan probe, 200 nM, suggested sequence FAM-CCGAGTAGTGTTGGGTYGCGAAAGG-BHQ1, position 254–278 according to the H77 reference strain of HCV (AF009606), Y refers to a 50:50 C/T wobble. Dilute the 100  $\mu\text{M}$  DNA TaqMan probe solution (Metabion International AG, Martinsried, Germany) 1:10 with water for a 10  $\mu\text{M}$  working solution and store at 4 °C (see Note 6).
5. Cy5-labeled TaqMan probe for the internal positive control, 200 nM (specific for the unique binding site of the HCV Internal standard RNA, see Table 1, Internal positive control).
6. Microcentrifuge for 25  $\mu\text{L}$  and 100  $\mu\text{L}$  SmartCycler Reaction Tubes (Cepheid, Sunnyvale, CA, USA, provided with the instrument).

## **2.3. Specific HIV-1 RNA Extraction**

1. HIV Lysis/hybridization buffer: 4.3 M bicine buffered guanidine hydrochloride, 20 mM EDTA, 10 % (v/v) Triton X-100, pH 8.5. For a 20 mL solution use 10.75 mL of an 8 M guanidine hydrochloride solution buffered with 0.05 M bicine (Sigma, Saint Louis, MO, USA), 2 mL of Triton X-100, and 0.8 mL of a 0.5 M EDTA Stock solution (pH=8.0), 6.45 mL water.
2. HIV 10 $\times$  Washing buffer: 1.5 M ammonium chloride, 10 mM EDTA, 100 mM Tris-HCl, 1 % (v/v) Tween-80. For 10 mL use 3 mL of a 5 M ammonium chloride stock solution, 1 mL

of a 1 M Tris-HCl stock solution (pH=7.4), 0.2 mL of a 0.5 M EDTA stock solution (pH=8.0), 0.1 mL of 100 % (v/v) polysorbate 80 (Tween-80), and 5.7 mL water.

3. HIV Washing buffer I: 150 mM ammonium chloride, 45 mM KCl, 1 mM EDTA, 50.5 mM Tris-HCl, 0.1 % (v/v) Tween-80. For 20 mL add 2 mL HIV 10× Washing buffer to 18 mL HIV Washing buffer II.
4. HIV Washing buffer II: 50 mM KCl, 45 mM Tris-HCl, 0.01 mM EDTA, pH 8.3. For 100 mL use 2.25 mL of a 2 M Tris-HCl stock solution (pH=8.3), 5 mL of 1 M KCl, 2 µL of 0.5 M EDTA stock solution (pH=8.0), and 92.75 mL water.
5. 34 µm streptavidin coated cross-linked agarose beads (Streptavidin Sepharose High Performance, GE Healthcare, Chalford St Giles, UK).
6. 1 M Trehalose. Dissolve 3.783 g D-(+)-trehalose-dihydrate (Sigma, Saint Louis, MO, USA) in 10 mL water.
7. Self-sealing, moldable and flexible Parafilm (Brand, Wertheim, Germany).
8. Biotinylated HIV antisense DNA capture probes mix each 25 µM: suggested sequences: probe 1 ATACTGTCTTACTTTGATAAAACCTCCAATTCCCCCTATCATTTTTGGTTTCATTTTCC-biotin, nucleotide position 2,429–2,370 according to the HXB2 reference strain of HIV-1 (K03455); probe 2 GCTGGTCCTTTCCAAATTGGGTCTCTGCTGTCCCTGTAATAAACCCGAAAATTTTGAATT-biotin, position 4,945–4,886; probe 3 CATCCTGTCTACCTGCCACACAATCATCACCTGCCATCTGTTTTCCATAATCCCTAATGA-biotin, position 5,088–5,029. For a working stock of 100 µL thaw and well mix the 100 µM stock solution of DNA (Metabion International AG, Martinsried, Germany), take 25 µL of each of the three HIV-1 capture oligonucleotides into one tube, add 25 µL water, and store at 4 °C (see Note 6).
9. Internal Standard RNA (in an appropriate concentration, see Table 1, Internal positive control).
10. 1 mL polypropylene columns with Luer-lock cap (Mobicols M1002, MoBiTec GmbH, Göttingen, Germany) equipped with a lower polyethylene filter (pore size 10 µm, diameter 2.7 mm, M2110, MoBiTec GmbH, Göttingen, Germany).

#### **2.4. Amplification and Detection of HIV-1 RNA**

1. RNA UltraSense One-Step Quantitative RT-PCR System (Invitrogen, Carlsbad, CA, USA).
2. Smart Cycler II System (Cepheid, Sunnyvale, CA, USA).
3. HIV Primers: suggested sequences 200 nM each: sense primer GCAGTGGCGCCCGAACAGG, position 632–650 according to the HXB2 reference strain of HIV-1 (K03455); antisense

primer 1 ACTGACGCTCTCGCACCCATCT, position 809–788; antisense primer 2 TGACGCTCTCGCACCCATCTCTC, position 807–785. Dilute the 100  $\mu\text{M}$  DNA primer stock solutions (Metabion International AG, Martinsried, Germany) 1:10 with water for a 10  $\mu\text{M}$  working solution and store at 4  $^{\circ}\text{C}$  (see Note 6).

4. FAM-labeled HIV TaqMan probes, 200 nM each: suggested sequences: probe A FAM-CTCTCGACGCAGGACTCGGCT-BHQ1, position 683–703 according to the HXB2 reference strain of HIV-1 (K03455); probe B FAM-CTCCGACGC AACGGGCTCG-BHQ1, positions 683–700. Prepare a 10  $\mu\text{M}$  working solution from a 100  $\mu\text{M}$  stock solution (Metabion International AG, Martinsried, Germany) by dilution with water 1:10 and store at 4  $^{\circ}\text{C}$  (see Note 6).
5. Cy5-labeled TaqMan probe for the internal positive control (200 nM) specific for the unique binding site of the HCV Internal standard RNA (see Table 1, Internal positive control).
6. Microcentrifuge for 25  $\mu\text{L}$  and 100  $\mu\text{L}$  SmartCycler Reaction Tubes (Cepheid, Sunnyvale, CA, USA, provided with the instrument).

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### 3. Methods

Carry out all procedures at room temperature unless otherwise specified.

#### 3.1. Specific HCV RNA Extraction

1. To prepare the streptavidin-coated beads for a total of  $n$  analyses pipette  $n \times 20$   $\mu\text{L}$  bead suspension into a 1.5 mL tube and put the tube into the magnetic separation stand. Remove the bead-free supernatant, add  $n \times 20$   $\mu\text{L}$  bead buffer, mix well and put the tube back into the magnetic separation stand. Repeat the washing step and resuspend the beads in  $n \times 20$   $\mu\text{L}$  bead buffer and use within the next hour.
2. Pipette 5  $\mu\text{L}$  of the 2.5  $\mu\text{M}$  biotinylated capture probe on the bottom of a new 1.5 mL safe lock tube. Add 65  $\mu\text{L}$  HCV Lysis/hybridization buffer, 10  $\mu\text{L}$  of the whole blood sample (see Note 7), and if available 2  $\mu\text{L}$  of HCV Internal Standard RNA (see Note 8).
3. Mix well and incubate the solution at 65  $^{\circ}\text{C}$  for 5 min for optimum denaturation of RNA secondary structures and hybridization to the capture probes with gentle shaking. Take the tubes out of the thermo mixer, shortly spin down, and let it cool down at room temperature for 2 min (see Note 9).

4. Add 20  $\mu\text{L}$  of the resuspended beads to the lysate. For the binding of biotin-labeled dimers to the streptavidin-coated superparamagnetic beads incubate the suspension for 10 min at 21  $^{\circ}\text{C}$  with shaking at 800 rpm (see Note 10).
5. Shortly spin the tubes and transfer them into the magnetic separation stand. Remove all traces of liquid, add 150  $\mu\text{L}$  HCV Washing buffer I, vortex, and put the tube back into the magnetic stand. Repeat the washing step once more with 150  $\mu\text{L}$  HCV Washing buffer I, twice with 150  $\mu\text{L}$  HCV Washing buffer II to remove the SDS. Resuspend the beads in 50  $\mu\text{L}$  HCV Washing buffer II and put the tube back into the magnetic stand, and remove all traces of liquid. Finally, resuspend the beads in 15  $\mu\text{L}$  water (total approximately 18  $\mu\text{L}$ ) and store on ice until subsequent qRT-PCR (see Note 11).

### **3.2. Amplification and Detection of HCV RNA**

1. Freshly thaw the 5 $\times$  reaction mix (contains dNTP) of the RNA UltraSense One-Step Quantitative RT-PCR kit from  $-20^{\circ}\text{C}$  before the preparation of the master mix.
2. Prepare the HCV master mix for a total volume of 75  $\mu\text{L}$  per reaction including 15  $\mu\text{L}$  5 $\times$  reaction mix, 3.75  $\mu\text{L}$  enzyme mix, 4.5  $\mu\text{L}$  of 10  $\mu\text{M}$  sense primer, 4.5  $\mu\text{L}$  of 10  $\mu\text{M}$  antisense primer, 1.5  $\mu\text{L}$  of 10  $\mu\text{M}$  TaqMan probe. If available, add the Cy5-labeled TaqMan probe to detect the HCV Internal Standard RNA (see Note 12).
3. Add the master mix to the bead suspension and transfer it to a 100  $\mu\text{L}$  Cepheid SmartCycler reaction tube. Shortly spin it in to arrange the beads on the edge of the rhomboid reaction tube (see Note 13).
4. Run the RT-PCR with the following program:
  - (a) Reverse transcription at 50  $^{\circ}\text{C}$  for 30 min.
  - (b) Initial denaturation step at 95  $^{\circ}\text{C}$  for 2 min.
  - (c) 45 cycles of denaturation at 95  $^{\circ}\text{C}$  for 5 s and annealing/extension at 67  $^{\circ}\text{C}$  for 45 s (see Notes 14 and 15).
5. Determine the cycle threshold (CT) by the reporter fluorescence FAM signal using a threshold of six relative fluorescence units with the Cepheid SmartCycler Software (see Note 16).
6. Since a HCV RNA standard does not exist for WB, generate a calibration curve with plasma samples that are calibrated against the WHO international standard HCV-RNA 96/790 (21) to determine the HCV RNA viral load in IU/mL (see Fig. 1).

### **3.3. Specific HIV RNA Extraction**

1. Prepare approximately 50 pellets of streptavidin-coated beads mix 750  $\mu\text{L}$  of streptavidin-coated cross-linked agarose beads with 83.5  $\mu\text{L}$  1 M trehalose. Arrange drops of 16.67  $\mu\text{L}$  to Parafilm, mix well after every six drops. Dry to pellets

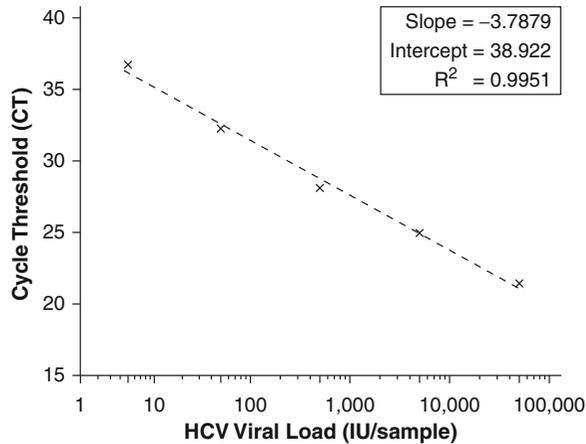


Fig. 1. HCV RNA qRT-PCR calibration curve of 10  $\mu\text{L}$  samples of a calibrated HCV RNA standard plasma panel containing HCV RNA viral loads from 5,000,000 IU/mL to 500 IU/mL. Mean cycle thresholds of five different runs are shown. The linear regression, the coefficient of determination ( $R^2$ ), slope, and intercept of the linear regression are indicated. The efficacy of extraction and amplification ( $E_{\text{assay}}$ ) including a competitive Internal standard RNA is 1.64 (82 %).

overnight at room temperature. These pellets can be stored at ambient temperatures (20–25 °C) in the presence of desiccants without loss of activity (see Note 17).

2. Place one pellet per test into a Mobicol 1 mL column containing a lower filter with 10  $\mu\text{m}$  pore size.
3. Pipette 5  $\mu\text{L}$  of the biotinylated HIV capture probe mix on the bottom of a 1.5 mL safe lock tube, add 125  $\mu\text{L}$  HIV Lysis/hybridization buffer, 25  $\mu\text{L}$  of the whole blood sample, and if available 5  $\mu\text{L}$  of HIV Internal Standard RNA (see Notes 7 and 8).
4. Mix well and incubate the solution at 65 °C for 5 min for optimum denaturation of RNA secondary structures and hybridization to the capture probes with gentle shaking. Take the tubes out of the thermo mixer and shortly spin down (see Note 9).
5. Transfer the lysate on the filter in the column containing the streptavidin-coated sepharose pellets. For the binding of biotin-labeled dimers to the streptavidin-coated beads incubate the suspension for 10 min at 21 °C with shaking (800 rpm) (see Note 10).
6. Remove the supernatant by applying the provided Luer-Lock cap to the column and pressing air through the filter with a syringe. To wash the beads apply 150  $\mu\text{L}$  HIV Washing buffer I to the beads on the filter and remove the supernatant with the help of the syringe (see Note 18). Repeat the washing step once more with 150  $\mu\text{L}$  HIV Washing buffer I, and twice with

150  $\mu\text{L}$  HIV Washing buffer II. Shortly spin down to remove all traces of supernatant for 1 min at  $600\times g$ .

7. Carefully resuspend the beads in 30  $\mu\text{L}$  water, remove the suspension from the filter, quickly transfer it to a tube, and store on ice until subsequent qRT-PCR (see Note 11).

### **3.4. Amplification and Detection of HIV-1 RNA**

1. Freshly thaw the  $5\times$  reaction mix reaction mix (containing dNTP) of the RNA UltraSense One-Step Quantitative RT-PCR kit from  $-20\text{ }^{\circ}\text{C}$  before the preparation of the master mix.
2. Prepare the HIV master mix for a total volume of 100  $\mu\text{L}$  per reaction including 20  $\mu\text{L}$   $5\times$  reaction mix, 5  $\mu\text{L}$  enzyme mix, 2  $\mu\text{L}$  of 10  $\mu\text{M}$  sense primer, 2  $\mu\text{L}$  of 10  $\mu\text{M}$  antisense primer 1, 2  $\mu\text{L}$  of 10  $\mu\text{M}$  antisense primer 2, 2  $\mu\text{L}$  of each of the 10  $\mu\text{M}$  TaqMan probes A and B, and 35  $\mu\text{L}$  water for a total of 70  $\mu\text{L}$  (see Notes 12 and 19). If available add an appropriate concentration of a Cy5-labeled TaqMan probe to detect the HIV Internal Standard RNA and reduce the amount of water accordingly.
3. Add 70  $\mu\text{L}$  the master mix to the 30  $\mu\text{L}$  of bead suspension and transfer it to a 100  $\mu\text{L}$  Cepheid SmartCycler reaction tube. Shortly spin it in to arrange the beads on the edge of the rhomboid reaction tube (see Note 13).
4. Run the RT-PCR with the following program:
  - (a) Reverse transcription at  $50\text{ }^{\circ}\text{C}$  for 15 min.
  - (b) Initial denaturation step at  $95\text{ }^{\circ}\text{C}$  for 2 min.
  - (c) 45 cycles of denaturation at  $95\text{ }^{\circ}\text{C}$  for 1 s, annealing at  $63\text{ }^{\circ}\text{C}$  for 30 s and extension at  $72\text{ }^{\circ}\text{C}$  for 30 s (see Note 14).
5. Determine cycle threshold (CT) by the reporter fluorescence FAM signal using a threshold of six relative fluorescence units with the Cepheid SmartCycler Software (see Note 16).
6. Generate a calibration curve with plasma samples that are calibrated against the HIV-1 first international standard (NIBSC Code 97/656) (22) to calculate the HIV-1 viral load in copies/sample.

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## **4. Notes**

1. We find that adding of 0.05 % (v/v) Tween-20 to the bead buffer reduces agglutination and improves the dispersion of the superparamagnetic beads.
2. If you experience inhibition in qRT-PCR, vary the number of washing steps and/or SDS concentration in washing buffer I

to fully remove PCR-inhibitors in red blood cells, such as hemoglobin (23), until the supernatant is colorless.

3. Decrease the salt concentration in washing buffer II to increase washing stringency. Try replacing ammonium chloride with potassium chloride if you experience problems with qRT-PCR sensitivity (see Table 1, Analytical sensitivity).
4. We tested different streptavidin-coated magnetic beads between 1  $\mu\text{m}$  and 50  $\mu\text{m}$  for indirect capturing and noticed a better extraction efficiency using smaller bead diameters or cavernous beads due to the increased total surface area. Use beads suitable for samples with a high chaotropic salt concentration that show low streptavidin leakage and low nonspecific binding.
5. We added 12.5 pmol hybridization probes to extract HCV RNA from 10  $\mu\text{L}$  samples. To optimize extraction yield try quantities of capture probes different to the recommended 12.5 pmol (ranging between 10 pmol and 500 pmol).
6. Please note that primers and probes directed to other regions of the HCV-genome and to the HIV-genome may work just as well.
7. The provided protocol also works with plasma or serum and with dried blood spots after elution in Lysis/hybridization buffer.
8. To avoid false negative results due to failure of the RNA isolation and/or amplification, we strongly recommend using an Internal Standard RNA containing capture binding sites and primer binding sites. For details on our HCV Internal Standard RNA and the respective Cy5-labeled TaqMan control probe, see Table 1. Besides using Internal Standard RNA as a positive control, we recommend the processing of a virus free whole blood sample as negative control.
9. For simultaneous lysis and indirect capturing of viral RNA in a chaotropic salt buffer we experienced a better extraction yield (lower CT value) at 65  $^{\circ}\text{C}$  than at room temperature. With respect to the nucleotide hybridization probe and its binding site try different hybridization temperatures between room temperature and 70  $^{\circ}\text{C}$  as well as different hybridization times.
10. Make sure that the suspension of streptavidin-coated beads and biotin-labeled nucleic acid hybrids is well mixed. The larger the beads the more shaking is needed to avoid sedimentation of the beads during incubation.
11. Alternatively, the elution of viral RNA from the hybrids is possible with deionized water at 95  $^{\circ}\text{C}$ , but in our hands it resulted in a lower extraction yield (higher CT value and lower limit of detection) than using all the beads as a template for subsequent

qRT-PCR steps. However, using beads as qRT-PCR template carries the risk of PCR inhibition. To validate the amount of inhibition add viral free processes beads to purified target RNA in qRT-PCR.

12. Keep the RNA UltraSense enzyme mix (contains reverse transcriptase, Taq DNA polymerase, ribonuclease inhibitor) at  $-20\text{ }^{\circ}\text{C}$  all the time. You do not need to use the provided bovine serum albumin, magnesium sulfate, and the reference dye in qRT-PCR regularly.
13. In our experience, the reduction of the qRT-PCR reaction volume (i.e., resuspending the beads in a smaller volume and perform the qRT-PCR in  $25\text{ }\mu\text{L}$ ) is possible but impairs the limit of detection, presumably due to a higher concentration of inhibitors in qRT-PCR.
14. Please note that the short denaturation time of 1 s is specifically recommend for PCR reactions performed in a Cepheid SmartCycler. The duration of the denaturation step needs to be adjusted for other thermal cyclers.
15. If you do not use an HCV Internal standard RNA you may also apply a less stringent annealing/extension temperature of  $63\text{ }^{\circ}\text{C}$  and a lower primer concentration of 400 nM instead of 600 nM.
16. Adjust the fluorescence signal threshold settings according to the noise fluorescence using the Cepheid SmartCycler Software to determine the cycle threshold of the reporter signal (FAM fluorescence). The threshold for CT determination should be set up as close as possible to the base of the exponential PCR phase. We used the threshold of six relative fluorescence units for the FAM-labeled target RNA and the threshold of two relative fluorescence units for the Cy5-labeled Internal Standard RNA (see Note 6).
17. Alternatively to the preparation of dried pellets containing streptavidin-coated sepharose beads, it is also possible to work with the bead slurry as provided by the manufacturer. After carefully mixing the slurry, place  $15\text{ }\mu\text{L}$  of slurry into the column, spin down shortly and remove the supernatant. Add  $200\text{ }\mu\text{L}$  of water, spin down again and remove the water. The beads and columns can now be used (continue from step 3 in Subheading 3.2 on specific HIV RNA extraction).
18. Alternatively, the supernatant can also be removed by centrifugation at  $1,500\times g$  for 30 s to 1 min.
19.  $0.2\text{ }\mu\text{L}$  T4 Gene 32 protein (New England Biolabs, Ipswich, MAS, USA) may be added per  $100\text{ }\mu\text{L}$  final qRT-PCR reaction volume to improve the efficiency of reverse transcription.

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## Guidelines for High-Resolution Genotyping of *Chlamydia trachomatis* Using Multilocus Sequence Analysis

Linus Christerson and Björn Herrmann

### Abstract

Chlamydia is one of the most common sexually transmitted infections worldwide and can cause ectopic pregnancies and infertility. It is therefore important to have adequate genotyping tools for investigating the spread of *Chlamydia trachomatis* among the population. Here, we describe a high-resolution multilocus sequence typing (MLST) system able to differentiate closely related clinical strains, which makes it ideal for short-term epidemiology and outbreak investigations. It is based on five highly variable but stable target regions which are PCR amplified and DNA sequenced.

**Key words:** *Chlamydia trachomatis*, Genotyping, High resolution, MLST, Multilocus sequence typing

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### 1. Introduction

Traditional typing of *Chlamydia trachomatis* has been performed using serotyping of the major outer membrane protein (MOMP) and later genotyping of the *ompA* gene encoding MOMP. The strain discrimination is however inadequate for high-resolution epidemiology (1).

Multilocus sequence typing (MLST) involves DNA purification, PCR amplification of several different genetic regions, DNA sequencing, and bioinformatic analysis. It is a widely used technique for bacterial genotyping and its success is partly due to the availability of free analysis tools, such as eBURST, and the practice of making data easily accessible through public online databases (<http://pubmlst.org>) (2).

MLST systems are usually based on slowly evolving housekeeping genes which make them suitable for evolutionary studies. Two such systems have been developed for *C. trachomatis* (3, 4). These two systems however offer a resolution comparable to that of *ompA*, which limits their usefulness in short-term epidemiology, where

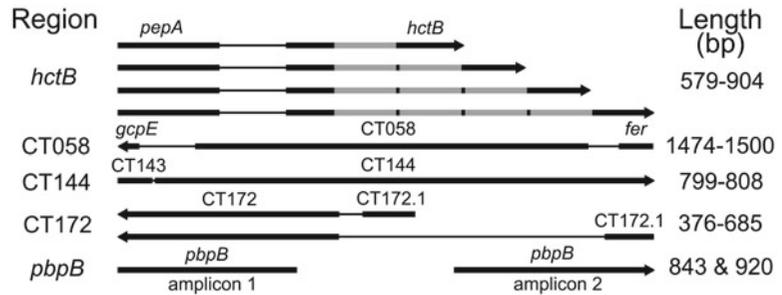


Fig. 1. Schematic overview of the five target regions of the high-resolution MLST system by Klint et al. *Bold lines* represent hypothetical or confirmed coding sequence. *Thin lines* represent noncoding sequence. *hctB* contains repetitive elements and the four major configurations are shown with a *gray color*. CT172 has an insertion/deletion and exists in two distinct length variants. The *pbpB* region has been divided into two amplicons and the middle part is no longer amplified.

high discrimination of strains is essential. Other MLST systems have instead been based on more variable genes to increase the epidemiological resolution. One such system exists for *C. trachomatis* and was developed by Klint et al. (5). There is also a multilocus variable number tandem repeat (VNTR) system, which is based on *ompA* and tandem repeats in three regions (6). This VNTR system offers a resolution similar to that of the MLST system by Klint et al. (7).

The high-resolution MLST system by Klint et al. is based on five highly variable genetic regions that are named after the gene or hypothetical open reading frame that constitute the majority of the region: *hctB*, CT058, CT144, CT172, and *pbpB* (Fig. 1). This MLST system provides threefold higher resolution than *ompA* (5) and has been able to separate a single predominating *ompA* genotype into 15 MLST types (8). DNA sequencing has limitations in detecting mixed infections with more than one genetic variant in a clinical sample, but occasionally such infections are found.

The MLST system by Klint et al. has been used in a variety of studies, including the following: contact tracing in trachoma epidemiology (9), determining the clonal spread of the new variant *C. trachomatis* (nvCT) (8, 10, 11), analysis of the clinical manifestations of urogenital infection (12), investigation of the high incidence of *C. trachomatis* in North Norway (13), and showing clonal spread of lymphogranuloma venereum (LGV) among men who have sex with men (MSM) in Europe (14). The public online database contains 714 specimens and 176 MLST genotypes (<http://mlstdb.bmc.uu.se/>, January 2011). The present chapter deals with the cornerstones of best practice-establishment of an MLST analysis using the example of *C. trachomatis*.

### 1.1. Selection of Target Regions for a MLST Assay

The purpose of the assay decides what kind of target regions is beneficial. As mentioned above housekeeping genes are used for analysis of evolutionary changes and Web sites as [pubmlst.org](http://pubmlst.org) and [mlst.net](http://mlst.net) contain such databases for many bacteria.

For short time epidemiology and outbreak investigations a higher discrimination is often needed and more variable genes should be selected. However, if the typing system is not stable enough due to a high mutation rate, erroneous differences may appear. When selecting target regions for a new typing assay the number of genomes available has been a limiting factor, but this problem is now rapidly decreasing for many bacteria species.

### **1.2. Specimen Quality Is Critical for Successful Genotyping**

In a MLST system aimed at analyzing clinical specimens of limited volume there are restrictions in the possibility to control parameters such as the DNA quality and the copy numbers of target regions. At the same time it is essential to optimize handling of the specimens to achieve sequences.

Our experience is that samples specifically collected and stored for MLST analysis gives the best yield with readable DNA sequences in up to 100 % of the cases. Urine has the advantage that bacteria can be concentrated from several milliliters by centrifugation ( $5,000 \times g$  for 10 min) before DNA extraction and thereby improve the sequencing result. Swab specimens from cervix, vagina, rectum, or conjunctiva are also possible to use and can be concentrated from the transport medium.

An organism load of 10,000 copies per specimen has been shown to result in successful genotyping of all five MLST target regions, but lower numbers may work as well. In case a quantitative estimation of the study specimens is needed copy numbers may be achieved by running a quantitative PCR, e.g., using an in-house PCR (15) or the artus *C. trachomatis* TM PCR Kit (Qiagen, Hilden, Germany).

Recommendations of specimen handling:

1. Minimize storage at room temperature or 4 °C.
2. Long-term storage at -70 °C in tubes with screw caps.
3. Minimize freezing–thawing by aliquoting the specimen.
4. Make sure you have 70 µL or more of purified DNA-solution to allow for reamplification of troublesome specimens.

### **1.3. Controls**

Especially when setting up a new assay, but also for monitoring the quality, some controls are useful:

1. Bacteria. Treatment of a defined suspension of bacteria covers both extraction and amplification of the DNA. The number of bacteria processed shall be high enough to result in reproducible results, but low enough to reveal failing reagents or assay conditions. Typically bacterial solution in the range of 1,000–20,000 cells, alternatively a defined volume of a bacterial suspension giving a cycle of threshold value between 27 and 32 in a quantitative PCR is used.

2. Purified DNA for control of amplification and PCR reagents. The amount needed shall result in a cycle of threshold value between 27 and 32 in a quantitative PCR.
3. Sequence control. A defined target DNA that is expected to give a readable sequence of certain length, typically 500–700 bp.

#### **1.4. Avoid Contamination**

1. Adhere to standard PCR-lab practices to avoid contamination.
2. Use disposable pipette tips with hydrophobic filters.
3. Do the DNA purification, PCR master mix, adding of non-PCR template, and post PCR handling in different rooms if possible.
4. Always wear lab coat and disposable gloves, and change them when moving from one room to another.

#### **1.5. Be Cautious When Analyzing Sequencing Data**

1. Use the online database (<http://mlstdb.bmc.uu.se>) to compare with known variants. Make sure your input sequence cover the complete target sequence.
2. If you do not get a 100 % match with a known variant, use a stand-alone sequence alignment editor. Cautiously compare your sequence with the known variants and manually read the chromatograms in the sequence assembler software on all positions that differ.
3. If you find novel mutations, present only in a single specimen, then the PCR amplification and DNA sequencing needs to be redone to assure that the novel mutations are not PCR artifacts or due to mistakes during DNA sequencing and analysis.

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## **2. Materials**

A list of standard lab equipment that is needed can be seen in Table 1.

### **2.1. DNA Purification**

1. QIAamp DNA Mini Kit (Qiagen) (see Note 1). Store proteinase K at 4 °C and the rest of the kit at room temperature.
2. Fresh 99 % (v/v) ethanol (see Note 2). Store at 4 °C.
3. Urine samples or flocked swabs (e.g., Floq swabs, Copan).

### **2.2. PCR Amplification**

1. HotStarTaq DNA polymerase (Qiagen) (see Note 3). The kit includes polymerase, 10x PCR buffer and 25 mM MgCl<sub>2</sub>. Store at –20 °C.
2. 2 mM dNTP (Fermentas). Store at –20 °C.
3. Primers, as specified in Table 2, in 10 μM working solutions. Store at –20 °C.
4. PCR grade water.
5. DNA samples (see Subheading 3.1).

**Table 1**  
**Standard lab equipment that is needed**

Analysis step		DNA purification (Subheading 3.1)	PCR amplification (Subheading 3.2)	Gel-electrophoresis (Subheading 3.3)	DNA cleanup (Subheading 3.4)	Sequencing PCR (Subheading 3.5)	Ethanol precipitation (Subheading 3.6)
Material							
Pipette 1–10 µL		x	x	x	x	x	x
Pipette 10–100 µL	x	x			x	x	x
Pipette 100–1,000 µL	x	x				x	
PCR-tubes or 96-well plate <sup>a</sup>		x	x		x	x	
1.5 mL eppendorf tubes		x	x		x		
2.0 mL eppendorf tubes (round bottom)		x					
Centrifuge							x
Vortex		x	x			x	
Table centrifuge		x	x				
Thermal heating block		x			x		
PCR-machine					x	x	
Refrigerator 4 °C		x	x	x	x	x	x
Freezer –20 °C			x		x	x	x

<sup>a</sup>Thermo-strip 8 well with cap or Thermo-fast 96 PCR detection plate (VWR International, West Chester, PA, USA)

**Table 2**  
**Primers used for PCR amplification and sequencing**

Region	Primer	Function	Sequence
<i>hctB</i>	hctB39F	PCR, sequencing	5'-CTCGAAGACAATCCAGTAGCAT-3'
	hctB794R	PCR, sequencing	5'-CACCAGAAGCAGCTACACGT-3'
CT058 <sup>a</sup>	CT058:222F	PCR, sequencing	5'-CTTTTCTGAGGCTGAGTATGATTT-3'
	CT058:1678R	PCR, sequencing	5'-CCGATTCTTACTGGGAGGGT-3'
	CT058:811F	Sequencing	5'-CGATAAGACAGATGCCGTTTTT-3'
	CT058:1022R	Sequencing	5'-TAAGCACAGCAGGGAATGCA-3'
CT144	CT144:248F	PCR, sequencing	5'-ATGATTAACGTGATTTGGTTTCCTT-3'
	CT144:1046R	PCR, sequencing	5'-GCGCACAAAACATAGGTACT-3'
CT172	CT172:268F	PCR, sequencing	5'-CCGTAGTAATGGGTGAGGGA-3'
	CT172:610R	PCR, sequencing	5'-CGTCATTGCTTGCTCGGCTT-3'
<i>pbpB1</i> <sup>b</sup>	pbpB:1F	PCR, sequencing	5'-TATATGAAAAGAAAACGACGCACC-3'
	pbpB:823R	PCR, sequencing	5'-CAGCATAGATCGCTTGCTAT-3'
<i>pbpB2</i> <sup>b</sup>	pbpB:1455F	PCR, sequencing	5'-GGTCTCGTTTTTGATGTTCTATTC-3'
	pbpB:2366R	PCR, sequencing	5'-TGGTCAGAAAGATGCTGCACA-3'

<sup>a</sup>CT058 needs two extra primers in the DNA sequencing

<sup>b</sup>*pbpB* is divided into two fragments that are amplified individually

### 2.3. Gel-Electrophoresis

1. Microwave oven.
2. Gel electrophoresis apparatus.
3. UV transilluminator.
4. Agarose for DNA fragment separation, e.g., SeaKem LE (VWR International).
5. Ethidium bromide solution 10 mg/mL. Ethidium bromide is toxic (see Note 4).
6. 5× TBE buffer, pH 8.3 (44.6 mM Tris, 44.5 mM boric acid and 1.2 mM EDTA Titriplex,). Make a 0.5× working solution using distilled water.
7. 6× DNA Loading Dye (e.g., Fermentas). Store at 4 °C.
8. O'GeneRuler Express DNA ladder (Fermentas). Store at 4 °C.
9. PCR product (see Subheading 3.2).

### 2.4. DNA Cleanup (See Notes 5 and 6)

1. FastAP Thermosensitive Alkaline Phosphatase (1 U/μL) (Fermentas). Store at -20 °C.
2. Exonuclease I (Exo I) (20 U/μL) (Fermentas). Store at -20 °C.
3. PCR product (see Subheading 3.2).

### 2.5. Sequencing PCR

1. BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The kit includes BigDyeMix and 5× BigDyeBuffer.

The BigDyeMix should be stored at  $-20^{\circ}\text{C}$  and aliquoted into smaller volumes to minimize freezing–thawing. The 5 $\times$  BigDyeBuffer should be stored at  $4^{\circ}\text{C}$ .

2. PCR grade water.
3. Cleaned PCR product (see Subheading 3.4).

### **2.6. Ethanol Precipitation**

1. Fresh 99 % (v/v) ethanol (see Note 2). Store at  $4^{\circ}\text{C}$ .
2. Fresh 70 % (v/v) ethanol (see Note 2). Store at  $4^{\circ}\text{C}$ .
3. Hi-Di Formamide (Applied Biosystems). Aliquot into smaller volumes to avoid refreezing. Formamide is toxic. Adequate ventilation is important to avoid inhalation. Store at  $-20^{\circ}\text{C}$ .
4. Rubber bands (if you are using PCR tubes).
5. PCR product from sequencing PCR (see Subheading 3.5).

### **2.7. Sequencing**

1. Standard reagents for your ABI sequencing instrument, as instructed by Applied Biosystems.

### **2.8. Analysis**

1. A sequence assembler software, for example DNA Baser (HeracleSoftware, Lilienthal, Germany).
2. <http://mlstdb.bmc.uu.se>.

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## **3. Methods**

Adhere to standard PCR-lab practices to avoid contamination, e.g., use filter-tips, wear gloves and set up the PCR master mix in an area free from template DNA and PCR products. All procedures are carried out at room temperature unless specified otherwise.

### **3.1. DNA Purification (See Note 7)**

1. Centrifuge  $\sim 500\ \mu\text{L}$  sample liquid in a 2 mL Eppendorf tube with round bottom at  $>5,000\times g$  for 10 min. Remove the supernatant with a pipette and resuspend the pellet in  $180\ \mu\text{L}$  ATL buffer that is part of the QiaAmp DNA Mini Kit.
2. Add  $20\ \mu\text{L}$  proteinase K and vortex for 5 s. Incubate at  $56^{\circ}\text{C}$  for 1 h. Centrifuge briefly to remove drops from the inside of the lid.
3. Add  $200\ \mu\text{L}$  AL buffer. Vortex for 5 s. Incubate at  $70^{\circ}\text{C}$  for 10 min. Centrifuge briefly.
4. Add  $200\ \mu\text{L}$  99 % (v/v) ethanol. Vortex for 10–15 s. Incubate at room temperature for 2 min.
5. Add a QIAamp Mini spin column to a 2 mL collection tube. Transfer the solution from step 4 to the column, including possible precipitation, without wetting the rim. Close the cap. Centrifuge at  $>5,000\times g$  for 1 min.

**Table 3**  
**Mastermix composition for the amplification PCR**

Reagent	Volume/ reaction ( $\mu\text{L}$ )	Final concentration
PCR grade $\text{H}_2\text{O}$	12.4	N/A
PCR buffer <sup>a</sup> , 10 $\times$	2.5	1 $\times$
dNTP (2 mM)	2.5	0.2 mM
$\text{MgCl}_2$ (25 mM)	0.5	0.5 mM
Forward primer (10 $\mu\text{M}$ )	1.0	0.4 $\mu\text{M}$
Reverse primer (10 $\mu\text{M}$ )	1.0	0.4 $\mu\text{M}$
HotStarTaq polymerase (5 U/ $\mu\text{L}$ )	0.1	0.5 U
Total (including 5 $\mu\text{L}$ template):	25.0	N/A

<sup>a</sup>Contains 15 mM  $\text{MgCl}_2$

6. Place the column in a new collection tube.
7. Add 500  $\mu\text{L}$  AW1 buffer to the column. Centrifuge at  $>5,000 \times g$  for 1 min. Place the column in a new collection tube.
8. Add 500  $\mu\text{L}$  AW2 buffer to the column. Centrifuge at  $20,000 \times g$  for 3 min. Place the column in a 1.5 mL eppendorf tube.
9. Carefully open the column and add 50  $\mu\text{L}$  AE buffer (or distilled water). Incubate for 5 min at room temperature. Centrifuge at  $>5,000 \times g$  for 1 min.
10. Repeat step 9.
11. Discard the column and store the eluted DNA in the 1.5 mL eppendorf tube and store in refrigerator up to 3 days or at  $-20^\circ\text{C}$  until use.

### 3.2. PCR Amplification (See Note 8)

1. Make a mastermix according to Table 3 (see Note 9) by multiplying the written volumes with  $x.1$ , where “ $x$ ” is the number of reactions you want to have. This will give you an extra 10 % in volume.
2. Add 5  $\mu\text{L}$  of template DNA. Mix by pipetting up and down.
3. Run the PCR according to the program in Table 4.

### 3.3. Gel- Electrophoresis

Gel-electrophoresis is used to check that the PCR amplification has worked before continuing with DNA sequencing. Remember to always wear gloves, since ethidium bromide is a toxic mutagen (see Note 4).

1. Make a 1 % (w/v) agarose solution using 0.5 $\times$  TBE-buffer.
2. Heat the solution in a microwave oven until boiling (see Note 10).

**Table 4**  
**Cycling conditions for the amplification PCR**

Temperature	Time	Cycles	Purpose
95 °C	15 min	1×	Initial activation
94 °C	45 s	40×	Denaturation
60 °C	45 s		Annealing
72 °C	1.5 min		Elongation
72 °C	10 min	1×	Final elongation
4 °C	∞		Cooling

- Let the solution cool off to about 60 °C or until you can hold the container in your hand without getting burned (see Note 11).
- Add 0.1 µg/mL ethidium bromide and stir the solution (see Note 4).
- Pour the gel into a gel rack, insert combs and let the gel solidify (about 20 min at room temperature or 10 min at 4 °C).
- Submerge the gel and gel rack into an electrophoresis tray filled with 0.5× TBE-buffer and remove the combs.
- Mix 5 µL PCR product with 2 µL loading dye and load on the gel.
- Load about 3 µL DNA ladder in one lane on each row.
- Run at 4 V/cm for ~40 min or until the first dye band has migrated at least 5 cm.
- Visualize the gel in UV light and document the results with a photo. A single clearly visible band of expected size normally results in a readable sequence.

### **3.4. DNA Cleanup** **(See Note 5)**

- Take 5 µL PCR product and add 1 µL FastAP Thermosensitive Alkaline Phosphatase and 0.5 µL Exonuclease I. Alkaline Phosphatase removes unincorporated dNTPs while Exonuclease I removes leftover primers.
- Incubate at 37 °C for 15 min and then inactivate the enzymes at 80 °C for 15 min, followed by cooling at 4 °C.

### **3.5. Sequencing PCR**

- Make a mastermix according to Table 5 (see Notes 9 and 12) by multiplying the written volumes with  $x$ , where “ $x$ ” is the number of reactions you want to have. This will give you an extra 10 % in volume. Only use one primer in every reaction.
- Add 2 µL of PCR product. Mix by pipetting up and down.
- Run the PCR according to the program in Table 6.

**Table 5**  
**Mastermix composition for the sequencing PCR**

Reagent	Volume/ reaction ( $\mu\text{L}$ )	Final concentration
PCR grade $\text{H}_2\text{O}$	13.18	N/A
BigDye buffer, 5 $\times$	3.5	0.875 $\times$
Primer (10 $\mu\text{M}$ )	0.32	0.16 $\mu\text{M}$
BigDye mix <sup>a</sup> , 2.5 $\times$	1.0	0.125 $\times$
Total (including 2 $\mu\text{L}$ template):	20	N/A

<sup>a</sup>Includes polymerase, buffer, dNTPs, and ddNTPs

**Table 6**  
**Cycling conditions for the sequencing PCR**

Temperature	Time	Cycles	Purpose
95 °C	90 s	1 $\times$	Initial denaturation
96 °C	10 s	30 $\times$	Denaturation
55 °C	5 s		Annealing
60 °C	1.5 min		Elongation
4 °C	$\infty$		Cooling

### 3.6. Ethanol Precipitation

All centrifugation steps are done at 4 °C.

1. Add 50  $\mu\text{L}$  99 % (v/v) ethanol directly to your PCR tubes or wells of the 96-well plate (see step 3 of Subheading 3.5).
2. Centrifuge at 3,000 $\times g$  for at least 30 min.
3. Immediately after centrifugation has stopped, remove your plate carefully. If you are using PCR tubes you should secure them to their rack with a rubber band before inverting. Flick the plate upside down over a sink to remove the supernatant. Keep the plate or tubes inverted! Put it on some paper towels back into the centrifuge (see Note 13).
4. Centrifuge the plate inverted at 190 $\times g$  for 1 min to remove residual liquid. Such a low speed centrifugation will keep the DNA precipitate attached to the plate.
5. Add 70  $\mu\text{L}$  70 % (v/v) ethanol.
6. Centrifuge at 1,650 $\times g$  for at least 30 min.
7. Repeat steps 3 and 4.

**Table 7**  
**An example of how a table with MLST results looks like**

Sample	MLST region				
	<i>hctB</i>	CT058	CT144	CT172	<i>pbpB</i>
Sample 1	7 <sup>a</sup>	12	1	3	2
Sample 2	7	14	1	3	18
etc...	...	...	...	...	...

<sup>a</sup>The numbers correspond to specific DNA sequences in the database. In this example the two samples differ in CT058 and *pbpB*

8. Incubate in the dark at room temperature for at least 10 min to evaporate residual ethanol.
9. Add 20  $\mu$ L Hi-Di Formamide and incubate in the dark at room temperature for at least 10 min (see Note 14).

### 3.7. Sequencing

1. The obtained sequence reactions are ready to be analyzed on a sequencer instrument according to the manufacturer's instructions. If an appropriate sequencer instrument is not available in your laboratory the reactions can be sent to a commercial DNA sequence service.

### 3.8. Analysis

1. Assemble the sequences from your forward and reverse primers into contigs using a sequence assembler such as DNA Baser (HeracleSoftware, Lilienthal, Germany). Make sure your sequences cover the amplicon by comparing with a reference sequence.
2. Go to the public MLST database located at <http://mlstdb.bmc.uu.se/>. Click the Profiles database and use the options under "Allele sequence queries" to assign MLST genotypes to your sequences (see Note 15). MLST results are compiled as a table with strings of digits representing different allele variants in the different target regions as seen in Table 7.

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## 4. Notes

1. The QIAamp DNA mini kit can be used when purifying DNA from culture, swabs or other biological liquids, urine excluded. When purifying DNA from urine it is instead recommended to use the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany). More automated purification methods might be needed to increase the throughput, for example using a

BioRobot M48 workstation (Qiagen, Hilden, Germany). Alternative DNA purification systems will also work.

2. The concentration of ethanol gradually decreases over time why it is recommended to use relatively fresh ethanol. It is especially important that the 70 % (v/v) ethanol have correct final concentration, since excessive loss of DNA might occur if the concentration is too low.
3. A less error-prone polymerase might be advisable if one does not plan to use the publically available MLST database. Using the database minimizes the risk of PCR introduced artifacts going unnoticed. The first 496 specimens in the database were amplified with the Expand High Fidelity PCR system (Roche Applied Science, Mannheim, Germany).
4. Ethidium bromide is a toxic chemical and a mutagen. Handle with care. Always wear gloves and immediately wash contaminated skin with water. It might be recommended to not work with the stock solution, but to prepare a lower concentration working solution instead. A nontoxic but more expensive alternative to ethidium bromide is staining with GelRed (VWR International, West Chester, PA, USA).
5. Cleanup of the PCR product is recommended to obtain optimal sequence results. However, our experience is that such a procedure has a limited effect and the cleanup after PCR amplification can be omitted.
6. Aliquots containing 2/3 FastAP Thermosensitive Alkaline Phosphatase and 1/3 Exonuclease I can be made to simplify the pipetting and minimize the time the enzymes are in room temperature. Store the aliquots at  $-20^{\circ}\text{C}$ .
7. Before using the AW buffers for the first time an appropriate amount of 99 % (v/v) ethanol needs to be added as indicated on the bottle. If a precipitate has formed in the AL buffer, dissolve it by incubating at  $56^{\circ}\text{C}$ , e.g., by using a water bath. Shake the AL bottle thoroughly before use. At all steps in the procedure it is important to avoid wetting the rim of the column and to avoid touching the membrane with the pipette tip.
8. Further optimization of the PCR amplifications may include nested PCRs to increase the success rate of troublesome specimens and/or making multiplex PCRs to simplify the workflow.
9. Mix all reagents thoroughly before use to avoid uneven concentrations. Add polymerase last and minimize the time it is kept at room temperature by keeping it in the freezer until needed. Always include a negative control. Amplification is preferably done in a 96-well plate if there are many reactions.
10. If you often do gels it might be a good idea to keep liquid gel in an incubator at  $56^{\circ}\text{C}$ . The gel should be consumed within a week to avoid growth of contaminants.

11. Wash the outside of the container with cold water while swirling to speed up the cooling.
12. The protocol for sequencing PCR in Table 3 uses 1/8 of the recommended amount of BigDye Mix. If the sequencing results are unsatisfactory the amount of BigDye Mix can be increased. The total amount of buffer (BigDye Buffer + BigDye Mix) should always be kept at 1×.
13. As soon as the centrifuge stops the DNA pellets start to dissolve, so time is of the essence. If the centrifuge has already stopped you need to recentrifuge to be sure the pellets are stuck at the bottom of the wells. It is important to then keep the tubes or plate inverted so that residual liquid do not go back into the bottom of the wells and dissolve the pellets.
14. To be sure that the DNA pellets have dissolved one can mix by pipetting up and down. Then you can transfer your samples from the PCR tubes to a 96-well plate, or load them directly into the sequencer if they are already in a plate.
15. It is recommended, but not necessary, to use a stand-alone sequence alignment editor such as the freeware program BioEdit (Ibis Therapeutics, Carlsbad, CA). From the Profiles Database in the public MLST database (<http://mlstdb.bmc.uu.se/>) you can download all allele variants in fasta format and align them in BioEdit together with your sequence. If you do not find a perfect match to any of the known variants you have to go back to the sequence assembler and manually read your chromatograms on all positions that differ. If the sequence still appears to be a novel variant you will have to redo the PCR amplification and DNA sequencing to assure that the novel mutations are not PCR artifacts or due to mistakes during DNA sequencing and analysis.

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## Guidelines for the Use of Molecular Tests for the Detection and Genotyping of Human Papilloma Virus from Clinical Specimens

Sin Hang Lee

### Abstract

Accurate genotyping of a human papilloma virus (HPV) isolated from clinical specimens depends on molecular identification of the unique and exclusive nucleotide base sequence in the hypervariable region of a highly conserved segment of the HPV L1 gene. Among other options, a heminested (nested) polymerase chain reaction (PCR) technology using two consecutive PCR replications of the target DNA in tandem with three consensus general primers may be used to detect a minute quantity of HPV DNA in crude proteinase K digestate of cervicovaginal cells, and to prepare the template for genotyping by automated direct DNA sequencing. A short target sequence of 40–60 bases excised from the computer-generated electropherogram is sufficient for BLAST determination of all clinically relevant HPV genotypes, based on the database stored in the GenBank. This chapter discusses the principle and the essential technical elements in performing nested PCR DNA amplification for the detection of HPV from clinical specimens and short target sequence genotyping for HPV, using standard molecular biology laboratory equipment and commercially available reagents.

**Key words:** HPV DNA, Nested PCR, Genotyping, DNA sequencing

### Abbreviations

ATCC	American Type Culture Collection
BLAST	Basic Local Alignment Search Tool
HPV	Human papilloma virus
NCBI	National Center for Biotechnology Information
PCR	Polymerase chain reaction

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## 1. Introduction

Papilloma viruses are members of the papovavirus family and possess a genome of about 8,000 base pairs (bp) consisting of a covalently closed circular DNA molecule. The L1 gene of the human papilloma virus (HPV) encodes the 56–60 kDa major capsid protein, and is about 1,600 bp in size and highly conserved. By definition, a genotype of HPV differs in the L1 gene DNA sequence by at least 10 % from every other known HPV type; HPV subtypes are those having DNA sequence similarities between 90 and 98 % with a prototype in its L1 gene; and variants of an HPV genotype are those having a DNA sequence identity of over 98 % of a prototype (1).

In the practice of laboratory medicine, to sequence the entire ~1.6 kb L1 gene for genotype determination of the HPV DNA detected in each and every positive clinical specimen is cost-prohibitive. Since sequence dissimilarities are unevenly distributed with scattered short sequence homologies between sequence variants of different papilloma virus genotypes positioned in various segments of the L1 gene (2), clinical laboratories usually determine a hypervariable short target DNA sequence in the L1 open reading frame (ORF) of the viral genome for HPV genotyping. Available methodologies for such sequence determination include restriction fragment length polymorphism (RFLP), probe hybridization, and direct Sanger DNA sequencing. Past experience has shown that interpretation of RFLP patterns and relating these patterns to specific HPV DNA sequences are often difficult (3). Probe hybridization methods cannot discriminate between the closely related HPV DNA sequences due to cross-reactivity of the binding nucleotides (4–6). Large differences in the detection and genotyping of HPV among the PCR-based hybridization methods are well known (7). These differences may be attributed to uneven efficiency in DNA amplification of various HPV genotypes in the polymerase chain reaction (PCR) or due to cross-reactivity in probe hybridization. To overcome the problems of cross-reaction, a bimolecular-beacon microarray technology which can discriminate a single-base mismatch by measuring the disruption of fluorescent resonance energy transfer (FRET) between a fluorophore attached to an immobilized DNA strand (“probe”) and a quencher containing a complementary sequence with an artificial mismatch at the site of interrogation (8) has been proposed for reliable HPV genotyping by hybridization (9). However, when used for testing HPV in clinical specimens, this technology may also fail to detect any sequence variants of a probe-targeted HPV genotype whose sequence contains a single-base mismatch with that of the probe because the discrimination against imperfectly matched sequences is absolute.

Facing a huge difference in the HPV positive rates generated by the two currently marketed HPV test kits (10), the US Food

**Table 1**  
**Reports on genotyping of HPV in clinical specimens by DNA sequencing**

Author (first)	Primary PCR primers	Nested PCR primers	Year (References)
Feoli-Fonseca	GP5+/GP6+ MY09/MY11	None None	1998 (13) 1998 (13)
Johnson	MY09/MY11	None	2003 (5)
Asato	L1C1/L1C2	None	2004 (14)
Speich	MY09/MY11 GP5+/GP6+	None None	2004 (6) 2004 (6)
Lee	MY09/MY11	GP5+/GP6+	2007 (15)
Lee	MY09/MY11	GP6/MY11	2009 (16)
Lee	MY09/MY11	GP6/MY11	2009 (17)
Lee	MY09/MY11	GP6/MY11	2009 (18)
Lee	MY09/MY11	GP6/MY11	2010 (19)
Carvalho	MY09/MY11	GP5+/GP6+	2010 (20)

*Notes:* Under Nested PCR primers

None = Primary PCR amplicon used as template for DNA sequencing

GP5+/GP6+ or GP6/MY11 = primer termini of nested PCR amplicon used as template for DNA sequencing

and Drug Administration (FDA) now requires the kit manufacturers “to perform an FDA-approved HPV test that detects the same genotypes as your test, or you may perform PCR plus sequencing (PCR/Sequencing) on your clinical specimens and compare these results to the results of your device (11)”. And the US National Cancer Institute (NCI) has decided to contract a foreign diagnostic laboratory to perform “Reliable genotyping using a PCR system with short target sequences” for testing HPV DNA in the cervical biopsy samples received at the NCI (12). Although DNA sequencing has been sporadically used as a standard tool to validate the genotyping results obtained by other HPV DNA assays, only a few laboratories in the world have developed DNA sequencing procedures for genotyping of HPV in clinical samples (Table 1). Only one of these procedures has been adapted as a routine assay to generate HPV genotyping results for consideration in patient management. This chapter explores the general guidelines in methodology development for the detection of HPV in clinical specimens by PCR and in performing reliable HPV genotyping by short target DNA sequencing of a hypervariable strand of the L1 gene, and shows a sample of procedure which is set up according to these guidelines using commercially available reagents.

## 2. Materials

The technology of HPV DNA detection and genotyping depends on preparation of suitable materials for DNA sequencing. The principle of PCR amplification and direct sequencing for molecular identification of DNA is simple. It involves enzymatic replication of a miniscule quantity of the target DNA molecules to a mass of identical copies for easy visual recognition at gel electrophoresis, and to be used as the template for direct Sanger sequencing. However, to apply this principle for the detection and genotyping of the HPV in clinical specimens needs careful integration of several important technical components, including selection of appropriate materials for the technology.

### 2.1. The Basics

#### 2.1.1. Reagents

1. Proteinase K (USB Corporation, Cleveland, OH, USA).
2. EDTA 0.5 M solution (GE Healthcare Biosciences, Piscataway, NJ, USA).
3. Tween 20 (GE Healthcare Biosciences, Piscataway, NJ, USA).
4. Tris-HCl 2 M solution (USB Corporation, Cleveland, OH, USA).
5. Molecular Biology (MB) grade water (Fisher Scientific, Pittsburgh, PA, USA).
6. Ethidium bromide (Sigma Chemical Co., St. Louis, MO, USA).
7. LoTemp® PCR master mix (HiFi DNA Tech, Trumbull, CT, USA. #8802).
8. Certified PCR Agarose (BioRad, Hercules, CA, USA).
9. 50× Tris/Acetic Acid/EDTA (TAE) buffer (BioRad, Hercules, CA, USA).
10. Nucleic acid sample loading buffer (BioRad, Hercules, CA, USA #161-0767).
11. EZ Load 100 bp Molecular Ruler (BioRad, Hercules, CA, USA).

#### 2.1.2. Working Solutions

1. TE buffer for general dilution of PCR primers and HPV DNA controls:  
(10 mM Tris-HCl, 1 mM EDTA, pH 7.5)

2 M Tris base	0.5 mL
0.5 M EDTA	0.2 mL
MB grade water	70 mL

Adjust to pH 7.5 with 1 N HCl and add MB grade water to 100 mL in a volumetric flask.

2. Tris-HCl buffer for cell washing and proteinase K digestion:  
(50 mM Tris-HCl, 1 mM EDTA, 0.5 % Tween 20, pH 8.1)

2 M Tris base	25 mL
Reagent grade water	800 mL
0.5 M EDTA	2.0 mL
Tween 20	5 mL

Adjust to pH 8.1 with 1 N HCl and add MB grade water to 1,000 mL.

3. Proteinase K digestion fluid:

Stock solution:

Add 10 mg proteinase K to 10 mL Tris-HCl buffer. Invert to mix. Store at -20 °C.

Working solution:

Dilute stock proteinase K 1:10 with Tris-HCl buffer. Good for 1 month at 4 °C.

#### 2.1.3. Oligonucleotide Primers

MY09 primer: (5'-CGTCCMARRGGAWACTGATC-3') in TE buffer (pH 7.5), 10 µM.

MY11 primer: (5'-GCMCAGGGWCATAAYAATGG-3') in TE buffer (pH 7.5), 10 µM.

GP5 primer: (5'-TTTGTTACTGTGGTAGATAC-3') in TE buffer (pH 7.5), 10 µM.

GP6 primer: (5'-GAAAAATAAACTGTAAATCA-3') in TE buffer (pH 7.5), 10 µM.

β-globin F primer: (5'-ACACAACTGTGTTCACTAGC-3') in TE buffer (pH 7.5), 80 µM.

β-globin R primer: (5'-CAACTTCATCCACGTTCCACC-3') in TE buffer (pH 7.5), 80 µM.

(These oligonucleotides are synthesized by Retrogen, Inc., San Diego, CA, USA)

Key to degenerate MY primer nucleotides: M=(A+C), R=(A+G), W=(A+T), Y=(C+T).

#### 2.1.4. Clinical Specimens

In the United States, the clinical specimens submitted to a clinical laboratory for HPV testing are mostly liquid-based cervicovaginal cell suspensions preserved in proprietary solutions provided by commercial suppliers (ThinPrep and Surepath) although cell suspensions preserved in 70–95 % ethanol are equally suitable for HPV DNA amplification by PCR. In general, an aliquot of 5 % of the collected specimen (i.e., about 1 mL of the ThinPrep or 0.5 mL of the Surepath vial volume) is adequate for a PCR assay. Small tissue fragments, not to exceed 1 mm in size, scraped or taken as

biopsies from the surface of a genital wart on the female or male anogenital skin or biopsied from a squamous epithelial tumor of the head and neck region and immediately fixed in 70–95 % ethanol, also have been used successfully for HPV detection. These alcohol-fixed cervicovaginal cell suspensions have no special handling or storage requirements. The small tissue fragments from a solid tumor fixed in ethanol are crushed with the unwrapped end of a sterile swab in a 1.5 mL microcentrifuge tube, and the crushed samples may be treated as cell suspensions for further processing. These clinical specimens are usually processed within 1 month after collection. However, HPV DNA can be readily recovered from archived specimens that have been stored for more than 1 year.

For the test results to be clinically meaningful, cervicovaginal cells must be collected by gynecologists or qualified health care professionals from the transformation zone of the uterine cervix under visual examination. The result of an HPV test on a vaginal specimen self-collected by the patient with a swab does not indicate the HPV infection status of the cervical epithelium.

Specimens collected after application of iodine, containing lubricants, or grossly bloody, should not be accepted for HPV DNA testing.

#### *2.1.5. Sample Preparation for PCR*

Since the quantity of HPV DNA is often minuscule among other nucleic acids in a cervicovaginal cell suspension, replication by primer-directed PCR is needed to increase the number of the target DNA copies to a molecular mass, the size of which would be large enough for visual detection by gel electrophoresis and for genotyping by direct automated Sanger sequencing.

A variety of methods and commercial kits have been used to concentrate and to purify the nucleic acids extracted from the cervicovaginal epithelial cells for PCR amplification (4–6, 20–22). In general, preparation of DNA sample for HPV PCR can be accomplished by classic phenol–chloroform extraction followed by ethanol precipitation (4), or by one of the proprietary commercial DNA extraction kits (5, 6, 20). However, the manufacturer-approved DNA extraction methods might not be the best for use in this assay. Different “front end” protocols have been reported to introduce variability into HPV genotyping results (23).

As the pathology of persistent HPV infection advances from a low-grade intraepithelial lesion (LSIL), which often contains numerous large koilocytes, to a high-grade intraepithelial lesion (HSIL) or a true malignancy, the size of the abnormal cells, their cytoplasm–nucleus ratio, and the viral load per abnormal cell all tend to decrease simultaneously (24, 25). Based on studies of keratinocyte cultures infected by HPV-16, it is estimated that the small cells contain approximately 100 episomal copies of HPV DNA per cell, whereas the large cells contain approximately 3,500 copies per cell (26). Therefore, it is important to be able to recover

a minute quantity of HPV DNA from specimens with a low viral load if the HPV test result is to be used as an aid to evaluate cervical cancer risk. Attempts to purify a small quantity of DNA in a clinical sample may risk losing the target template all together. The use of crude proteinase K digestate without purification for PCR amplification may avoid false negative results due to loss of DNA, and can reduce the cost of sample preparation (15–19).

#### *2.1.6. Optimization of PCR Conditions*

There are more than 200 known genotypes of HPV, and about 40 of them have been reported worldwide to be clinically relevant and detected in the anogenital region with varying frequencies among different human populations. Due to the diversity of the L1 gene DNA sequences in the individual HPV genotypes, the use of type-specific primer PCR to amplify all possible clinically relevant HPV genotypes is impractical and uneconomical. Most scientists in the field use a pair of degenerate general primers or consensus general primers to amplify a short segment of target DNA in the L1 gene for detection of all these clinically relevant HPV genotypes, and then use the PCR amplicon as the template for genotyping by DNA sequencing. As reported in the literature, the most robust and most commonly used PCR primers are the MY09/MY11 primer pair (27) and the GP5/GP6 primer pair (28), or their modified versions.

Successful construction of a practical PCR system capable of amplifying the various sequences derived from all clinically relevant HPV genotypes must integrate many essential factors into the procedure in addition to selecting the appropriate PCR primers. Many interdependent factors need to be optimized to achieve the expected results. The efficiency and specificity of the *in vitro* enzymatic cycle primer extensions need to be balanced in designing a procedure for DNA polymerization. For PCR detection of HPV DNA in clinical specimens, specificity is sacrificed for efficiency. The specificity of target DNA PCR amplification can be validated by DNA sequencing of the PCR amplicon. Theoretically, the efficiency of oligonucleotide priming and extension depends on the kinetics of association and dissociation of the primer–template duplexes at the annealing and extension temperatures, the effects of mismatched bases and their location on duplex stability, and the properties of the DNA polymerase (29, 30). Since a DNA polymerase performs its function in a solution of chemicals, the effects of these chemicals on the kinetics of primer–template association and dissociation should also be considered.

#### *2.1.7. Selection of PCR Polymerase*

The major difference between PCR detection of HPV DNA in clinical samples and other PCR DNA amplifications in general is that the primer–template duplex in an HPV PCR mixture almost always contains base mismatches, which may even be positioned at the 3'-terminus of the primer; for example, when a GP6 primer binds to the HPV-52 L1 gene template (Fig. 1). To amplify a target

HPV Type	ID Locus	GP6 site	Nucleotide Sequence (3' - 5')	MY11 site	Total bases	Location (5' - 3')
6	AF092932	GAAAAATAAAATTTGTAATCA	-----	CCATTGTTATGTCCTGGGC	181	6723 - 6903
11	EU918768	GAAAAATAAACTGTAAATCA	-----	CCATTGTTATGTCCTGAGC	181	6707 - 6887
16	FJ006723	GAAAAATAAACTGTAAATCA	-----	CCATTATTGTGGCCCTGTGC	184	6641 - 6824
18	GQ180792	GAAAAATAAACTGCAAAATCA	-----	CCATTGTTATGACCCTGTGC	187	6558 - 6744
26	NC_001583	GAAATATAAAATTTGTAATCA	-----	CCATTATTATGACCCTGTGC	187	6533 - 6719
31	PPH31A	GAAATATAAAATTTGTAATCA	-----	CCATTATTGTGTCCTGAGC	184	6500 - 6683
32	X74475	GAAATATAAACTGTATATCA	-----	CCATTATTGTGGCCTGTGC	181	6817 - 6997
33	EU_918766	GAAAAACAACTGTAGATCA	-----	CCATTATTATGACCCTGTGC	181	6542 - 6722
35	PPH35CG	GAAAAATAAACTGTAAATCA	-----	CCATTATTATGGCCTGTGC	184	6522 - 6705
39	PPHT39	GAAATATAAAATTTGTAATCA	-----	CCATTGTTGTGGCCCTGGGC	187	6585 - 6771
40	X74478	GAAAAATAAACTGCAAAATCA	-----	CCATTGTTATGGCCCTGGGC	187	6718 - 6904
44	HPU31788	GAAACATAAAATTTGTAAGTCA	-----	CCATTATTGAGGCCCTGCGC	187	6627 - 6813
45	EF202167	GAAAAATAAACTGTAAATCA	-----	CCATTGTTATGGCCCTGGGC	187	6562 - 6748
51	PPHDNA	GAAAAATAAAATTTGCAATCA	-----	CCATTATTGTGACCCTGCGC	184	6466 - 6649
52	GQ472848	GAAAAATAAAATTTGTAATCG	-----	CCATTATTGTGGCCCTGCGC	181	6631 - 6811
53	NC_001593	GAAACACAAATTTGTAATCA	-----	CCATTATTATGTCCTGGGC	181	6594 - 6774
54	AF436129	GAAATATAAAATTTGTAATCA	-----	CCATTGTTCTGACCCTGGGC	181	6509 - 6689
55	HPU31791	GAAACATAAACTGTAAAGTCA	-----	CCATTATTGTGGCCCTGCGC	187	6625 - 6813
56	X74483	GAAAAACAAATTTGTAATCA	-----	CCATTATTATGGCCTGGGC	181	6539 - 6719
58	FJ407217	GAAAAACAACTGTAAAGTCA	-----	CCATTGTTATGACCCTGTGC	181	6588 - 6768
59	EU918767	GAAATATAAACTGCAAAATCA	-----	CCATTGTTTAAACCCTGAGC	187	6551 - 6737
61	HPU31793	GAAAAATAAAATTTGCAAAATCA	-----	CCATTGTTGTGGCCCTGGGC	184	6712 - 6895
62	AY395706	GAAATATAAAATTTGCAAAATCA	-----	CCATTATTATGGCCCTGCGC	181	6725 - 6905
66	EF177191	GAAACACAACTGTAGTTCA	-----	CCATTATTATGGCCCTGTGC	181	6590 - 6770
67	D21208	GAAATATAAACTGCAAAATCA	-----	CCATTGTTATGACCCTGTGC	181	6564 - 6744
68	EU918769	GAAATATAAAATTTGCAAAATCA	-----	CCATTGTTGTGTCCTGTGC	187	6453 - 6639
69	AB027020	GAAATATAAACTGTAAATCA	-----	CCATTATTATGACCCTGGGC	187	6489 - 6675
70	HPU21941	GAAATATAAAATTTGTAATCA	-----	CCATTATTGTGTCCTGGGC	187	6529 - 6715
71	AY330623	GAAATATAAAATTTGCAAAATCA	-----	CCATTGTTTGTGCCCTGTGC	181	6791 - 6971
72	X94164	GAAATATAAACTGCAAAATCA	-----	CCATTGTTGTGACCCTGGGC	184	6738 - 6921
73	X94165	GAAAAACAACTGTAAATCA	-----	CCATTATTGTGTCCTGTGC	190	6433 - 6622
74	AF436130	GAAAAATAAAATTTGCAAAATCA	-----	CCATTATTGTGGCCTGTGC	190	6524 - 6713
81	AJ620209	GGAAAATAAACTGCAAAATCA	-----	CCATTATTATGGCCCTGTGC	181	6809 - 6989
83	AF151983	GCAATATAACCTGTAAAGTCA	-----	CCATTATTATGTCCTGGGC	180	6693 - 6872
84	AF293960	GGAAATATAAACTGCAAAATCA	-----	CCATTGTTATGACCCTGGGC	181	6619 - 6799
86	AF349909	GAAAAATAAAATTTGCAAAATCA	-----	CTATTATTGTGTCCTGCGC	181	6615 - 6795
87	AJ400628	GAAAAATAAACTGTAAATCA	-----	CCATTATTGTGGCCCTGGGC	181	6712 - 6892
89	AF436128	GGAAATATAAACTGTAGTCA	-----	CCATTATTATGGCCCTGTGC	180	6706 - 6885
90	AY057438	GGAAAATAAACTGCAAAATCA	-----	CCATTGTTAGGCCCTGTGC	181	6746 - 6926
91	AF419318	GAAATATAAACTGTAAATCA	-----	CCGTTATTATGCCCTGTGC	187	6810 - 6996

Fig. 1. HPV L1 gene base heterogeneity at the GP6 and MY11 primer-binding sites. *Footnote:* This chart shows the native sequences at the GP6 and MY11 primer-binding sites of the 40 HPV genotypes isolated from cervicovaginal cells of patients in southern Connecticut, USA. Data were retrieved from the National Center for Biotechnology Information (GenBank). Note: All GP6 primer-binding sites end as 5'---TCA 3' except for HPV-52. All MY11 primer-binding sites end as 3' CCATT---5', except for HPV-86 and HPV-91.

DNA under this condition, one of the important factors that affects PCR product yield is the relative efficiency with which the DNA polymerase extends from a mismatched primer-template duplex (29, 30). Once extension from a mismatched primer occurs, the resultant product and the complement synthesized in subsequent cycles are fully matched with both primers (29). In the PCR mixture, the DNA molecules with termini defined by both primers accumulate exponentially. On the other hand, products of extensions from the native template have only one defined terminus and accumulate linearly (29).

Selective amplification of a minute quantity of target HPV DNA among a complex DNA pool—as in a crude cellular proteinase

K digestate—may be greatly facilitated by a polymerase with high processivity and a 3′–5′ exonuclease proofreading activity, such as a *Bacillus stearothermophilus* (*Bst*) DNA polymerase, which has been genetically modified by DNA recombination and site-directed mutagenesis (31). However, the latter enzyme is rapidly inactivated at temperatures above 75 °C—a temperature far below 94 °C that is required for thermal DNA dissociation for PCR amplification. To use the modified *Bst* DNA polymerase for PCR requires a high concentration of melting agents in the reaction mixture, which is inconvenient for clinical laboratory applications. One of the proprietary DNA polymerases (15–19) that shares many of the essential characteristics of the modified *Bst* DNA polymerase and is more heat-tolerant is preferred. This new moderately thermostable enzyme in working solutions can withstand repeated heating to 85 °C in the presence of protective agents and is stable at 4 °C, as well as room temperature, for several weeks and even months. It is supplied as a ready-to-use low temperature PCR master mix with its magnesium and dNTP concentrations optimized for a broad range of high-efficiency and high-specificity primer extensions. According to the manufacturer, proprietary melting agents have been added to force double-stranded DNA strands to dissociate at 85 °C. The optimal temperature for enzymatic primer extension is 65 °C. The annealing temperature can be set at 40–50 °C. No hot start is necessary. For amplification of target DNA in crude clinical samples, a low ramp rate (0.9 °C/s) in the thermal cycling program is selected for its optimal function. This DNA polymerase is about ten times more efficient than *Taq* DNA polymerase in amplifying HPV-16 plasmid DNA by single-run MY09/MY11 PCR, and about 100–1,000 times more efficient when the first MY09/MY11 PCR product is reamplified by a GP5+/GP6+ nested PCR in tandem. Nested PCR with this DNA polymerase can detect 1–10 copies of plasmid DNA of HPV types-16, -18 or -6B purchased from ATCC (15).

To achieve maximum sensitivity and specificity in PCR amplification of a target DNA with a pair of specific primers, the concentrations of the DNA polymerase, the magnesium, and the dNTPs in the final reaction mixture must be individually optimized after careful titration of each component. However, in a clinical laboratory such individualized PCR optimization for every target template is impractical. For convenience, a PCR master mix may be selected for amplification of all target DNA templates. Amplification of the HPV sequences with a low  $T_m$  of primer–template combination may be accomplished by lowering the annealing temperature of the thermal cycling program to 40 °C. For amplification of other templates, such as the DNA extracted from *Chlamydia trachomatis*, *Neisseria gonorrhoeae* (32), and *Borrelia burgdorferi* (33), the annealing temperature can be simply raised for more specific primer extensions.

Most clinical laboratories perform HPV DNA PCR amplification using a *Taq* DNA polymerase. For PCR, it is important to determine the appropriate concentrations of the enzyme, the magnesium chloride and the dNTPs for the chosen *Taq*, with or without cosolvents added to the final reaction mixture.

All thermophilic DNA polymerases have an absolute requirement for divalent cations. Optimal activity of *Taq* DNA polymerase is obtained with 10 mM Mg<sup>2+</sup> (34). However, for PCR amplification of HPV DNA an optimal *Taq* activity may not be desirable. Using a pair of GP/MY primers (35) which is a modified version of the MY09/MY11 primers for study, the commonly used concentration of 1.5 mM of magnesium chloride has been found to be efficient for PCR amplification of HPV-16 and HPV-18, but sub-optimal for amplification of HPV-31, HPV-33, HPV-35, or HPV-56 (36). Increasing the magnesium chloride concentration to 3 mM leads to optimal detection for most HPV genotypes, but is associated with a poor signal-to-noise ratio and frequent false positive results at gel electrophoresis (36). One of the choices in optimization of PCR conditions for HPV detection is to maintain the magnesium concentration at 1.5 mM for all genotypes to reduce nonspecific amplifications while using a GP5+/GP6+ nested PCR to increase the sensitivity of detection (6). On the other hand, some scientists have found it necessary to increase the concentration of magnesium chloride to 2 mM in the MY09/MY11 primary PCR mixture, and to 2.5 mM in the GP5+/GP6+ nested PCR mixture for a high amplification efficiency (5). Sample purification is required for all *Taq* PCR amplifications of HPV DNA in clinical specimens (5, 6, 20–23).

HPV detection rates differ when different DNA polymerases are used. For example, a *Taq* nested PCR containing 2 mM magnesium chloride may fail to detect four of seven (4/7) HPV isolates in clinical specimens whereas all of the seven specimens have been confirmed to contain various genotypes of HPV by a low temperature PCR polymerase procedure (Fig. 2a, b). This example also shows that there is no apparent difference in PCR amplification between the two PCR systems when purified plasmid HPV-16 control is used as the template for amplification. The low efficiency of HPV amplification by certain polymerases becomes obvious only when clinical materials, especially if not purified, are used as the starting PCR templates. As shown in this example, the *Taq* PCR polymerase failed to detect one of the two HPV-16 isolates in crude clinical sample digestates.

### 2.1.8. Selection of PCR Primers

The MY09 and MY11 degenerate primers (27) were originally designed in a conserved region of the L1 open reading frame with the intent of amplifying the DNA of HPV-6, HPV-11, HPV16, HPV-18, and HPV-33 in a single PCR. Degenerate base sites were included into the primers to accommodate the nucleotide base

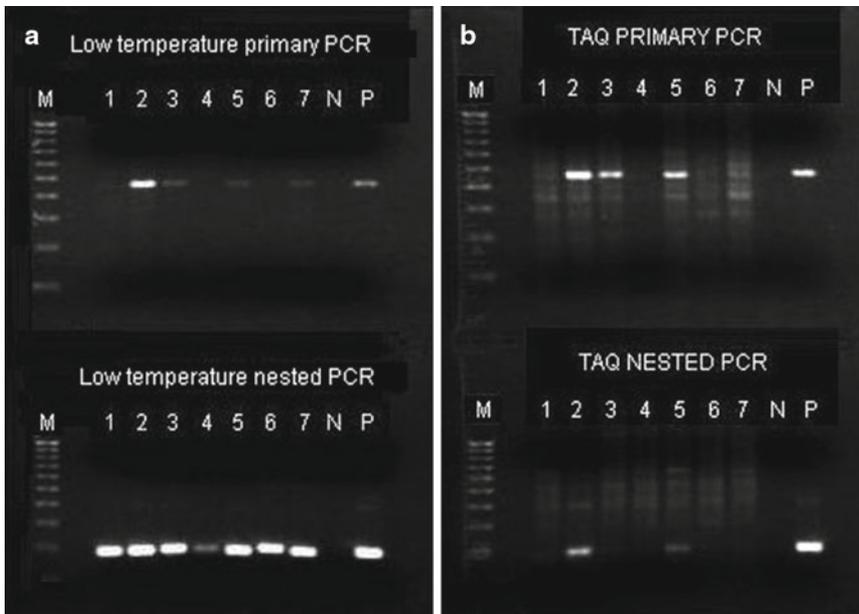


Fig. 2. (a) HPV detection in clinical samples by a low temperature PCR polymerase. *Footnote:* Agarose gel electrophoresis plate showing detection of HPV DNA in 1  $\mu$ L of crude proteinase K digestate of liquid-based cervicovaginal cytology specimen from seven patients by a low temperature nested PCR. Each PCR mixture contained forward primer 1  $\mu$ L, reverse primer 1  $\mu$ L, PCR master mix 20  $\mu$ L, template, and water added to final volume 25  $\mu$ L. Primary PCR primers were MY09/MY11, and the nested PCR primers GP6/MY11. Primary PCR products were transferred by micro-glass rods. All nested PCR amplicons were validated by DNA sequencing: Lane 1, HPV-16; Lane 2, HPV-16; Lane 3, HPV-39; Lane 4, HPV-68; Lane 5, HPV-54; Lane 6, HPV-59; Lane 7, HPV-54; N, negative control; P, plasmid HPV-16 DNA control; M, molecular ruler. *Note:* Primary PCR did not show a visible PCR amplicon in 3 of 7 HPV-positive specimens. Thermocycling steps: initial heating at 85  $^{\circ}$ C for 10 min, followed by 30 cycles, each set at 85  $^{\circ}$ C for 30 s, 40  $^{\circ}$ C for 30 s, and 65  $^{\circ}$ C for 1 min. The final extension was 65  $^{\circ}$ C for 10 min. (b) HPV detection in clinical samples by a *Taq* PCR polymerase. *Footnote:* Comparative parallel primary and nested PCR detection of HPV DNA in 1  $\mu$ L crude proteinase K digestate from the seven patients as for Fig. 2a, using a *Taq* DNA polymerase. The *Taq* PCR protocol: GE *rTaq* DNA polymerase, 2.5 units in 0.25  $\mu$ L, was used in each PCR with 10 $\times$ PCR buffer 2.5  $\mu$ L, 25 mM MgCl<sub>2</sub> 2  $\mu$ L, 5 mM dNTPs 1  $\mu$ L, forward primer 1  $\mu$ L, reverse primer 1  $\mu$ L, template, and water added to final volume 25  $\mu$ L. The nested PCR mixture contained the same ingredients. Primary PCR products were transferred to nested PCR tubes by micro-glass rods as described in this chapter. Primary PCR primers were MY09/MY11 and the nested PCR primers GP6/MY11, as for Fig. 2a. Thermocycling steps were set as preheating for 1 min at 94  $^{\circ}$ C, followed by 30 cycles, each set at 94  $^{\circ}$ C for 30 s, 54  $^{\circ}$ C for 2 min and 72  $^{\circ}$ C for 1 min. The final extension was 72  $^{\circ}$ C for 10 min for both primary and nested PCR. *Note:* Although the *Taq* PCR failed to detect HPV DNA in four of the seven unpurified clinical samples known to be positive for HPV, it amplified pure plasmid HPV-16 DNA quite efficiently (lane P).

heterogeneity in this region among these five genotypes. The sequences of other HPV genotypes were not known at that time. The resultant degenerate primers, commonly referred to as the MY09 and the MY11, are actually composed of a mixture of 24 unique oligonucleotide sequences in variable proportions which may differ from lot to lot. When used properly, these two degenerate primers are capable of amplifying almost all known HPV genotypes, with varying degrees of efficiency. Some scientists added an extra sequence-specific HMB01 oligonucleotide to the MY09/MY11 primer pair (35, 37), specifically targeting HPV-51 amplification.

However, when a moderately thermostable DNA polymerase was used for nested PCR amplification, there appeared to be no need to add another nucleotide just for HPV-51 amplification (18, 19) because the degenerate primers have a 75 % perfect base match at the MY09 binding site (Locus PPHDNA 6898-6917) and a 90 % perfect base match at the MY11 binding site (Locus PPHDNA 6466-6485). In enzymatic extension of DNA duplexes with a low  $T_m$  of primer–template combination, the properties of a DNA polymerase may play a significant role (29, 30).

During the early phase of PCR, when the starting templates are low in concentration or have a low primer–template  $T_m$  due to base mismatches, a processive DNA polymerase is crucial to complete replication of the template strands which are needed to allow subsequent rounds of exponential amplification (29, 30, 38). To enhance the processivity of thermostable DNA polymerases has been an ongoing quest for many researchers with only limited success (38). In general, DNA polymerases with a lower heat tolerance are more processive than those with high heat resistance (39).

The ~450 bp amplicon of the primary MY09/MY11 PCR may or may not be visible at gel electrophoresis. For high-efficiency HPV detection a nested (or heminested) PCR is necessary in which the products of the first PCR that may contain the amplified target DNA are transferred to a new second PCR reaction mixture with at least one of the two primers positioned internal to the MY09 and MY11 termini of the first amplicon. Usually, one or two of the GP5 and GP6 primers or their elongated version referred to as the GP5+ and GP6+ are used as the new primer(s) in the second PCR. However, the shorter GP5/GP6 primer pair is more efficient in generating nested PCR products for detection and for DNA sequencing. It is well known that reducing the length of the primers used in PCR leads to higher efficiency in duplex extension. For HPV DNA amplification, a heminested GP6/MY11 combination as the routine nested PCR primer pair is preferred because the degenerate MY11 primer covers a broader spectrum of sequence variants at its priming site than the GP5 primer. For example, all three—MY09/MY11, GP5+/GP6+, and GP5/GP6—primer pairs are able to amplify the standard plasmid HPV-6, HPV-16, and HPV-18 DNA purchased from ATCC, but fail to amplify the plasmid HPV-11 DNA under identical conditions. As a result, the ATCC purified plasmid DNA of HPV-6, HPV-16, and HPV-18, but not that of HPV-11, should be used as standard controls for method development (15). The plasmid DNA of HPV-11 maintained at the ATCC can only be successfully amplified by a pair of GP6+/MY11, or GP6/MY11, primers, in a primary PCR or in a nested PCR setting. Failures to amplify various HPV genotypes in clinical specimens by the GP5+/GP6+ primers are well known (15, 37, 40). In particular, the failure to detect HPV-52 in clinical specimens by GP5+/GP6+ PCR has raised special concerns because

HPV-52 is considered to be a common “carcinogenic” genotype (37, 40, 41). HPV-52 has been confirmed by a GP6/MY11 nested PCR system to be the second most prevalent “high-risk” HPV genotype in a representative US female population (18, 19).

The combined use of an optimized PCR system and the GP6/MY11 nested PCR primer pair has facilitated generation of a suitable PCR amplicon for direct DNA sequencing of all clinically relevant HPV genotypes (17–19), in spite of a 3′-terminal mismatch in the GP6/HPV-52 duplex (Fig. 1). Using MY11 as one of the primers for both HPV primary and nested PCR amplifications in a heminested setting has the added advantage that the primer–template duplex at the MY11 binding site is fully matched in the second PCR run because both termini of the template molecule in the nested PCR have been largely defined by the MY09/MY11 primers at the primary PCR (29). The GP6/MY11 heminested PCR may have contributed to a higher rate of HPV-52 detection (18, 19).

Occasionally, the MY09/MY11 primary PCR product of an HPV isolate may not be reamplified by the GP6/MY11 nested PCR primers—presumably due to mutation at the GP6 binding site—and needs a GP5/MY09 nested PCR primer pair for amplification (15, 18). Fortunately, these incidents are relatively uncommon.

It is still possible that some sequence variants of clinically relevant HPV genotypes may evade detection by both the MY09/MY11 primary and the GP6/MY11 nested PCR. To detect these theoretical variants, a second nested PCR with a GP5/MY09 primer pair would have to be added to the procedure to run in parallel with the GP6/MY11 nested for every clinical specimen.

The regions of the MY09, MY11, GP5 and GP6 general primer-binding sites are the most conserved of the open reading frame of the L1 gene. Since these PCR primers are consensus general primers, the sensitivity in the detection of different HPV genotypes varies greatly. The sensitivity in detecting HPV-16 is the highest, with a detection limit of 1–5 copies of HPV DNA per PCR. For clinical specimens, under low stringency PCR conditions, an initial primary MY09/MY11 PCR amplification to be followed by a GP6/MY11 or GP5/MY09 nested (heminested) PCR can detect 40 clinically relevant anogenital genotypes of HPV, including HPV-6, 11, 16, 18, 26, 31, 32, 33, 35, 39, 40, 44, 45, 51, 52, 53, 54, 55, 56, 58, 59, 61, 62, 66, 67, 68, 69, 70, 71, 72, 73, 74, 81, 83, 84, 86, 87, 89, 90, and 91, all confirmed by short target sequence genotyping, including some new sequence variants of these genotypes when the mutation occurs in the inter-primer region. However, it is not known if mutations which occur in one of the primer-binding sites, especially in the region of the GP5- and GP6-binding sites, may lead to failure of the final nested PCR, thus causing a false negative result.

*2.1.9. Nested PCR  
Products as Sequencing  
Template*

For a typical nested PCR, two pairs of primers are used for amplification of a target DNA in two consecutive runs of PCR, referred to as the primary PCR and the nested PCR, in tandem. The primary PCR primers form two primer–template duplexes located on the outside (upstream) of the target DNA. After the first PCR run, a small fraction of the reaction mixture is transferred from the first PCR tube to a second PCR mixture containing a pair of nested PCR primers, which now form two new primer–template duplexes located internal to (downstream of) the termini of the primary PCR amplicon. If an amplicon containing the target DNA sequence is present in the primary PCR mixture, the amplicon will be reamplified, yielding a nested PCR amplicon which is smaller in size than the primary PCR amplicon and readily recognized at gel electrophoresis. Therefore, the nested PCR technology greatly increases the efficiency and specificity of *in vitro* target DNA replication by PCR. In hemi-nested PCR, one of the two termini of the nested PCR amplicon is defined by an oligonucleotide which has been used as a primer to amplify both the primary and nested PCR products.

In general, a significant portion of the cost in performing PCR/DNA sequencing in a clinical laboratory is the expense for sample preparation. DNA purification and quantification are standard steps to prepare templates for PCR and for the Sanger sequencing reaction.

Other than increasing the efficiency in HPV detection, the nested PCR technology has an added advantage in eliminating the need for “front end” sample purification if an efficient DNA polymerase is selected for the cycle primer extensions. When the conditions are optimized, a proteinase K crude cell digestate without DNA purification can be suitable for PCR detection of HPV and for preparation of sequencing templates. For example, in performing nested PCR in a total volume of 25  $\mu\text{L}$  per reaction, the non-target DNA and impurities in the crude digestate are diluted by 625-fold ( $25 \times 25$ ). The trace of irrelevant DNA carried over from the diluted digested sample does not interfere with the Sanger sequencing reaction since the overwhelmingly dominant DNA is that of the nested PCR amplicon, now being used as the sequencing template. When 100 copies of plasmid HPV-16 DNA in 1  $\mu\text{L}$  of proteinase K digestate of cervicovaginal cell suspension are used for MY09/MY11 primary PCR, 1  $\mu\text{L}$  of the primary PCR products even after further serial dilutions in water to 1,024-fold is always sufficient as the template for nested PCR amplification. An aliquot of 0.05–1.0  $\mu\text{L}$  of the nested PCR products, without further purification, is adequate as the target template for a direct automated Sanger sequencing reaction.

*2.1.10. Cross-  
contamination Control  
in Nested PCR*

The greatest danger that may threaten the life of a clinical molecular diagnostic laboratory is contamination by target DNA PCR amplicons. In performing diagnostic PCR an identical target DNA

strand from each positive specimen is repeatedly replicated by the same pair of primers over and over again in a confined working space. Billions or trillions of copies of identical target DNA strands are replicated exponentially in each PCR every day. If these target DNA amplicons are discharged accidentally into the circulating air in the laboratory or are allowed to contaminate the reagents and laboratory equipment, especially the micropipettors, the consequence can be disastrous because every contaminating target DNA molecule can be amplified to a mass of identical target DNA strands in any sample PCR mixture, leading to false positive results.

The nested PCR/DNA sequencing technology carries an even greater danger of target DNA cross-contamination because all post-amplification PCR products are routinely transferred from micro-test tube to micro-test tube for reamplification and for analysis by gel electrophoresis or by a Sanger sequencing reaction. The rates of cross-contamination can be so high in some laboratories that a few scientists have openly questioned the reliability of all positive test results generated by nested PCR (42). As a result, very few scientists use nested PCR amplicons as templates for their HPV genotyping by DNA sequencing even though they recommend nested PCR for its higher sensitivity in detection of the HPV DNA in clinical specimens (5, 6, 13).

However, sample cross-contamination is not an inherent part of the nested PCR technology. It is rather a function of the clinical laboratory that performs PCR. Cross-contamination in a PCR laboratory can be readily monitored and largely eliminated by meticulous selection of technical staff, proper training, implementation of strict operational rules, and elimination of all micropipetting procedures that may induce aerosol of PCR amplicons. Naturally, all PCR products must be transferred within a special workstation equipped with ultraviolet light to destroy any unwanted DNA contaminants in the station.

Nested PCR can be performed in 0.2 mL PCR tube-strips with individually attached caps. After all components for the primary PCR are added and mixed, the PCR tubes are briefly centrifuged to collect the fluid in the bottom of each tube before the PCR tubes are placed into the thermocycler so that there is little or no reaction mixture caught between the cap and the rim of the PCR tube. This step is important to prevent amplicon aerosol when the cap is opened for transferring the primary PCR products to the nested PCR tubes after the primary PCR thermal cycling is completed. A folded piece of disposable tissue paper may be laid over the caps of the PCR tubes, placing the paper between the opening finger and each cap, when the caps are being opened. This maneuver is to prevent any PCR product droplets from getting into the air or from contaminating the adjacent test tubes as the individual caps are opened.

Transferring of the primary PCR products to the nested PCR tubes, or the nested PCR products to the Sanger sequencing

reaction tubes, can be accomplished by using a disposable wettable micro-glass rod of ~1.2 mm in diameter. The micro-glass rod carrying a trace of the PCR products is always removed from or inserted into the mixture in the PCR tubes perpendicularly without touching the rim or the cap of any tube. Mixing of the new reaction mixture in each nested PCR tube and Sanger sequencing reaction tube is accomplished by stirring the contents with the micro-glass rod in a twisting motion. The rims and caps of all nested PCR tubes and the tubes for Sanger sequencing reaction are always kept dry to reduce the chance of liquid aerosol, which might occur when the caps are opened and closed if the under surface of the cap is wet. With these technical precautions, sample cross-contamination in nested PCR can be practically eliminated. After training, a technologist may be required to pass a final practical proficiency test with a 100 % correct result before being allowed to perform unsupervised PCR. This in-house proficiency test may consist of performing nested PCR on a single batch of 50 simulated samples, about one third (1/3) of which have been randomly spiked with a target DNA (33).

A “trace” of the nested PCR product carried on the surface of a micro-glass rod, equivalent to 0.2–0.5  $\mu$ L in volume, is adequate as the template for a Sanger sequencing reaction. When the clinical specimen yields an unusually thick band on the MY09/MY11 primary PCR gel—which indicates a very high load of target DNA in the clinical sample—its nested PCR product can be diluted 1/10 in water before being transferred by micro-glass rod for Sanger DNA sequencing reaction to avoid template overload.

#### 2.1.11. Sample Adequacy Assurance

Since HPV is an intracellular infective agent, whose hosts may be the basal cells, the metaplastic cells, the precancerous cells and cancer cells of the cervical epithelium, almost always showing certain cytopathologic characteristics, one assurance of sample adequacy is to demonstrate that these morphologically abnormal host cells are indeed included in the materials submitted for HPV testing. However, in practice, this cytological proof is difficult to obtain in most molecular diagnostic laboratories because the concurrent Pap cytology test is invariably performed in another department.

The concentration and quantity of HPV DNA in a purified preparation are usually too low to measure in a clinical laboratory to assure specimen adequacy. All purification processes are associated with loss of target DNA. As a result, PCR amplification of a host cell DNA, for example, part of the  $\beta$ -globin gene, which is present in all human cells at one copy per cell, is customarily used as a surrogate molecular internal control for specimen adequacy (4–6, 20). It is generally assumed that if the  $\beta$ -globin gene is amplified by a pair of specific PCR primers, the sample is considered adequate for evaluation because the cells in the specimens are primarily epithelial cells at the transformation zone of the

uterine cervix. A 110 bp DNA segment of the  $\beta$ -globin gene can be amplified by a pair of specific primers (43).

Therefore, three polymerase chain reactions are needed for each clinical specimen received for HPV testing, one for MY09/MY11 primary PCR, one for GP6/MY11 nested PCR, and one for  $\beta$ -globin gene PCR with their amplicons measuring ~450, ~185 and ~110 bp in size, respectively, at gel electrophoresis.

Since one infected host cell may harbor hundreds or thousands of copies of HPV genomic DNA, and each epithelial cell only has one copy of  $\beta$ -globin gene, some hypocellular clinical specimens may be positive for HPV by nested PCR without demonstrable  $\beta$ -globin gene amplification. These samples are obviously adequate for HPV detection even in the absence of  $\beta$ -globin gene amplification.

Current world literature seems to indicate that human cervical precancerous or cancerous cells must contain HPV (44), and that all truly HPV-negative specimens are free of high-grade precancerous or cancerous cells (45). However, there are very few studies examining the HPV status of women with negative cytology. Data from individual laboratories seem to suggest that the status of endocervical/transformation zone component in a cytology sample and its HPV DNA positivity appear to be independent (46). For HPV detection, relying on the presence of normal endocervical cells or cells from the transformation zone as the criteria for specimen adequacy may not be appropriate.

## **2.2. Equipment and Supplies for PCR**

1. Microcentrifuge 5424 w/rotor 022620444 (Eppendorf North America, Westbury, NY, USA).
2. Microcentrifuge 5430 w/2-place plate rotor 022620568 (Eppendorf North America, Westbury, NY, USA).
3. Mini Spin plus with Rotor F-42-12-11 (Eppendorf North America, Westbury, NY, USA).
4. Micropipettors covering the full range of 1–1,000  $\mu$ L (Eppendorf North America, Westbury, NY, USA).
5. pH meter.
6. Incubator set for 45–55 °C.
7. Heat Block Model BR6B for 1.5 mL tubes set to 95–99 °C (Laboratory Supplies Co., Inc., Hicksville, NY, USA).
8. Sub-Cell GT UV-Transparent Mini Gel170-4436 (BioRad, Hercules, CA, USA).
9. Electrophoresis transformer and cell (BioRad, Hercules, CA, USA).
10. Cassettes and combs for agarose gel slab formation and electrophoresis (BioRad, Hercules, CA, USA).
11. 15-Well Comb170-4465 (BioRad, Hercules, CA, USA).

12. Mini Ready Sub-Cell GT Cell170-4487ED (BioRad, Hercules, CA, USA).
13. Mini-Gel Caster170-4422 (BioRad, Hercules, CA, USA).
14. PowerPac Basic Power Supply164-5050 (BioRad, Hercules, CA, USA).
15. Microwave oven.
16. Photodyne UV viewbox with ethidium bromide filter and digital camera (Fotodyne, Delafield, WI, USA).
17. Computer using Photodyne software (Fotodyne, Delafield, WI, USA).
18. 0.2 mL thin-walled PCR tube-trips with attached caps, 8-tube strip with individual caps (MED supply partners, Atlanta, USA).
19. Transfer Pipette, 5.8 mL, Fine Tip, 147 mm (MED supply partners, Atlanta, GA, USA).
20. Mini centrifuge C-1202 for 0.2 mL 8-strip tubes (Labnet International, Inc., Woodbridge, NJ, USA).
21. AC632LFUVC 32" PCR workstation X2 (AirClean® Systems, Raleigh, NC, USA).
22. Techne TC-412 Thermocycler X2 (Bibby Scientific US, Burlington, NJ, USA).
23. Calibrated micro-glass rods (HiFi DNA Tech, Trumbull, CT, USA. #3324).

**2.3. Equipment  
and Supplies  
for Automated DNA  
Sequencing**

1. ABI 3130 Genetic Analyzer with integrated computer (Applied Biosystems, Foster City, CA, USA. #3130).
2. 96-well sequencing tray, holder, septa, and cover (Applied Biosystems, Foster City, CA, USA).
3. BigDye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA. #4337450).
4. POP-7™ Polymer for 3130/3130xl Genetic Analyzers (Applied Biosystems, Foster City, CA, USA).
5. Hi-Di™ Formamide (Applied Biosystems, Foster City, CA, USA).
6. 5× sequencing buffer (Applied Biosystems, Foster City, CA, USA).
7. Centri-Sep 8 well strip for Dye Terminator Clean-up (Princeton Separations, Adelphia, NJ, USA).
8. Centri-Sep 96-well wash plates (Princeton Separations, Adelphia, NJ, USA).
9. Eppendorf 96-well plate centrifuge (Eppendorf North America, Westbury, NY, USA).

### 3. Methods

PCR products, generated by nested PCR or single run PCR, can either be cloned or used directly for Sanger sequencing. The former procedure, when used for HPV DNA genotyping, involves cloning, transformation, bacterial incubation, and DNA isolation steps to prepare the DNA template for sequencing, using the ~450 bp HPV MY09/MY11 primer PCR products derived from the clinical specimens as the material for subcloning (47). Direct Sanger sequencing of PCR products is a much more practical approach for clinical laboratories to follow. If a single sequence of HPV DNA is amplified, the PCR amplicon can be easily sequenced directly.

#### 3.1. HPV Positive Control

The commercially available purified HPV-16 phagemid DNA (ATCC, Manassas, VA, USA. #45113D) is dissolved and diluted with TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5) to a working standard solution with an HPV DNA concentration of 1 pg/ $\mu$ L to be used as the positive control in all HPV detection and genotyping tests.

#### 3.2. PCR Procedure

##### 3.2.1. DNA Extraction from Fixed Cells

1. Centrifuge 1 mL of Thin-Prep or 0.5 mL of SurePath cell suspension (usually  $10^5$ – $10^6$  cells) in a 1.5 mL tube at  $\sim 16,000 \times g$  for 5 min.
2. Discard supernatant with a disposable micropipette without disturbing the pellet.
3. Add 1 mL reagent grade water into the tube, vortex and centrifuge at  $\sim 16,000 \times g$  for 5 min.
4. Discard supernatant as above.
5. Add 1 mL Tris-HCl buffer (pH 8.1) into the tube, vortex, and centrifuge at  $\sim 16,000 \times g$  for 5 min.
6. Remove all supernatant as above.
7. Add 0.1 mL working proteinase K digestion fluid.
8. Vortex and incubate samples at 45–55 °C overnight.
9. Heat the tube in the 95 °C block for 10 min.
10. Vortex and centrifuge at  $\sim 16,000 \times g$  for 5 min.
11. Harvest the supernatant digestate for PCR (or for storage at  $-20$  °C).

##### 3.2.2. Primary MY09/MY11 PCR (in PCR Room I)

1. Prepare one PCR tube for each digestate, plus one negative control tube and one positive control (HPV-16) tube for every ten specimens. The total number of PCR tubes needed for  $X$  number of specimens is:  $X + (2X/10)$ ; or 2 when  $X < 10$  =  $\mathcal{Y}$ .

2. Prepare MY09/MY11 primary PCR master mix in a 1.5 mL tube by adding the following (value of “ $\gamma$ ” from above formula):

Water	$2 \times (\gamma + 0.1 \gamma) \mu\text{L}$
10 $\mu\text{M}$ MY09	$\gamma + 0.1 \gamma \mu\text{L}$
10 $\mu\text{M}$ MY11	$\gamma + 0.1 \gamma \mu\text{L}$
Master PCR Mix	$20 \times (\gamma + 0.1 \gamma) \mu\text{L}$

3. Vortex the primary PCR master mix and centrifuge briefly to collect all fluid to the bottom of the tube.
4. Add 1  $\mu\text{L}$  of sample digestate into the bottom of each empty PCR tube (taking extreme care not to cross-contaminate adjacent tubes).
5. Add 1  $\mu\text{L}$  water into the negative control tube, and 1  $\mu\text{L}$  of HPV-16 DNA solution into the positive control tube for every ten clinical specimen tubes.
6. Add 24  $\mu\text{L}$  of the MY09/MY11 primary PCR master mix into each PCR tube containing clinical sample digestate, negative control, or positive control.
7. Make sure that all caps are tightly closed, then mix well, centrifuge briefly to bring all liquids in each tube to the bottom.
8. Bring all tubes to the thermocycler (ramp rate set at 0.9  $^{\circ}\text{C}/\text{s}$ ), programmed for an initial heating at 85  $^{\circ}\text{C}$  for 10 min, followed by 30 cycles, each set at 85  $^{\circ}\text{C}$  for 30 s, 40  $^{\circ}\text{C}$  for 30 s, and 65  $^{\circ}\text{C}$  for 1 min. The final extension is 65  $^{\circ}\text{C}$  for 10 min (30-cycle lo-temp program).

### 3.2.3. $\beta$ -Globin Gene Control PCR (in PCR Room I)

1. Prepare one PCR tube for each sample digestate for  $\beta$ -globin gene amplification, plus one negative control tube for each ten specimens. The  $\beta$ -globin F and R primer sequences are listed under Subheading 2.1.3. The total number of PCR tubes needed for  $X$  specimens is:  $X + (X/10 \text{ or } 1) = Z$ .
2. Prepare  $\beta$ -globin gene PCR master mix in a 1.5 mL tube by adding the following:

Water	$2 \times (Z + 0.1Z) \mu\text{L}$
80 $\mu\text{M}$ $\beta$ -globin F	$Z + 0.1Z \mu\text{L}$
80 $\mu\text{M}$ $\beta$ -globin R	$Z + 0.1Z \mu\text{L}$
Master DNA Polymerase PCR Mix	$20 \times (Z + 0.1Z) \mu\text{L}$

3. Add 1  $\mu\text{L}$  of sample digestate into the bottom of each empty PCR tube.
4. Add 1  $\mu\text{L}$  water into a negative control tube for every ten clinical specimen tubes.

5. Add 24  $\mu\text{L}$  of the  $\beta$ -globin PCR mix into each PCR tube containing clinical sample digestate, or negative control.
6. Make sure that all caps are tightly closed, then mix well, centrifuge briefly to bring all liquids in each tube to the bottom.
7. Bring all tubes to the thermocycler and run the “30-cycle lo-temp” program.

#### 3.2.4. Nested GP6/MY11 PCR (in PCR Room II)

The number of tubes needed for nested PCR amplification is identical to that for MY09/MY11 primary PCR ( $=Y$ ).

1. Prepare HPV nested PCR master mix in a 1.5 mL by adding the following:

Water	$3 \times (\gamma + 0.1 \gamma) \mu\text{L}$
10 $\mu\text{M}$ MY11	$\gamma + 0.1 \gamma \mu\text{L}$
10 $\mu\text{M}$ GP6	$\gamma + 0.1 \gamma \mu\text{L}$
Master DNA Polymerase PCR Mix	$20 \times (\gamma + 0.1 \gamma) \mu\text{L}$

2. Vortex and centrifuge briefly to collect all fluid to the bottom of the tube.
3. Transfer 25  $\mu\text{L}$  of the master mix to each empty PCR tube.
4. Transfer a trace of the MY09/MY11 primary PCR products by dipping a micro-glass rod (using the sealed end only) into the contents of each MY09/MY11 PCR tube and “inoculating” a trace of the primary PCR products into the corresponding nested PCR tube prepared above. Mix the contents with the glass rod which is disposed with extreme care into the waste container while avoiding contact with the rim or the cap of the PCR tube.
5. Make sure that all the caps are tightly closed. Bring all tubes to the thermocycler for the “30-cycle lo-temp” program.

#### 3.2.5. Agarose Gel Electrophoresis (in Analysis Room)

This is conducted in the Analysis Room which is considered the “most DNA-contaminated”. Observe the “One Way Out” rule, which means all equipment items designated to be exclusively used in this room and all wastes must not be brought to other parts of the laboratory. *Always* handle all electrophoresis components with disposable gloves. Wash hands between each glove change.

#### Agarose Gel Slab Preparation

1. Dilute 20 mL of 50 $\times$  TAE buffer to 1,000 mL.
2. Place 8 g of agarose gel powder into 400 mL of TAE buffer in Pyrex bottle.
3. Add 16  $\mu\text{L}$  ethidium bromide solution into the bottle.
4. Heat Pyrex bottle containing PCR-Agarose Gel in microwave until liquefied (*Do not boil-over*).

5. Once liquefied, let bottle stand until it cools to 60 °C.
6. When bottle has reached 60 °C and all the bubbles have disappeared from the liquefied gel, pour into gel-electrophoresis molds.

#### Electrophoresis

1. Place a freshly prepared gel slab in the electrophoresis chamber and flood to the “buffer” line with electrophoresis buffer.
2. Pipette 3  $\mu$ L molecular ruler into the first well of every gel slab row being used.
3. Pipette 5  $\mu$ L of the PCR product mixed with 2  $\mu$ L loading dye into each corresponding well in the gel.
4. When all samples, controls, and molecular ruler are pipetted into their respective wells, press the chamber cover down firmly over the chamber and connect the electrical leads.
5. Set the voltage to 100, and the timer to 40 min.
6. After electrophoresis is finished, place the gel on the viewing tray in the Fotodyne light box. Slide the tray into the center of the light box and close and latch the door.
7. Double click on the “Image” icon on the desktop of the computer attached to the Fotodyne, and, following your worksheet, number each lane and save the electrophoresis image into the appropriate file on the hard drive.

#### 3.2.6. Interpretation of PCR Results

The presence of a 110 bp PCR product amplified by the  $\beta$ -globin gene primers indicates sample adequacy. The presence of a 181–190 bp GP6/MY11 nested PCR product constitutes the presumptive evidence for HPV DNA, pending sequencing validation. The position of the nested PCR product band does not accurately reflect the molecular size of the amplicon. The ~450 bp MY09/MY11 PCR product, even in a specimen positive for HPV DNA, may or may not be visualized.

- All putative HPV DNA nested PCR amplicons of 181–190 bp in size are to be validated by DNA sequencing and for genotyping, using the GP6 oligonucleotide as the sequencing primer with the result confirmed by BLAST with the GenBank database (see below).
- Specimens which do not show a  $\beta$ -globin gene PCR amplicon, a primary HPV PCR amplicon, or a nested HPV PCR amplicon, are considered to be inadequate for evaluation.
- Specimens that show a  $\beta$ -globin amplification band, but no bands on the primary or the nested PCR lanes, are considered to be negative for HPV.
- Specimens that show a positive ~450 bp MY09/MY11 primary PCR amplicon, but no companion 181–190 bp GP6/MY11 nested PCR amplicon, will be subjected to an additional

long-nested PCR, in which a trace of the primary MY09/MY11 PCR products is amplified by a pair of GP5/MY09 nested PCR primer pair, replacing the routine GP6/MY11 nested PCR primers. This would generate a ~380–395 bp PCR product for DNA sequencing, using the GP5 oligonucleotide as the sequencing primer. Since nontarget DNA may be amplified by the MY09 and MY11 degenerate primers, not all ~450 bp PCR products are HPV DNA amplicons.

- The primary and nested PCR protocol presented in this chapter for the detection of HPV DNA in crude clinical sample digestates may not be suitable for other heat-resistant DNA polymerase PCR systems. When a heat-resistant *Taq* DNA polymerase-based PCR formulation is used in place of the low temperature DNA polymerase-based PCR Mix for a parallel comparison study, more than half of the HPV-positive clinical specimens were erroneously classified as “negative” (Fig. 2).
- Amplicon cross-contamination is a potentially serious problem of using nested PCR technology in routine clinical diagnostic laboratories. Micropipetting transfer of PCR amplicons is prohibited because of the danger of aerosol of the PCR products. Instead, transferring of primary PCR products and nested PCR products is routinely accomplished by micro-glass rods. All used glass rods are immediately put into a dedicated container located in the PCR Workstation for proper disposal without contaminating any objects in the laboratory. Avoid touching the rims of the PCR tubes and caps with the working gloves in the PCR Room II.

### **3.3. Direct Automated Sanger DNA Sequencing**

Traditionally, a successful Sanger sequencing reaction using fluorescent dye-labeled ddNTPs as the chain terminators requires a purified DNA template at a specified concentration and an optimal concentration of the sequencing primer for the cycle primer extension/termination reaction to generate a ladder of DNA fragments for automated capillary electrophoresis and computer-based analysis, according to the reagent manufacturer’s instructions (4–6, 20). However, the “front end” cost of HPV DNA template purification may be too high for a clinical laboratory to absorb if the protocol of the manufacturer is followed strictly. One alternative approach is to design a modified reproducible robust protocol to use unpurified nested PCR products as the sequencing template without the need to determine its DNA concentration and purity. The nested PCR technology serves the dual purpose of augmenting HPV detection sensitivity and template preparation for direct automated Sanger DNA sequencing. The unwanted molecules carried over from the original crude sample digestate to the Sanger sequencing reaction mixture would have been diluted by about 12,500-fold ( $25 \times 25 \times 20$ ), if a total volume of 25  $\mu$ L mixture is

used in the primary and nested PCR, and a total volume of 20  $\mu$ L is used for the modified Sanger reaction.

Most scientists performing DNA sequencing use a BigDye<sup>®</sup> Terminator kit from Applied Biosystems (Foster City, CA) for automated Sanger DNA sequencing reactions, according to the manufacturer's instructions. However, some components used in the reaction mixture may need to be adjusted for each laboratory and according to the template used for sequencing.

### 3.3.1. Selection of a Target Sequence for Genotyping

The principle of short target sequence genotyping of HPV takes advantage of the fact that there is a hypervariable DNA sequence, downstream of the MY11 degenerate primer-binding site (Fig. 1) in the highly conserved region of the L1 gene of various genotypes of HPV. Based on the database stored in the GenBank, algorithmic analysis of a sequence of 40–60 nucleotide bases in this region will reliably distinguish all known clinically relevant HPV genotypes. Alternatively, a segment downstream of the MY09 degenerate primer-binding site can also be used for genotyping. Since the MY09 and MY11 primers are degenerate, each composed of numerous individual nucleotides, neither of them can serve as a primer to generate high quality base-calling electropherograms. In contrast, the GP5 or GP6 consensus primer, substantially complementary to a DNA sequence located internal of the MY09 and MY11 termini, is composed of one single nucleotide. Therefore, either GP5 or GP6 can function as a suitable sequencing primer provided it has been used to pair with a degenerate primer (MY09 or MY11) for generating a target PCR amplicon which is to be used as the sequencing template.

Theoretically, anyone of the five nested (heminested) PCR amplicons, namely a nest defined by the primer pair of GP5/GP6, GP5 (reverse)/MY11, GP6/MY11, GP5/MY09, or GP6 (reverse)/MY09, of an MY09/MY11 PCR product, can be used as a DNA sequencing template. However, the GP5/GP6 (or GP5+/GP6+) PCR primer pair is known for its failure to amplify the target DNA of some high-risk HPV genotypes (15, 37, 40, 41), and the GP5 (reverse)/MY11 nest is too short to be used for automated sequencing because the sequence of the 22 inter-primer bases in the amplicon cannot be resolved with the current automated Sanger sequencing technology. The nest flanked by the GP6 and MY11 primers has been chosen for routine short target sequencing (18, 19) because this nest is comparatively small in size and because there are ~140 inter-primer bases in the nested PCR amplicon (Fig. 1), which easily allows a readable sequence of 40–80 bases on the computer-generated electropherogram for reliable genotyping of all clinically relevant HPVs isolated from clinical specimens (18). The nest flanked by the GP5/MY09 primer pair (15, 18), or by the GP6 (reverse)/MY09 primer pair, is also a suitable candidate as a sequencing template for short target sequence HPV genotyping.

3.3.2. *Adjustments  
for Sequencing  
Optimization*

The chemistry of most commercial cycle sequencing kits has been optimized for long readings of 500–800 bases on a base-calling electropherogram. This kind of chemistry is preferred by their large-scale sequencing customers. For a long read in DNA sequencing, the Sanger reaction mixture must have a low ddNTPs/dNTPs ratio, which is not suitable for short target sequence HPV genotyping where the entire PCR-amplified template is only ~180 bp in size. The higher the concentration of the ddNTPs in the reaction, the shorter the terminated products will be, hence, the readable sequence is closer to the primer. However, the first few dozen bases close to the primer are very difficult to resolve with the current automated sequencing technology.

For direct sequencing of PCR-generated short DNA templates derived from clinical materials, the commercial proprietary reagents are usually diluted to optimize the ratios between ddNTPs/dNTPs–template–primer. This process is entirely empirical because the ddNTPs–dNTPs ratio itself is proprietary information, and fixed. One must bear in mind that the DNA polymerase and other ingredients essential for the Sanger reaction to proceed are also reduced when the vendor-provided reagent is diluted. As a result, the only variables that can be adjusted without adversely affecting the performance of the vendor-provided sequencing reaction mixture are the template and the primer.

Among the ABI BigDye® Terminator Sequencing kits, the Terminator v1.1 Cycle Sequencing Kit for short sequencing is preferred. The ABI protocol recommends for sequencing a ~200 bp double stranded DNA template to use 2–7 ng of purified PCR amplicon free of residual PCR primers, salts, DNA polymerase and nontarget DNA molecules. The template should form a single band on agarose gel electrophoresis, and have an A260/A280 ratio from 1.7 to 1.9 when read in a spectrophotometer. For a typical modified Sanger reaction in a total volume of 20 µL, 3.2 pmol sequencing primer which is fully matched with the template at the annealing site is recommended. For short target sequencing, the primer may need to be doubled or tripled for successful sequencing of various HPV genotypes from clinical specimens. However, the sequencing results using a purified HPV-16 plasmid DNA control as template may not show any difference when the concentration of sequencing primer in the modified Sanger reaction is varied.

For direct DNA sequencing of HPV nested PCR products, the quantity of the primer used in each modified Sanger reaction probably needs to be upwardly adjusted over the level recommended in the vendor protocol, for another two reasons.

First, the GP6 (or GP5) nucleotide may not be fully matched in sequence with the HPV template at the annealing site, which may affect the normal enzymatic primer extension/termination reaction. The Sanger cycle sequencing reaction is usually conducted under high stringency conditions whereas PCR for HPV

detection generally uses low stringency amplification, in which enzymatic primer extension is allowed to proceed effectively even with mismatched bases in a primer–template duplex. Under high stringency conditions, a higher concentration of primer if not fully matched with the template may be required for the extension/termination reaction to proceed “normally.” In support of this interpretation about the influence of fully matched versus partially matched primer on the quality of DNA sequencing, is the observation that the electropherogram for HPV-16 sequencing is always cleaner and has a longer read toward the primer site than those for many other HPV genotypes isolated from clinical specimens probably because the HPV-16 sequence at its annealing site is fully matched with the GP6 primer (48). Additional support comes from the observation that there is always a cleaner and longer readable length of a base-calling sequence on the electropherograms for *Chlamydia trachomatis* cryptic plasmid DNA (32) and *Borrelia burgdorferi* 16S rDNA nested PCR amplicons where specific primers with fully matched bases are used for both primary and nested PCR, and for DNA sequencing (33).

Secondly, one complication in performing direct DNA sequencing on the PCR products which have been amplified by a pair of degenerate MY09/MY11 primers under low stringency PCR conditions in order to detect the various HPV genotypes in clinical specimens is that some nontarget DNA fragments are always coamplified in the primary PCR or even in the nested (heminested) PCR, although not exponentially as for the target template. These coamplified nontarget DNA fragments are not readily separated from the target DNA even by column purification because they may have a size similar to that of the target DNA amplicon, and may competitively bind (consume) sequencing primers in the Sanger sequencing reaction mixture.

Each clinical laboratory must decide if the commercial sequencing reaction mixture should be diluted for routine applications, depending on the preparation of the sequencing templates. In this chapter, an example protocol is given to show that the ABI BigDye® Terminator v1.1 Cycle Sequencing Kit is routinely diluted 1:8 for DNA sequencing diagnoses of several infective agents, including HPV. Once the dilution factor of a commercial cycle sequencing reagent mixture kit has been titrated with varying concentrations of primer against varying quantities of PCR-generated HPV templates, the quantity of the sequencing primer used for each Sanger sequencing reaction can be fixed. The appropriate quantity of template to be used for each Sanger sequencing reaction is a fraction of 1  $\mu$ L of unpurified nested PCR products. Since only a short readable sequence of 40–60 bases is needed for accurate molecular diagnoses, the range of quantitative tolerance for a sequencing template is quite wide. In general, the technologies of automated DNA sequencing are quite mature, and covered by numerous

overlapping patents and proprietary intellectual property rights that any gross adjustments of the recommended protocol should be administered with caution.

**3.3.3. Cycle Sequencing  
Reaction (in PCR Room II)**

1. All sequencing primers are aliquoted and kept at  $-20\text{ }^{\circ}\text{C}$  to avoid frequent repeated freezing and thawing cycles.
2. Each sequence cycle run must have 4 samples, or one of its multiple numbers (8, 12, 16, etc.). Each sequence cycle reaction mixture must consist of:  $14.5\text{ }\mu\text{L}$  water,  $3.5\text{ }\mu\text{L}$   $5\times$  buffer,  $1\text{ }\mu\text{L}$  BigDye Terminator v1.1, and  $1\text{ }\mu\text{L}$  sequencing primer. Therefore, prepare the master mix for “ $S$ ” number of sequencing samples as follows:

Water	$14.5\text{ }\mu\text{L}\times(S+1)$
$5\times$ Buffer	$3.5\text{ }\mu\text{L}\times(S+1)$
BigDye 1.1	$1\text{ }\mu\text{L}\times(S+1)$
Primer	$1\text{ }\mu\text{L}\times(S+1)$

3. Distribute  $20\text{ }\mu\text{L}$  of the master mix into each  $0.2\text{ mL}$  thin-walled PCR tube labeled with the patient or control number.
4. Carefully transfer a “trace” of the nested PCR product as template with the calibrated micro-glass rod into the sequencing mix in the corresponding thin-walled PCR tube. Stir-mix without the glass rod touching the rim of the PCR tube.
5. Place tubes in order into the Techne thermocycler programmed for sequencing (ramp set at  $1.0\text{ }^{\circ}\text{C}/\text{s}$ ).
6. Close the thermocycler and set it for the 20-cycle BigDye program, consisting of an initial heating at  $96\text{ }^{\circ}\text{C}$  for 4 min, followed by  $96\text{ }^{\circ}\text{C}$  10 s,  $50\text{ }^{\circ}\text{C}$  5 s and  $60\text{ }^{\circ}\text{C}$  4 min per cycle for a total of 20 cycles with final hold at  $4\text{ }^{\circ}\text{C}$

**3.3.4. Sample Desalting  
for DNA Sequencing  
(in Analysis Room)**

1. Cut bottom tabs off of a Centri-Sep strip (use enough strips for the number of samples in the run).
2. Remove top foil cover and place strips into the wash plate (be sure to balance the pair of wash plates)
3. Spin for 2 min at  $750\times g$  to remove liquid from the columns.
4. Fill columns with reagent grade water and spin for 2 min at  $750\times g$ .
5. Place appropriately labeled empty PCR tubes in wash plate and place the Centri-Sep columns in the PCR tubes.
6. Remove the specimen tubes from the thermocycler and place them in order into the tube rack.
7. Pipette  $10\text{ }\mu\text{L}$  of each specimen reaction product into the corresponding numbered Centri-Sep column. Note: pipette into the center of the Centri-Sep filter, without actually touching the filter.

8. Follow by pipetting an aliquot of 13  $\mu\text{L}$  of formamide (which has been removed from freezer and allowed to thaw at room temperature for about 10 min) directly into each Centri-Sep column.
9. Place the balanced wash plates back into the centrifuge with the PCR tubes and columns.
10. Spin again for 2 min at  $750 \times g$ .
11. Remove the wash plate/spin column assemblies from the centrifuge and discard the spin columns. Place the labeled PCR tubes in order into the tube rack.

*3.3.5. Automated DNA Sequencing (in Analysis Room)*

(If necessary, contact an ABI representative for technical assistance)

1. Pipette 5 mL of  $10\times$  capillary electrophoresis buffer into 45 mL of reagent grade water and mix, to make working buffer.
2. Remove the buffer cup and buffer well from the ABI 3130 sequencer and rinse with reagent grade water.
3. Refill with capillary electrophoresis buffer up to the lines. Do not overflow.
4. Remove the three water wells from the ABI 3130 sequencer, rinse with reagent grade water and refill to the lines with reagent grade water.
5. Replace buffer cup, buffer well, and the three water wells into the sequencer. Note: capillaries should not be allowed to dry out, so work quickly.
6. Into a 96-well plate with plate holder attached, pipette 10  $\mu\text{L}$  of reaction-formamide mixture from each numbered PCR tube into the corresponding well of the 96-well plate.
7. Place septum on top of the 96-well plate and spin again for 2 min at  $750 \times g$ .
8. Cover with the white plastic cover plate.
9. Place assembled 96-well plate into the plate holder position in the ABI 3130 sequencer.
10. Open the ABI program on the computer to connect to the sequencer.
11. On the screen click "Plate Manager", then "New". Enter Name and Date.
12. Enter the following: Application: Sequencing analysis  
Plate type: 96  
Owner name  
Operator name: (technician name)

13. Click: OK
14. Go to the Sequencing Analysis Plate Editor  
Enter case number, then “template dilutions bigdye”  
Select: UltraSeq 36\_POP7\_E  
Select: v1.1 PCR  
Click: OK  
Click: Plate view (to link plates)  
Click: Find All Plates  
Click on the Date or Name you want to run
15. With the plate holder in the sequencer and the doors closed, the gray plate grid on the computer screen will turn yellow. Click on the grid position corresponding to your plate position with the mouse arrow. The grid will turn green.
16. Click the “play” button and the program will ask you “would you like to proceed”. Click “yes” and sequencing will begin.

*3.3.6. BLAST Sequence  
Alignment for HPV  
Genotyping*

1. When sample processing is finished, the data will be stored in a file that was named in steps listed above. Before submitting the sequence to NCBI for assignment of genotype you must review and edit the sequence by “trimming” extraneous bases (all but the 40–60 bases immediately downstream of the MY11 primer binding site), then clicking on the bases coded “W” or “Y” and examining the shape and color of the curve tracing to determine whether the base is A, T, C, or G. When you have a clean sequence, highlight it and click “copy” on the toolbar.
2. Open NCBI BLAST and click on “nucleotide”.
3. Click “paste” on the toolbar and your edited sequence will be pasted into BLAST.
4. You must then “choose database” by clicking on “others”.
5. “Nucleotide collection (nr/nt)” will appear, in the data section.
6. Click on the “BLAST” bar, and your sequence will be aligned with the sequences of all viruses in the database.
7. The species and genotype of your pasted sequence, along with percentage match to established database genotypes will appear on screen. You must highlight the entire data section and click “copy” in the toolbar.
8. Open Microsoft Word and click “paste” and your copied data from BLAST will appear on the Word screen. Click “file” and “print” to print out the NCBI alignment data for attachment to the report.

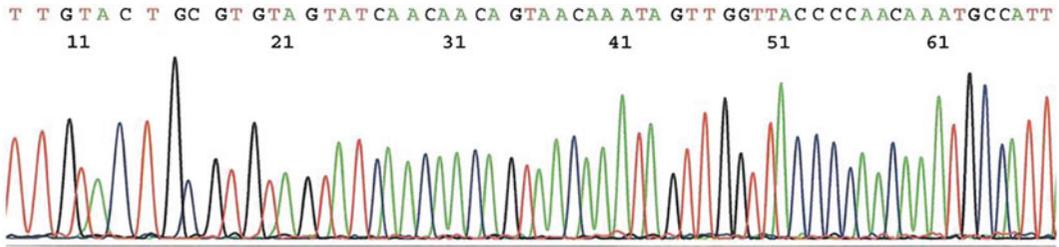


Fig. 3. Computer-generated electropherogram showing the sequence of a target HPV DNA. *Footnote:* A segment of DNA sequencing electropherogram with its ABI computer-generated base-calling results, consisting of the last five bases (3'CCATT--5') of the MY11 primer-binding site on the right and 56 bases downstream thereof. The triad “AAA” sequence, which is invariably positioned 23 bases downstream of the MY11 primer-binding site, is seen for all known HPV genotypes of clinical relevance.

```

gb|HM057182.1| Human papillomavirus type 16 isolate Amazonian, complete
genome Length=7915

Score = 102 bits (112), Expect = 1e-19
Identities = 56/56 (100%), Gaps = 0/56 (0%)
Strand=Plus/Minus

Query 1      TTGTACTGCGTGTAGTATCAACAACAGTAACAAATAGTTGGTTACCCCAACAAATG 56
            |||
Sbjct 6644   TTGTACTGCGTGTAGTATCAACAACAGTAACAAATAGTTGGTTACCCCAACAAATG 6589
    
```

Fig. 4. Copy of online report from the GenBank after submission of the sequence in Fig. 3. *Footnote:* The 56-base inter-primer sequence illustrated in Fig. 3 was submitted to the GenBank for BLAST alignment algorithm. The online report from the NCBI is shown here, confirming the molecular diagnosis of an HPV-16 DNA in this clinical specimen beyond a reasonable doubt.

9. Type the patient name in front of the sequence and date numbers, and store the file in the sequence folder on the “C” drive, or on an external hard drive.
10. The contents of the sequence folder are backed up daily on a flash drive to be held apart from the laboratory as record keeping.

3.3.7. Interpretation of Sequencing Results

When GP6 and MY11 are used as the HPV nested (heminested) PCR primers, the native sequence at the MY11 binding site (Fig. 1) of the nested PCR amplicon which is also the sequencing template, has now been replaced by the sequences defined by the MY11 degenerate nucleotides. Therefore, it is an easy task to visually locate the end of the MY11 primer site 3'-CCATT-5' on the electropherogram, reading from the right of the color tracing (Fig. 3). A short stretch of 40–60 bases downstream of the MY11 primer-binding site, excised from the computer-generated base-calling sequence, is sufficient for HPV BLAST genotype determination. A 100 % identities match is required for reliable HPV genotyping (Figs. 3 and 4).

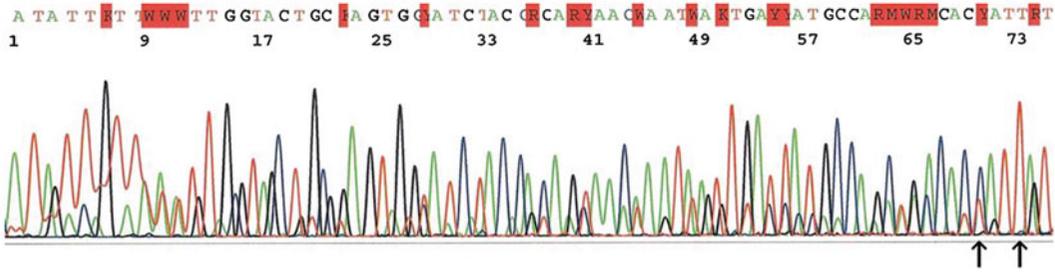


Fig. 5. Sequencing electropherogram of more than one HPV target DNA in specimen. *Footnote:* When the infection is caused by two HPV genotypes with different amplicon sizes (HPV-16 and HPV-18), one end sequence 3'CCATT--5' of the MY11 primer site is positioned ahead of the other, as shown in this mixed HPV DNA sequence tracing. The first T of each end segment is indicated by an arrow. This is due to the fact that the size of the GP6/MY11 nested PCR amplicon of HPV-18 is 3-bp longer than that of HPV-16 (see Fig. 1).

Failure in base-calling by the computer analytical system may be caused by mixed HPV infections (more than one genotype of HPV DNA in the sample). A visual analysis of the 3'-CCATT-5' end piece of the MY11 primer-binding site and the key bases downstream thereof may help confirm a mixed HPV infection (Fig. 5).

Without subsequent new infections, a mixed HPV infection usually clears up completely or becomes persistent. In the latter case, a persistent HPV infection tends to be maintained by one of the genotypes present in the initial mixed infection as often found in repeat testing in 6–12 months (Fig. 6).

### 3.4. Key Points

- A 40–60 base short target sequence downstream of the MY11 primer-binding site, excised from the sequencing electropherogram, is sufficient for HPV BLAST genotype determination. A 100 % identities match is required for genotyping.
- Failure of base-calling by the ABI computer system may be caused by mixed HPV infections (more than one genotype of HPV DNA in the sample). A visual analysis of the (3'CCATT--5') end piece of the MY11 primer-binding site and the key bases downstream thereof may help confirm a mixed HPV infection.
- Recognizing the highly conserved MY11 primer ending of the HPV target DNA and the variable sizes of the GP6/MY11 nested PCR amplicons between different HPV genotypes may help identification of a mixed HPV infection.
- Without subsequent new infections, a mixed HPV infection usually clears up completely or becomes persistent. In the latter case, the persistent infection tends to be maintained by one of the genotypes present in the initial mixed infections as often found in repeat testing in 6–12 months.

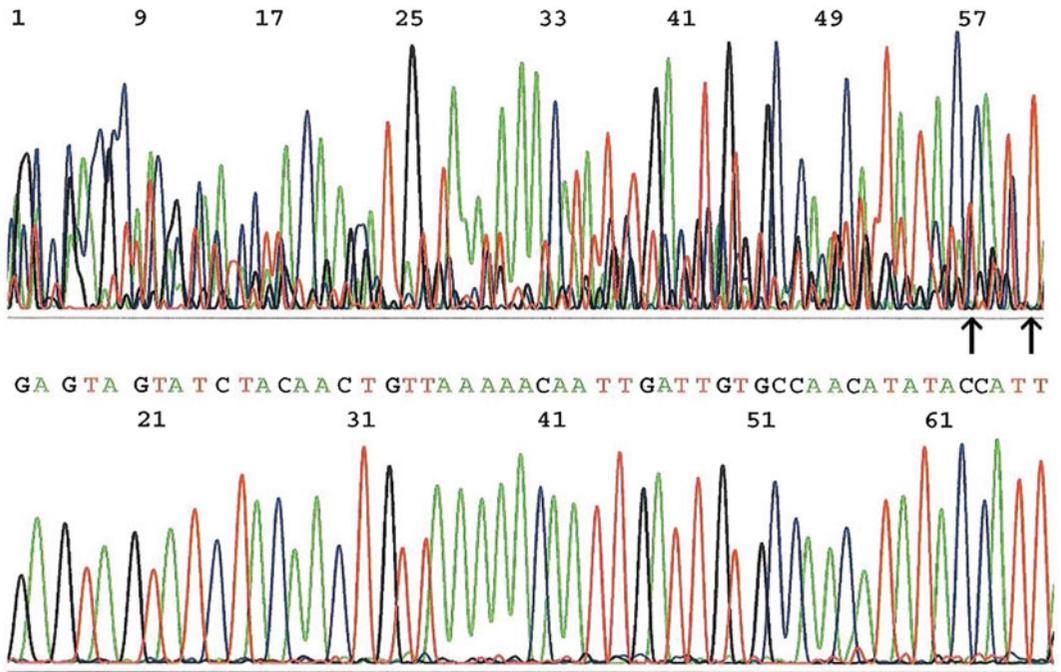


Fig. 6. Mixed HPV infection turning into single persistent HPV infection on follow-up. *Footnote:* Mixed HPV infections are usually transient. If not totally cleared on follow-up, they often turn into a single HPV infection, as the case in this 49-year-old woman with a history of reactive Papanicolaou cytology result. In December, 2009, her cervicovaginal cells were positive for a mixed HPV infection with two superimposing staggering 3'CCATT--5' MY11 endings on the right of the upper sequence tracing (the first T of each ending is indicated by an *arrow*). A repeat HPV test in June 2010 detected a single HPV-59 infection as demonstrated in the lower sequence tracing. Comparison of the two tracings reveals that a DNA sequence characteristic of that of an HPV-59 was overshadowed in the background of the mixed HPV sequences in the electropherogram recorded 6 months earlier.

#### 4. Notes

Since persistent HPV infection is a necessary but not a sufficient factor in determining the outcome of a pathologic process which may lead to cervical cancer development, the first usage of testing HPV in clinical cervicovaginal cell specimens is for its negative predictive value (*npv*) in assessing the cervical cancer risk of a patient. It is generally agreed that no HPV infection means almost zero risk for cervical cancer. Therefore, an HPV test should be as sensitive as possible in detecting all the 13 generally accepted high-risk or “carcinogenic” HPV genotypes, including their sequence variants, and at least 90 % of the approximately 40 clinically relevant HPV genotypes. In general, a low HPV viral load does not limit the sensitivity of detection because each infected cell may harbor about 100–3,700 episomal copies of HPV DNA (26). An efficient PCR should be able to detect less than 10 copies of target HPV DNA per reaction.

The second important usage of an HPV test is for identifying the patients with persistent infection by a high-risk HPV. Although the role of persistent infection by HPV as a tumor promoter in cervical cancer induction has been recognized (49–51), the issues of persistence of an HPV infection can be adequately studied only when HPV type-specific or perhaps even variant-specific methods are available to the clinical laboratories which perform routine HPV tests for patient management. There is no alternative to DNA sequencing if reliable genotyping is the goal in HPV testing (12).

The gateway to DNA sequencing is PCR in the practice of laboratory medicine. If a proper PCR-generated template is obtained, HPV genotyping by automated Sanger DNA sequencing is straightforward because the manufacturers of the DNA sequencing reagent kits and equipment have already optimized most of the technical steps for a variety of usages requiring reliable, accurate DNA sequence data in scientific research and in industries.

Most of the published PCR protocols for HPV amplification are designed to prepare samples for probe hybridization in which nonspecific DNA products may not affect the binding between the target DNA and the probe. As a result, high concentrations of magnesium chloride ranging from 4 to 6.5 mM (35, 52) are often used in a *Taq* PCR mixture to increase the efficiency of the enzyme in extension of the HPV DNA duplexes with a low  $T_m$  of primer-template combination. Occasionally, a high concentration of magnesium chloride up to 10 mM (28) is used to amplify certain HPV genotypes. Since increasing the magnesium chloride concentration from 1.5 to 3 mM to artificially raise the efficiency of the *Taq* function for HPV DNA detection also generates nonspecific PCR products from the clinical samples (36), these PCR-generated non-target DNA fragments are bound to cause difficulties in the Sanger sequencing reactions because they may have the same degenerate termini as the target amplicon and a size close to that of the target amplicon. These DNA fragments are difficult to separate from the desired template even using various commercial purification kits.

As demonstrated by others the first consideration in setting up a protocol for PCR/sequencing HPV testing is to choose a DNA polymerase that can amplify all HPV genotypes at a magnesium chloride concentration between 1.5 mM (6) and 2–2.5 mM (5).

The second consideration is to determine the method for sample preparation—for example, to use a commercial cell lysis kit or a proteinase K digestion to free the HPV DNA from the cells—a DNA extraction and purification protocol, or no purification at all. The samples so prepared must be compatible with the DNA polymerase and the concentration of magnesium chloride selected for making the PCR master reaction mixture. Most scientists use a purified DNA preparation from the cell suspension for PCR/DNA sequencing (5, 6, 20), but a crude proteinase K digestate may be an alternative (15–19).

The third consideration is to select the primer pair or primer pairs for the PCR. For the L1 gene amplification, there are four proven effective consensus general primers reported, namely, the GP5 (or GP5+), GP6 (or GP6+), the MY09, and the MY11 nucleotides. The selected pair or pairs of primers must be capable of amplifying the selected target sequences of the standard full-length plasmid HPV-6, HPV-11, HPV-16, and HPV-18 DNA, which are the only HPV DNA controls available commercially from ATCC. Since there are approximately 40 clinically relevant HPV genotypes reported worldwide, the protocol should be capable of detecting at least 36 HPV genotypes (90 % of 40)—including the 13 generally recognized “high-risk” HPV genotypes and their local variants—in a pool of liquid-based cervicovaginal cytology specimens collected from a local population.

Finally, the HPV DNA PCR amplicon used as the template must be optimized with the sequencing primer, with or without purification, for genotyping by automated direct Sanger DNA sequencing.

For quality assurance, it is highly desirable to enroll in a national HPV survey program for testing the ability and competence of the individual clinical laboratories in the detection and genotyping of various genotypes of HPV in blind-coded simulated clinical specimens. Unfortunately, as of October 2010 the College of American Pathologists (CAP) has only a token survey program to set a minimal performance standard for HPV testing, based on two commercial probe-hybridization test kits which do not identify specific genotypes, or identify HPV-16 and HPV-18 only. The blind-coded positive samples dispatched by the CAP to various laboratories for proficiency surveys invariably contain a heavy load of HPV-16 and do not have other high-risk HPV genotypes, such as HPV-52 which has been reported to be the most prevalent genotype infecting young women in the United States (53, 54). A semiofficial regulatory proficiency test with such low analytical standards can hardly stimulate further technical development of sensitive HPV detection and reliable genotyping technologies in clinical laboratories. The problem of the CAP HPV program is further compounded by the authoritative opinions that detection and genotyping of HPV from clinical specimens need both analytical and clinical validation (55). According to the experts, clinical validation means a 92 % detection rate for cervical intraepithelial neoplasia 3 (CIN3) as the clinical sensitivity and an 85 % CIN3 confirmation as the clinical specificity for validation of all HPV assays (56). However, histologically CIN3 itself is a heterogeneous group of morphological changes (57), which are bound to generate great confusions when used as the “gold standard” to validate the DNA tests based on nested PCR and Sanger sequencing. The directors of HPV-testing laboratories are charged with the professional responsibilities for rigorously validating all HPV test results.

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## The Molecular Diagnosis of Sexually Transmitted Genital Ulcer Disease

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### Abstract

Highly sensitive and specific nucleic acid amplification tests (NAATs) have emerged as the gold standard diagnostic tests for many infectious diseases. Real-time PCR has further refined the technology of nucleic acid amplification with detection in a closed system and enabled multiplexing to simultaneously detect multiple pathogens. It is a versatile, fast, and high-throughput system for pathogen detection that has reduced the risk of PCR contamination, eliminated post-PCR manipulations, and improved the cost-effectiveness of testing. In addition, real-time PCR can be applied to self-collected noninvasive specimens. Here, we describe an *in-house* developed TaqMan-based real-time multiplex PCR (M-PCR) assay for the diagnosis of sexually transmitted genital ulcer disease (GUD) and discuss briefly on issues associated with validation of assay performance.

**Key words:** Polymerase chain reaction, PCR, Nucleic acid amplification test, NAAT, Genital ulcer disease, GUD, Real-time multiplex PCR, M-PCR

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### 1. Introduction

The predominant etiologic agents of genital ulcer disease (GUD) are *Haemophilus ducreyi* (chancroid), *Treponema pallidum* (syphilis), and herpes simplex virus (HSV) types 1 and 2 (genital herpes). Additional, but rare causes of GUD include *Klebsiella granulomatis* (donovanosis) and the lymphogranuloma venereum (LGV) biovars of *Chlamydia trachomatis*. GUD has long been recognized as a potential risk factor for HIV acquisition and transmission (1–6); however, the etiology of GUD can vary temporally and geographically (7, 8). Today, HSV-2 is the leading cause of GUD worldwide, but a rise in relative prevalence of HSV-1 as a cause of genital herpes

has been noted (9). A diagnosis of GUD based solely on clinical presentation is not dependable, since atypical presentations are common, mixed infections frequently occur, and the treatment of individual causative agents differs; thus, it is important to accurately identify the etiology. The conventional laboratory methods for the detection of causative agents of GUD utilize various specialized techniques including bacteriological and tissue cultures, serologic tests, histopathology, and dark-field microscopy. Culture of etiologic agents of GUD can be difficult, time-consuming, and technically demanding, while dark-field microscopy which requires to be performed within 20 min of specimen collection by a highly trained microscopist has both a lower sensitivity and specificity than detection of *T. pallidum* by PCR (10). Serologic testing exhibits low sensitivity in early stages of infection with syphilis, genital herpes, and LGV, while the tests are unable to distinguish between current and previous infection. In the case of LGV serology, there is considerable cross-reactivity between LGV and non-LGV biovars rendering the test relatively nonspecific.

Several GUD etiological studies (11–16) concurred that PCR-based detection methods provide a more definitive diagnosis than both clinical diagnosis and traditional laboratory methods. Traditional laboratory methods used for comparison with molecular detection of *T. pallidum* by M-PCR include dark-field microscopy and both nontreponemal and treponemal serologic tests such as Rapid Plasma Reagin (RPR) or Venereal Disease Research Laboratory (VDRL), the Tolidine Red Untreated Serum Test (TRUST, New Horizons Diagnostic Corp., Columbia, MA), the *Treponema pallidum* Passive Particle Agglutination Assay (TPPA), and fluorescent treponemal antibody absorption test (FTA-ABS). Bacterial culture was the only reported method for the validation of the detection of *H. ducreyi* by M-PCR. In addition to virus culture, herpes serology that include HerpChek ELISA (Dupont, Wilmington, DE), Kalon ELISA (Kalon Biological Ltd., Guilford, UK), home-brew and commercial Western blots (Euroimmune, Lübeck, Germany) were used to compare the molecular diagnosis of genital herpes by M-PCR. As already stated, clinical features of the ulcerations alone often had poor agreement with GUD M-PCR results, while the standard laboratory tests had limitations associated with poor sensitivity (culture and direct detection) or specificity (serologic methods).

Currently, there are no commercial PCR or M-PCR tests available for the diagnosis of syphilis, chancroid, or multiple GUD etiology. The only FDA-cleared real-time PCR test is for the detection and typing of HSV using swab specimens from vaginal lesions (EraGen Biosciences Inc., Madison, WI.). The use of *in-house* M-PCR/real-time M-PCR assays for the laboratory diagnosis of GUD has become more common as a result of their reproducibility and the speed of detecting multiple etiologic agents in a single, active

ulceration with a high level of sensitivity and specificity. Although real-time M-PCR is regarded as a superior platform for the diagnosis of GUD, to appropriately validate the performance of the assays remains a significant challenge for the laboratory. Genital ulcer disease caused by *T. pallidum* or *H. ducreyi* is rare in most industrialized societies. The collection and sampling of cases of chancroid for both culture and PCR for *H. ducreyi* is particularly challenging simply because this infection has been declining, even in previously endemic areas in developing countries, since the introduction of syndromic management for GUD. Even if a sufficient number of GUD cases could be located, the contribution of using relatively insensitive microscopic, culture and serologic tests to validate an M-PCR assay remains questionable. The lack of commercially available PCR tests for *T. pallidum* and *H. ducreyi*, and the absence of different detection technologies/platforms with comparable sensitivity for head to head comparison also increase the difficulty in performing clinical validation studies. As an alternative, the use of a similar real-time M-PCR format with a combination of supplemental/confirmatory PCR targets should be considered as an appropriate approach toward validating a suitable NAAT for the diagnosis of GUD.

Prior to the implementation of an M-PCR assay for diagnostic use, the Clinical and Laboratory Standards Institute (CLSI) guideline for the analytical verification and validation of multiplex nucleic acids assays (17) recommends the establishment of the assay performance characteristics including accuracy, precision, analytical sensitivity and specificity, reportable range and normal values. The limit of detection (LOD) and PCR efficiency of the *in-house* developed real-time M-PCR assay for GUD were first determined for the individual targets by a monoplex PCR assay using tenfold serial dilutions of either commercial HSV quantitated DNA, *H. ducreyi* genomic DNA purified from culture or *T. pallidum* genomic DNA harvested and purified from infected rabbit testes; ranging from 1 to 10<sup>5</sup> or 10<sup>6</sup> genomic copies per reaction to establish a standard curve for quantitation. The LOD and PCR efficiency for each target were subsequently determined in a multiplex format. The results obtained were similar to those obtained in the monoplex assays. Overall, the analytical sensitivity of this *in-house* developed M-PCR assay is approximately 10–100 copies per reaction with a PCR efficiency ranging from 90 to 110 %. The analytical specificity was assessed using DNA from a panel of organisms including commensal and pathogenic microbes found in the genitourinary tract and as part of the normal skin flora.

This chapter describes the methodology of a TaqMan-based diagnostic real-time M-PCR for the simultaneous detection of *H. ducreyi*, *T. pallidum*, and HSV using DNA extracted from ulcer specimens. Human ribonuclease P gene was incorporated in the assay as an internal control to monitor sample adequacy and PCR inhibition.

## 2. Materials

The QIAamp DNA Mini Kit (Qiagen Inc., Valencia, CA) is used for DNA extraction from genital ulcer specimens. All reagents in the kit are stored at room temperature. PCR reagents require refrigeration (4–8 °C) but can also be frozen. *Taq* polymerase must be stored at –20 °C in a non-frost-free freezer. Hazardous waste should be disposed in accordance with local waste disposal regulations. Additional information on specific chemicals can be obtained from the material safety data sheets provided by the product supplier. Appropriate specimens include ulcer swabs (dry or in transport medium), tissue biopsies (frozen or paraffin-embedded or thin sections on slides that have not been stained). Ideally, specimens should be stored at –20 °C or colder prior to shipping to the laboratory for NAAT. If sending swabs or frozen tissue blocks, overnight express shipment with cold packs is usually acceptable, but shipment with dry ice is preferred. Suitable transport media include 1 mL of specimen transport medium (Roche Amplicor STM, Roche Diagnostics, Indianapolis, IN), Genelock (Sierra Molecular Corp., Sonora, CA), 10 mM Tris–HCl pH 8.0, saline, 2SP, or M4 (Remel Products, Lenexa, KS).

### 2.1. DNA Extraction

1. QIAamp® DNA Mini Kit.
2. Water bath or dry bath at 56 °C.
3. Sterile 1.5- to 2.0-mL microcentrifuge tubes.
4. Ethanol (96–100 %).

### 2.2. Real-Time Multiplex PCR

All PCR reagents (except *Taq* polymerase) must be thawed prior to use and kept on ice during master mix preparation. Designate a clean, DNA-free preamplification area or use a PCR workstation equipped with UV lamps to prepare the master mix (see Note 1).

1. DNA template.
2. Positive controls (genomic DNA purified from a laboratory strain of *T. pallidum*, or *H. ducreyi*, or HSV) (see Note 2).
3. HSV DNA target for both type 1 and 2: glycoprotein D gene (*gD*).
  - (a) Forward primer: 5'CCCCGCTGGAAGTACTATGACA3'.
  - (b) Reverse primer: 5'GCATCAGGAACCCCAGGTTA3'.
  - (c) Probe: 5'FAM-TTTAGCGCCGTCAGCGAGG-BHQ3'.
4. *H. ducreyi* DNA target: hemolysin gene (*hhdA*).
  - (a) Forward primer: 5'AATCGTAACTGCGGGATTAGG3'.
  - (b) Reverse primer: 5'CAATAGACACATTATCGCCCTTAA3'.

- (c) Probe: 5'JOE- ATGGCCATGGTAGTGAGGTAAATCAGGCTGT-BHQ3'.
- 5. *T. pallidum* DNA target: 47-kDa lipoprotein gene (*tpn47*).
  - (a) Forward primer: 5'CAACACGGTCCGCTACGACTA3'.
  - (b) Reverse primer: 5'TGCCATAACTCGCCATCAGA3'.
  - (c) Probe: 5'ROX-ACGGTGATGACGCGAGCTACACCA-BHQ3'.
- 6. Internal DNA control: Human ribonuclease P gene (*RNase P*).
  - (a) Forward primer: 5'CCAAGTGTGAGGGCTGAAAAG3'.
  - (b) Reverse primer: 5'TGTTGTGGCTGATGAACTATAAAAGG3'.
  - (c) Probe: 5' CY5-CCCCAGTCTCTGTCAGCACTCCCTTC-BHQ3'.
- 7. DNA polymerase (5 U/ $\mu$ L, AmpliTaq Gold<sup>®</sup>, Applied Biosystems, Foster City, CA).
- 8. GeneAmp<sup>®</sup> deoxyribonucleoside 5'-triphosphate mix with dUTP (2.5 mM each of dATP, dCTP, dGTP, and 5.0 mM dUTP, Applied Biosystems).
- 9. AmpErase<sup>®</sup> Uracil-*N*-glycosylase (1 U/ $\mu$ L, UNG, Applied Biosystems).
- 10. GeneAmp<sup>®</sup> 10 $\times$  PCR buffer (supplied with AmpliTaq Gold<sup>®</sup>).
- 11. Magnesium chloride (25 mM, supplied with AmpliTaq Gold<sup>®</sup>).
- 12. DNase and RNase-free water.
- 13. 0.2 mL-microcentrifuge tubes (flat top, thin walled).
- 14. Cold block.
- 15. Real-time PCR instrument (see Note 3).

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### 3. Methods

When using the QIAamp DNA Mini Kit for DNA extraction, it is necessary to follow the manufacturer's protocol and adhere to their instructions unless otherwise stated below. Extracted DNA should be used immediately or placed at 4 °C if PCR is performed on the same day otherwise, samples should be stored at -20 °C or -70 °C. Avoid repeated freezing and thawing, as this will lead to degradation of the DNA.

### 3.1. DNA Extraction

#### 3.1.1. QIAamp DNA Mini Kit

1. Pipette 20  $\mu\text{L}$  protease or proteinase K into a 1.5 mL microcentrifuge tube.
2. Add 200  $\mu\text{L}$  sample to the tube. (If sample volume is less than 200  $\mu\text{L}$ , add the appropriate volume of PBS)
3. Add 200  $\mu\text{L}$  Buffer AL and mix by pulse-vortexing for 15 s.
4. Incubate at 56  $^{\circ}\text{C}$  for 10 min.
5. Briefly centrifuge the tube to remove drops from the inside of the lid.
6. Add 200  $\mu\text{L}$  ethanol to the sample and mix by pulse-vortexing for 15 s. Briefly centrifuge the tube to remove drops from the inside of the lid.
7. Carefully apply the mixture from step 6 to the QIAamp Mini spin column (in a 2 mL collection tube) without wetting the rim. Close the cap and centrifuge at  $6,000\times g$  for 1 min. Place the spin column in a clean 2 mL collection tube and discard the tube containing the filtrate.
8. Carefully open the spin column and add 500  $\mu\text{L}$  Buffer AW1 without wetting the rim. Close the cap and centrifuge at  $6,000\times g$  for 1 min. Place the spin column in a clean 2 mL collection tube and discard the tube containing the filtrate.
9. Carefully open the spin column and add 500  $\mu\text{L}$  Buffer AW2 without wetting the rim. Close the cap and centrifuge at  $20,000\times g$  for 3 min. Place the spin column in a new 2 mL collection and discard the old collection tube with filtrate. Centrifuge at full speed for 1 min to ensure that there is no carryover of Buffer AW2.
10. Place the spin column in a clean 1.5 mL microcentrifuge tube and discard the tube containing the filtrate. Carefully open the spin column and add 100  $\mu\text{L}$  Buffer AE or TE buffer pH 8.0. Allow the spin column to stand at room temperature for 5 min (see Note 4) and then centrifuged at  $6,000\times g$  for 1 min.

#### 3.2. Real-Time M-PCR (See Note 5)

1. Set up a 25  $\mu\text{L}$  of master mix containing 4  $\mu\text{L}$  deoxyribonucleoside triphosphate mix (2.5 mM of dATP, dCTP, dGTP, and 5.0 mM dUTP), 10  $\mu\text{L}$   $\text{MgCl}_2$  (25 mM), 1U UNG, 10U AmpliTaq Gold polymerase, 5  $\mu\text{L}$  10 $\times$  PCR buffer, a final concentration of 400 nM forward primer, 600 nM reverse primer, and 400 nM probe for HSV and *H. ducreyi* target; 600 nM each primer and 400 nM probe for *T. pallidum* target; 160 nM each primer and probe for *RNase P*.
2. Add 25  $\mu\text{L}$  sample DNA.
3. Include a positive (*H. ducreyi*, *T. pallidum*, and HSV DNA) and no template control (NTC, using TE or water instead of sample DNA) for each run (see Note 6).
4. Initial hold cycle: 50  $^{\circ}\text{C}$  for 2 min (see Note 7).

**Table 1**  
**Interpretation of real-time M-PCR results**

	FAM channel	JOE channel	ROX channel	CY5 channel
HSV DNA detected	pos	neg	neg	pos
<i>H. ducreyi</i> DNA detected	neg	pos	neg	pos
<i>T. pallidum</i> DNA detected	neg	neg	pos	pos
HSV and <i>H. ducreyi</i> DNA detected	pos	pos	neg	pos
HSV and <i>T. pallidum</i> DNA detected	pos	neg	pos	pos
<i>H. ducreyi</i> and <i>T. pallidum</i> DNA detected	neg	pos	pos	pos
HSV, <i>H. ducreyi</i> , and <i>T. pallidum</i> DNA detected	pos	pos	pos	pos
Negative	neg	neg	neg	pos
Invalid	neg	neg	neg	neg

5. Initial denaturation: 95 °C for 10 min (see Note 8).
6. The following steps are repeated for 45 cycles:
  - (a) Denaturation at 95 °C for 20 s.
  - (b) Primer annealing and extension at 60 °C for 1 min. (Program the real-time PCR instrument to acquire fluorescent signals from all four channels at this step)
7. Interpretation of results (see Table 1)
  - (a) HSV-positive sample: A positive fluorescent signal indicated by a threshold cycle (Ct) value above the manually set cutoff in both the FAM and CY5 channels (see Note 9). Similarly, *H. ducreyi*-positive sample is indicated by positive fluorescent signals and Ct values in JOE and CY5 channels, and positive signals in ROX and CY5 channels for *T. pallidum*-positive sample.
  - (b) HSV-negative sample: No fluorescent signal or Ct value in the FAM channel but having a positive fluorescent signal in the CY5 channel. Similarly, no Ct values in JOE or ROX channel indicates the sample is tested negative for *H. ducreyi* or *T. pallidum* DNA, respectively.
  - (c) Invalid result: No fluorescent signal in the CY5 channel indicates the possibility of PCR inhibition and/or inadequate sample. Repeat DNA extraction and real-time M-PCR testing. In addition, the test run should be considered invalid if the NTC tube had a positive fluorescent signal or Ct value. Prepare new batch of NTC and repeat the test run.

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## 4. Notes

1. This real-time GUD M-PCR assay is designed to detect both HSV-1 and -2. The typing of HSV can be established as a second round real-time M-PCR assay or incorporated into the existing assay to become a 5-plex M-PCR assay. This chapter has excluded the methodology on the molecular diagnosis of LGV and donovanosis. Laboratories interested in the detection of LGV using real-time PCR/M-PCR can refer to publications in *Emerging Infectious Disease* (2005), 11: 1311–1312; *Sexually Transmitted Disease* (2007), 34: 451–455; and *Sexually Transmitted Infection* (2008), 84: 273–276. The methodology for the molecular diagnosis of donovanosis using PCR, restriction enzyme digestion, and/or nucleotide sequencing can be found in reference (16). Gene targets, primers, and probes for the GUD real-time M-PCR assay are also listed in Table 4, Chapter 57: “*Treponema* and *Brachyspira*, Human Host-Associated Spirochetes” of the Manual of Clinical Microbiology published in 2011.
2. With the exception of *T. pallidum*, culture and purified genomic DNA are commercially available from American Type Culture Collection, Manassas, VA, and Advanced Biotechnologies, Inc., Columbia, MD.
3. The real-time TaqMan assay is run on a Rotor-Gene 3000/6000/Q (Qiagen Inc.). If a different instrument or fluorescent reporter molecule is used, additional validation or optimization may be required.
4. This step increases the elution efficiency. Elution with a smaller volume of Buffer AE (50–100  $\mu$ L) will increase the DNA concentration but might decrease the overall yield.
5. The use of 47-kDa gene as the PCR target for *T. pallidum* cannot distinguish *T. pallidum* subsp. *pallidum* (venereal syphilis) from the causes of nonvenereal treponematoses (*T. pallidum* subsp. *pertenue*, and subsp. *endemicum*). This Real-time M-PCR assay is not FDA-cleared and should be used for research and epidemiologic purpose only, and not for direct patient management without thorough validation processes. The real-time PCR detects DNA from both dead and live organisms, thus when used as a test-for-cure, the testing should be delayed until after 3 weeks. A positive test result after appropriate treatment and the necessary interval for clearing of antigen or nucleic acid is a sign of incomplete treatment or reinfection.

6. This assay is to generate qualitative positive/negative results, thus the concentration or copy number of positive control DNA used is not as critical as in the Q-PCR assays. However, we recommend the use of similar amounts of individual positive control DNA that can produce a Ct value in the range of 25–30. Alternatively, individual target DNA can be PCR amplified and cloned into a suitable plasmid vector and used as a positive control.
7. In the presence of alkaline pH and heat, UNG is able to cleave dU-containing PCR products, thus preventing carryover contamination from previous PCR amplification using dUTP in the master mix. We highly recommend the use of UNG. In the event that UNG is not used in the reaction, the initial PCR hold cycle of 50 °C for 2 min can be omitted.
8. This step is to activate AmpliTaq Gold® DNA polymerase and simultaneously inactivate the UNG.
9. The Ct value is where the threshold line crosses the amplification curve. By setting a threshold line, the Ct value for each sample is established. Depending on the instrument, the threshold can be set manually and/or automatically, in addition, the Ct value can also be affected by the software features of individual instrument for data normalization and analysis. Regardless of fluorescent signal strength, the presence of a Ct value indicates a positive PCR amplification of target. Pay special attention to samples with atypical amplification curve (e.g., linear) or extremely weak signal strength. Consider repeating the individual sample run or confirming with a monoplex PCR assay.

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## Validation of a Sensitive and Specific Real-Time PCR for Detection and Quantitation of Hepatitis B Virus Covalently Closed Circular DNA in Plasma of Chronic Hepatitis B Patients

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### Abstract

Hepatitis B virus (HBV) covalently closed circular DNA (cccDNA) serves as a template for viral replication and plays a role in persistence of HBV infection. The origin and significance of cccDNA in plasma, however, are not well understood. A sensitive, specific, and reproducible real-time PCR for detection and quantitation of cccDNA in plasma of chronic hepatitis B patients was developed and validated. Four HBV DNA reference panels and 96 plasma samples of chronic hepatitis B patients are analyzed. Results are compared with total HBV DNA levels. This cccDNA assay had a lower limit of detection at 15 copies/PCR, a lower limit of quantitation at 91 copies/PCR, and a correlation coefficient ( $R$ ) of 0.98 ( $p < 0.0001$ ). HBV cccDNA can be detected in two of four international panels. Significant correlation is found between cccDNA and total HBV DNA levels in both panels ( $R = 0.96$  and  $R = 0.43$ ) and in samples of the chronic hepatitis B patients ( $R = 0.88$ ,  $p < 0.0001$ ). In 57 % of these samples cccDNA can be detected. Mean level of cccDNA is 0.16 % of total HBV load. Plasma HBV cccDNA levels are higher in HBeAg-positive samples than in HBeAg-negative samples ( $p < 0.0001$ ). Total HBV DNA levels and HBV genotype do not influence cccDNA detection.

**Key words:** cccDNA, HBV, Plasma, Quantitation, Real-time PCR

### List of Abbreviations

HBV	Hepatitis B virus
cccDNA	Covalently closed circular DNA
ALT	Alanine transaminase
HAI	Histology activity index
PCR	Polymerase chain reaction
BSA	Bovine serum albumin
q-PCR	Quantitative real-time PCR
SC	Silica particles
SiO <sub>2</sub>	Silicon dioxide
GuSCN	Guanidinium thiocyanate

HCl	Hydrochloric acid
EDTA	Ethylenediaminetetraacetic acid
Tris buffer	Trisamine (hydroxymethylaminoethane) buffer
TE buffer	Tris/HCl/EDTA buffer

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## 1. Introduction

Hepatitis B virus (HBV) is a member of the Hepadnaviridae group and its infection is an important cause of self-limiting or chronic hepatitis. Approximately 350 million people in the world are chronically infected with HBV (1). A proportion of these chronic hepatitis B patients develop liver cirrhosis and hepatocellular carcinoma (2, 3).

In viral particles, the 3.2-kb-long HBV genome consists of a partially double-stranded relaxed circular DNA. Upon infection, this relaxed circular DNA is transported to the nucleus of the hepatocyte to form a covalently closed circular DNA (cccDNA). There, cccDNA is transcribed into viral RNA, which serves either as viral pregenomic RNA, or as mRNA coding for the polymerase, core, X, and surface proteins. Pregenomic RNA is transported to the cytosol and encapsulated by core particles, which after reverse transcription by the polymerase accounts for the formation of the single-stranded negative DNA strand. Subsequently, pregenomic RNA is degraded and the negative DNA strand serves as a template for the positive DNA strand, forming relaxed circular DNA. The HBV genome can either form new viral particles, or recycle back into the nucleus to maintain a pool of cccDNA (4, 5). In chronic hepatitis B, cccDNA persists as a stable microsome in the nuclei of infected hepatocytes, and serves as a template for viral replication. This results in a steady-state population of 5–50 cccDNA molecules per infected hepatocyte (6, 7). Eradication of (chronic) HBV infection requires the elimination of cccDNA from infected hepatocytes (4).

Besides qualitative analysis, the introduction of real-time PCR has made it possible to reliably quantify cccDNA. This quantification is important in order to understand the natural history and management of chronic hepatitis B. Studies have shown that HBeAg-positive chronic hepatitis B patients contain significantly more cccDNA molecules per hepatocyte than HBeAg-negative patients, and that intrahepatic cccDNA is strongly correlated with total plasma HBV DNA levels (8–13). In HBeAg-negative patients the lower HBV viral load is due to lower intrahepatic cccDNA levels, and to impaired total HBV DNA production (12). Antiviral therapy causes reduction of intrahepatic cccDNA and studies showed that lower intrahepatic cccDNA levels after therapy can result in a sustained response (9, 10, 13, 14). Until now, most studies have focused on intrahepatic cccDNA. Five studies also describe the role

of cccDNA in serum of chronic hepatitis B patients (11, 15–18). However, from these studies it is unclear if circulating cccDNA in plasma is correlated with inflammation in the liver. Therefore, we developed a sensitive real-time PCR for the detection of cccDNA in plasma.

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## 2. Materials

### 2.1. Construction of an HBV cccDNA Positive Control (cccDNA Plasmid)

- 100  $\mu\text{L}$  of a plasma sample containing HBV cccDNA and approximately  $10^8$  copies/mL of total HBV DNA as earlier determined by COBAS<sup>®</sup> TaqMan 48<sup>®</sup> assay [CAP/CTM] (Roche, Branchburg, NJ, USA).
- Proteinase K solution (1 mL 5 M NaCl, 2 mL 10% SDS, 1 mL 1 M Tris/0.1 M EDTA (pH 8.0), and 1 mL proteinase K (20 mg/mL)).
- For extraction by Boom method (19):
  - Silica particles (SC) ( $\text{SiO}_2$ ).
  - Lysis buffer (0.1 M Tris/HCl, GuSCN, Triton X-100, and 0.2 M EDTA).
  - Wash buffer (0.1 M Tris/HCl, GuSCN).
  - 70 % Ethanol.
  - 100 % Acetone.
  - TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]) (19).
  - Heat block.
- Selective primers for cccDNA (9):
  - Forward primer: HBV CCCF (5'-CTCCCCGTCTGT GCCTTCT-3') (Biolegio, Nijmegen, The Netherlands).
  - Reverse primer: HBV CCCR (5'-GCCCCAAAGCCAC CCAAG-3') (Biolegio, Nijmegen, The Netherlands).
- PCR mix: 1 $\times$  PCR II buffer, 2.5 mM  $\text{MgCl}_2$ , 0.05 U/ $\mu\text{L}$  Amplitaq Gold, 1.0 mM dNTP, and 0.1  $\mu\text{g}/\mu\text{L}$  BSA.
- TOPO TA Cloning<sup>®</sup> Kit (pCR<sup>®</sup> II TOPO<sup>®</sup>: Invitrogen, Breda, The Netherlands).
- Restiction enzyme: *Bam*H I.
- MEGAscript<sup>®</sup> T7 Kit (Ambion).
- Thermocycler.
- NanoDrop<sup>®</sup> ND-1000 Spectrophotometer V3.0.0 (Labtech International Ltd, Ringmer, United Kingdom).
- Didioxy sequencing using the SQL Lims<sup>®</sup> system (Applied Biosystems; Nieuwerkerk aan de IJssel, The Netherlands).

## **2.2. Construction of a Noncompetitive DNA Internal Control**

1. Hybridization of the complementary sequences 5'-TGA CCT TAT CAG TGT AAT GAA CCG CCG CAT TGA GGA GAT CTG CAC CCT TTA CAT CTT TCT GAA GTA GGG G-3' and 5'-CCC CCT ACT TCA GAA AGA TGT AAA GGG TGC AGA TCT CCT CAA TGC GGC GGT TCA TTA CAC TGA TAA GG-3' (Advanced Biotechnologies Inc., Columbia, MD) (20).
2. Primers CMV-517 (5'-GAT GAG GAG AGA GAC AAG GTG C-3') and CMV-1113 (5'-CTC AGA CAC TGG CTC AGA CTT G-3').
3. PCR mix: 1× PCR II buffer, 2.5 mM MgCl<sub>2</sub>, 0.05 U/μL Amplitaq Gold, 1.0 mM dNTP, and 0.1 μg/μL BSA.
4. Thermocycler.
5. Vector (pCRII; 3,932 bp; Promega, Leiden, The Netherlands).
6. Restriction enzymes *Hha*I, *Aoc*I, and *Hind*III.
7. NanoDrop® ND-1000 Spectrophotometer V3.0.0 (Labtech International Ltd, Ringmer, United Kingdom).
8. Didioxy sequencing using the SQL Lims® system (Applied Biosystems; Nieuwerkerk aan de IJssel, The Netherlands).

## **2.3. DNA Purification with IC-DNA**

1. MagNA Pure LC (Roche Lifescience, Penzberg, Germany).
2. MagNA Pure LC Total Nucleic Acid Isolation Kit (Roche Diagnostics, Mannheim, Germany).
3. Construct of the noncompetitive DNA internal control (IC-DNA) from Subheading 2.2 (21).
4. Construct of HBV cccDNA positive control (cccDNA plasmid) from Subheading 2.1.
5. Panels:
  - (a) The WHO international standard of hepatitis B (NIBSC, Potters Bar, United Kingdom).
  - (b) A quantitative control panel (QC-panel, Sanquin, Amsterdam, The Netherlands).
  - (c) A 12-member OptiQuant® HBV DNA quantitation panel (AcroMetrix Europe BV, Alkmaar, The Netherlands).
  - (d) A 15-member HBV genotype panel (Teragenix; HemaCare BioScience, Ft Lauderdale, USA).
6. Clinical samples.

## **2.4. Real-Time PCR of HBV cccDNA with IC-DNA**

1. Lightcycler® 480 Real-Time PCR System (LC480) (Roche Diagnostics, Almere, The Netherlands).
2. PCR master mix (Lightcycler® 480 Probes Master, Roche Diagnostics, Mannheim, Germany).

3. Selective primers for cccDNA and IC-DNA:
  - (a) Forward primer cccDNA: HBV CCCF (5'-CTCCCC GTCTGTGCCTTCT-3') (Biolegio, Nijmegen, The Netherlands).
  - (b) Reverse primer cccDNA: HBV CCCR (5'-GCCCAAAGC CACCCAAG-3') (Biolegio, Nijmegen, The Netherlands).
  - (c) Forward primer IC-DNA: IC F1 (5'-CAA GCG GCC TCT GAT AAC CA-3') (Biolegio, Nijmegen, The Netherlands).
  - (d) Reverse primer IC-DNA: IC R1 (5'-ACT AGG AGA GCA GAC TCT CAG AGG AT-3') (Biolegio, Nijmegen, The Netherlands).
4. Probes:
  - (a) HBV cccDNA probe: 5'-6FAM-CGTCGCATGGARACC ACCGTGAACGCC-BHQ1-3' (Biolegio, Nijmegen, The Netherlands).
  - (b) IC-DNA probe: 5'-HEX-TBRCCCTTTACATCTTTCTG AAGTAGGG-BHQ-3' (Biolegio, Nijmegen, The Netherlands).

**2.5. Assessment of Lower Limit of Detection, Linearity, and Reproducibility of HBV cccDNA Real-Time PCR**

1. MagNA Pure LC (Roche Lifescience, Penzberg, Germany).
2. PCR master mix (Lightcycler® 480 Probes Master, Roche Diagnostics, Mannheim, Germany).
3. MagNA Pure LC Total Nucleic Acid Isolation Kit (Roche Diagnostics, Mannheim, Germany).
4. HBV, HCV, and HIV negative plasma (Sanquin, Amsterdam, The Netherlands).
5. 12–15 Replicates of tenfold serial dilution of HBV cccDNA positive control from Subheading 2.1.
6. The noncompetitive IC-DNA with a constant amount of  $10^3$  copies/extraction from Subheading 2.2.

**2.6. Specificity of the HBV cccDNA Assay**

1. MagNA Pure LC (Roche Lifescience, Penzberg, Germany).
2. PCR master mix (Lightcycler® 480 Probes Master, Roche Diagnostics, Mannheim, Germany).
3. MagNA Pure LC Total Nucleic Acid Isolation Kit (Roche Diagnostics, Mannheim, Germany).
4. Plasma from an HBV cccDNA negative sample with high total HBV DNA.
5. Background of HBV, HCV, and HIV negative plasma (Sanquin, Amsterdam, The Netherlands).
6. A noncompetitive IC-DNA with a constant amount of  $10^3$  copies/extraction from Subheading 2.2.
7. Amount of  $10^5$  HBV cccDNA plasmid copies in extraction from Subheading 2.1.

### **2.7. HBV cccDNA in HBV DNA Reference Panels**

1. Reference panels:
  - (a) Plasma samples of chronic HBV-positive samples.
  - (b) The WHO international standard of Hepatitis B (NIBSC, Potters Bar, United Kingdom).
  - (c) A quantitative control panel (QC-panel, Sanquin, Amsterdam, The Netherlands).
  - (d) A 12-member OptiQuant® HBV DNA quantitation panel (AcroMetrix Europe BV, Alkmaar, The Netherlands).
  - (e) A 15-member HBV genotype panel (Teragenix; HemaCare BioScience, Ft Lauderdale, USA).
  - (f) A tenfold serial dilution of the HBV cccDNA positive control (corresponding with  $10^4$  down to 10 copies/PCR of total HBV DNA).
2. MagNA Pure LC (Roche Lifescience, Penzberg, Germany).
3. MagNA Pure LC Total Nucleic Acid Isolation Kit (Roche Diagnostics, Mannheim, Germany).

### **2.8. HBV cccDNA in Plasma Samples of Chronic Hepatitis B Patients**

1. Plasma samples of chronic hepatitis B patients were analyzed for the presence of cccDNA.
2. Total HBV DNA will be determined by the COBAS® TaqMan 48® assay (Hoffmann-La Roche Ltd, Basel, Switzerland), which has a lower limit of detection 20 IU/mL and a dynamic range up to  $1.70 \times 10^8$  IU/mL.
3. MagNA Pure LC (Roche Lifescience, Penzberg, Germany).
4. MagNA Pure LC Total Nucleic Acid Isolation Kit (Roche Diagnostics, Mannheim, Germany).

### **2.9. Influence of HBV Genotypes on cccDNA Detection and Quantification**

1. Plasma samples of chronic hepatitis B patients were analyzed for HBV genotype.
2. HBV genotype determined by didoxy sequencing using the SQL Lims® system (Applied Biosystems; Nieuwerkerk aan de IJssel, The Netherlands).
3. MagNA Pure LC (Roche Lifescience, Penzberg, Germany).
4. PCR master mix (Lightcycler® 480 Probes Master, Roche Diagnostics, Mannheim, Germany).
5. MagNA Pure LC Total Nucleic Acid Isolation Kit (Roche Diagnostics, Mannheim, Germany).
6. A 15-member HBV genotype panel (Teragenix; HemaCare BioScience, Ft Lauderdale, USA).

### 3. Methods

#### **3.1. Construction of an HBV cccDNA Positive Control (HBV cccDNA Plasmid)**

The selected clinical plasma sample with approximately  $10^8$  copies/mL of total HBV DNA is used for the construction of an HBV cccDNA positive control (HBV cccDNA plasmid), because it is known from the literature that HBV cccDNA is present in a lower concentration than total HBV DNA (16). One-hundred microliters of plasma is added to 100  $\mu$ L proteinase K solution and incubated for 30 min at 56 °C. After incubation the material is purified by adding the 900  $\mu$ L lysis buffer and 20  $\mu$ L SC and is incubated at room temperature for 10 min. then washed twice with wash buffer, twice with 70 % ethanol, and once with acetone; then dried and subsequently eluted in 100  $\mu$ L TE buffer (19). Selective primers for HBV cccDNA (HBV CCCF en HBV CCCR; Subheading 2.1) are used, targeting the gap region that is only present in HBV cccDNA between the two Direct Repeat Regions (DR1 and DR2). PCR is performed with 15  $\mu$ L of the extracted plasma sample and 10  $\mu$ L of PCR mix: 1 $\times$  PCR II buffer, 2.5 mM MgCl<sub>2</sub>, 1.0 mM dNTP, 0.1  $\mu$ g/ $\mu$ L BSA, 0.05 U/ $\mu$ L Amplitaq Gold. Amplification of cccDNA is performed as follows: 50 °C for 2 min, then 95 °C for 10 min, followed by 45 cycles of 95 °C for 10 s, 58 °C for 5 s, 10 s at 63 °C, and 20 s at 72 °C (see Note 1). Two microliters of PCR product are then used in the TOPO TA cloning<sup>®</sup> mix containing the plasmid vector and 3  $\mu$ L of the TOPO<sup>®</sup> cloning reaction are used for transformation into the TOP10 competent cells. The plasmid is purified from bacterial cultures, and in vitro transcription is performed on *Bam*H I-linearized plasmid using the T7 promoter (see Note 1), resulting in an HBV cccDNA positive control (HBV cccDNA plasmid). The concentration of the HBV cccDNA plasmid is measured by OD calibration with the NanoDrop<sup>®</sup> ND-1000 Spectrophotometer V3.0.0 (see Note 1). Finally, the integrity of the amplicon must be determined by didoxy sequencing, using the SQL Lims<sup>®</sup> system (see Note 1).

#### **3.2. Construction of a Noncompetitive DNA Internal Control**

A 597-bp amplicon was generated by PCR from CMV AD169 DNA (Subheading 2.2) with primers CMV-517 (Subheading 2.2; nt positions 2,021–2,042) and CMV-1113 (Subheading 2.2; nt positions 2,596–2,617). The amplicon was digested with *Aoc*I, and a 227-bp fragment (nt positions 2,021–2,248) was purified from the gel. The amplicon was also digested with *Alw*I, and a 296-bp fragment (nt positions 2,321–2,617) was purified from the gel. A double-stranded DNA sequence was obtained from in vitro-synthesized single-stranded DNAs (by hybridization of the complementary sequences). This double-stranded DNA sequence contained DNA overhangs and was ligated to the *Aoc*I site of the 227-bp fragment and to the *Alw*I site of the 296-bp fragment, generating a 597-bp fragment (20). This fragment was purified

from the gel and amplified by PCR with primers CMV-517 and CMV-1113, and the amplicon was cloned into a plasmid vector PCRII by adding 2  $\mu$ L of PCR product to the cloning mix containing the plasmid vector PCRII and 3  $\mu$ L of the cloning reaction are used for transformation into the competent cells. The plasmid is purified from bacterial cultures, and in vitro transcription is performed on *Hind*III-linearized plasmid using the T7 promoter (see Note 1), resulting in the noncompetitive IC-DNA which will serve as the IC-DNA (20). The concentration of the IC-DNA plasmid is measured by OD calibration with the NanoDrop<sup>®</sup> ND-1000 Spectrophotometer V3.0.0 (see Note 1). Finally, the integrity of the amplicon must be determined by didoxy sequencing, using the SQL Lims<sup>®</sup> system (see Note 1). For real-time PCR a target of 597 bp is too large; therefore a 126 bp fragment of the 597-bp plasmid was chosen, using the primers IC F1 and IC R1 for real-time PCR (Subheading 2.4). Negative results for HBV cccDNA were considered truly negative when the CP values for co-extracted IC-DNA were all positive.

### **3.3. DNA Purification with IC-DNA**

Plasma samples, the WHO international HBV DNA standard, reference standards (WHO-, QC-, AcroMetrix-, and Teragenix-panel), and tenfold serial dilutions of the HBV cccDNA positive control from Subheading 3.1 (corresponding with  $10^4$  down to 10 copies/PCR of total HBV DNA) are isolated using the MagNA Pure LC (see Note 2). The noncompetitive IC-DNA (from Subheading 3.2) of  $10^3$  copies/extraction is used to monitor extraction and amplification efficiency.

### **3.4. Real-Time PCR of HBV cccDNA with IC-DNA**

Real-time PCR is performed using the Lightcycler<sup>®</sup> 480 (LC480) using 10  $\mu$ L of the eluate and 20  $\mu$ L of PCR mix, using selective primers and probes for HBV cccDNA and IC-DNA (Subheading 2.4). Final primer concentrations should be 0.9  $\mu$ M for the target and IC-DNA primers. The final probe concentration for HBV cccDNA should be 0.4  $\mu$ M and the probe concentration for IC-DNA 0.2  $\mu$ M (see Note 2). Amplification is performed as follows: 50 °C for 2 min, then 95 °C for 10 min, followed by 55 cycles of 95 °C for 10 s, 58 °C for 5 s, 10 s at 63 °C, and 20 s at 72 °C. Every run must include a negative plasma sample, water, and a positive control with a known concentration of HBV cccDNA ( $10^3$  copies/PCR). The results are analyzed with LightCycler<sup>®</sup> 480 software v1.5.0.39 (see Note 2).

### **3.5. Assessment of Lower Limit of Detection, Linearity, and Reproducibility of HBV cccDNA Real-Time PCR**

The lower limit of detection is defined as the lowest concentration of HBV cccDNA that yields a positive result. To determine the lower limit of detection, linearity, and reproducibility, tenfold serial dilutions of the HBV cccDNA positive control from Subheading 3.1 (containing  $10^3$  copies IC-DNA from Subheading 3.2) are extracted using the MagNA Pure<sup>®</sup> LC (see Note 3). The HBV cccDNA is

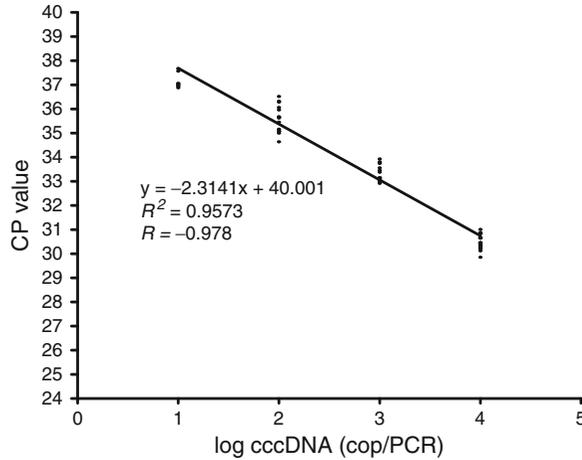


Fig. 1. Linearity of the HBV cccDNA assay. CP values in 15 replicates of tenfold serial dilutions of cccDNA plasmid after extraction in the MagnaPure<sup>®</sup> and amplification in the LC480<sup>®</sup>.

eluted in 100  $\mu$ L elution buffer using the MagNA Pure LC Total Nucleic Acid Isolation Kit and 10  $\mu$ L of the extracted HBV cccDNA is used in real-time PCR. All samples must be analyzed in triplicate (intra-assay variation) and on five consecutive days (inter-assay variation); thus a total of 15 samples are analyzed by real-time PCR per dilution. Figure 1 shows the results of the linearity assessment of the HBV cccDNA assay. The linear regression plot has a slope of  $-2.31$  with a correlation coefficient ( $R$ ) of  $-0.98$  ( $p < 0.0001$ ) (see Note 3). The most rigorous and classical method of determining amplification efficiency is by examining the slope of the dynamic range. An assay with 100 % efficiency yields a  $-3.33$  curve slope, a relatively narrow standard deviation about the mean, and a correlation coefficient ( $R$ ) of 1.000 (see Note 2). This results in a lower limit of detection with a 50 % rate of 15 copies/PCR (95 % CI 5–50 copies/PCR) and a lower limit of quantitation with a 95 % rate at 91 copies/PCR (95 % CI 55–98 copies/PCR) (Table 1).

### 3.6. Specificity of the HBV cccDNA Assay

To test whether the primers and probe of the HBV cccDNA assay cross-react with HBV relaxed circular (total) DNA, the following experiment is performed (see Note 3). Tenfold serial dilutions of plasma from an HBV cccDNA negative sample with a high total HBV DNA content ( $>1.70 \times 10^8$  IU/mL) are performed in HBV-, HCV-, and HIV-negative plasma and to half of the serial dilution a constant amount of  $10^5$  HBV cccDNA copies is added before extraction. One hundred microliters of this are extracted in the MagNA Pure LC and eluted in 100  $\mu$ L of the elution buffer. Finally, 10  $\mu$ L of the extracted samples are used for the detection of HBV cccDNA by real-time PCR (LC480). Results are shown in Fig. 2. The flat line represents the serial dilution of total HBV DNA sample without HBV cccDNA. The curved lines represent

**Table 1**  
**Lower limit of detection and quantitation of the HBV cccDNA assay, as determined in tenfold serial dilutions**

cccDNA (copies/mL)	cccDNA in extraction <sup>a</sup> (copies/100 µL)	cccDNA in PCR <sup>a</sup> (copies/10 µL)	<i>n</i>	Positive (%)
1,000,000	100,000	10,000	15	15 (100)
100,000	10,000	1,000	15	15 (100)
10,000	1,000	100	15	15 (100) <sup>b</sup>
1,000	100	10	15	7 (47) <sup>c</sup>
100	10	1	15	0 (0)

*n* number of replicates

<sup>a</sup>Assuming 100 % extraction and amplification efficiency

<sup>b</sup>95 % Hit rate at 91 copies/PCR (95 % CI 55–98 copies/PCR)

<sup>c</sup>50 % Hit rate at 15 copies/PCR (95 % CI 5–50 copies/PCR)

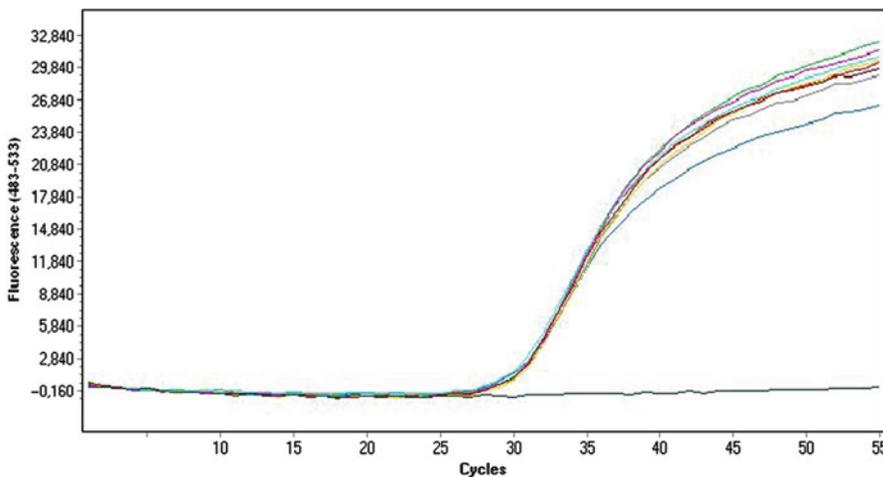


Fig. 2. Specificity of the cccDNA assay. The *flat line* represents the serial dilutions of total HBV DNA sample without HBV cccDNA. The *curved lines* represent tenfold serial dilutions of total HBV DNA spiked with constant concentration of HBV cccDNA ( $10^5$  copies/extraction).

tenfold serial dilutions of total HBV DNA spiked with HBV cccDNA. Only the samples spiked with  $10^5$  copies HBV cccDNA produce an amplification curve (CP-value). The assay is specific for only HBV cccDNA and no differences between CP values of spiked HBV cccDNA could be obtained in the different concentrations of total HBV DNA, indicating that no cross-reactivity exists between HBV cccDNA and total HBV DNA (see Note 3).

**3.7. HBV cccDNA in HBV DNA Reference Panels**

As reference panels the WHO international standard of hepatitis B, a quantitative control panel (QC-panel), a 12-member OptiQuant<sup>®</sup> HBV DNA quantitation panel (AcroMetrix-panel), and a 15-member

HBV genotype panel (Teragenix-panel) are used. One hundred microliters of each panel member (spiked with  $10^3$  copies IC-DNA from Subheading 3.2) are extracted using the MagNA Pure LC and eluted in 100  $\mu\text{L}$  elution buffer and 10  $\mu\text{L}$  of the extraction are used for real-time PCR. Samples are analyzed in triplicate on two consecutive days and thus a total of six replicates are analyzed with the LC480. The WHO—and QC—panel tests negative for HBV cccDNA. Only in the Teragenix and Acrometrix panel members can HBV cccDNA be detected. The negative results in the WHO- and QC-panel for HBV cccDNA may be due to the composition of the panel material (low viral loads of total HBV DNA). The viral loads of total HBV DNA in the WHO- and QC-panels are much lower compared with the Teragenix- and Acrometrix-panels who have a high total HBV DNA load ( $1.25 \times 10^7$  to  $2.0 \times 10^9$  copies/mL) and it is known from the literature that HBV cccDNA is present in a lower concentration than total HBV DNA (16).

### 3.8. HBV cccDNA in Plasma Samples of Chronic Hepatitis B Patients

In plasma samples of chronic hepatitis B patients to be analyzed for the presence of HBV cccDNA, the total HBV DNA load is determined using the COBAS<sup>®</sup> TaqMan 48<sup>®</sup> assay, which has a lower limit of detection of 20 IU/mL and a dynamic range up to  $1.70 \times 10^8$  IU/mL. To convert IU/mL into copies/mL a 5.82 conversion factor is applied. Analysis for HBV cccDNA is done as follows: 100  $\mu\text{L}$  of each plasma sample (including  $10^3$  copies IC-DNA from Subheading 3.2) are extracted in the MagNA Pure LC and eluted in 100  $\mu\text{L}$  of the elution buffer. Ten microliters of the extracted sample are used in real-time PCR for the detection of HBV cccDNA. As shown in the example in Fig. 3 plasma samples

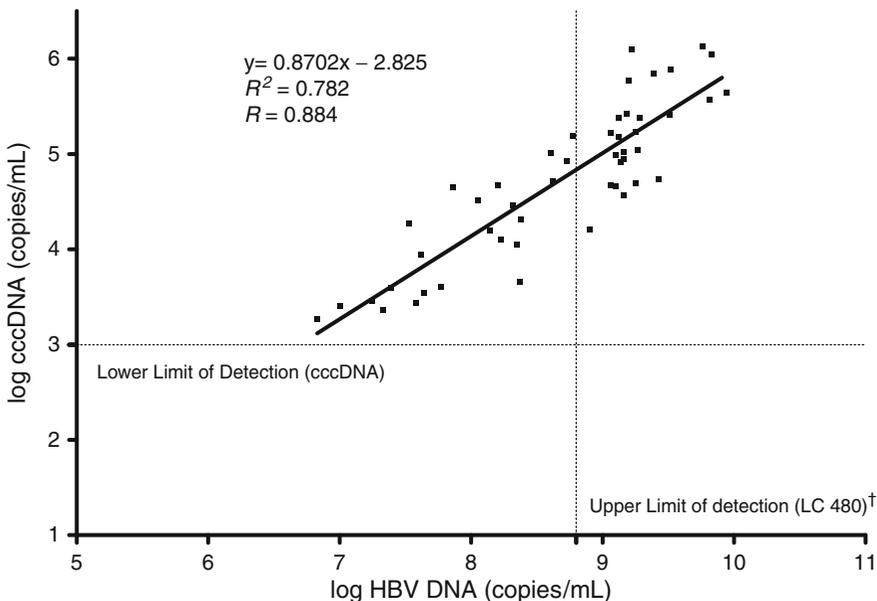


Fig. 3. Correlation between HBV cccDNA and total HBV DNA in plasma samples of chronic hepatitis B patients.

of 96 chronic hepatitis B patients were analyzed for HBV cccDNA and in 55/96 (57 %) samples cccDNA was detectable. In these samples a good linearity between the HBV cccDNA (log copies/mL) and total HBV DNA (log copies/mL) is seen ( $R$  of 0.88;  $p < 0.0001$ ) (see Note 3).

### **3.9. Influence of HBV Genotypes on cccDNA Detection and Quantitation**

To determine whether the HBV genotype influences HBV cccDNA quantitation, quantified HBV cccDNA levels are compared using different genotypes. HBV genotypes are determined by sequencing using the didoxy technology, with the SQL Lims<sup>®</sup> system software. The HBV cccDNA assay detected HBV cccDNA independent of the genotype. Genotypes A through E were present in these plasma samples and in the Teragenix panel members HBV cccDNA was detected in the panel samples with genotype A, C, D, and F.

## **4. Notes**

1. Potential pitfalls in the construction of plasmid/a positive control (see Table 2).
2. Potential pitfalls in the construction of a q-PCR (see Table 3).
3. Potential pitfalls in the validation of a new assay (see Table 4).

**Table 2**  
**Potential pitfalls in the construction of plasmid/a positive control**

<b>Preparing plasmid/ positive control workflow</b>	<b>Factors affecting plasmid results</b>	<b>Problems</b>
Sample	Primer designs PCR conditions ↓	Check primer design (BLAST), concentration of primers, amplicon length and PCR program.
Cloning	Agar plates TA competent cells SOC medium Colony PCR ↓	Were agar plates at room temperature with appropriate antibiotics and checked for self life before use ? Were TA competent cells stored at $-80^{\circ}\text{C}$ at arrival ? Use SOC medium at room for culturing cloned plasmids. Check cloned products by PCR either by universal M13 primers or specific primers used for cloning.

(continued)

**Table 2**  
(continued)

Preparing plasmid/ positive control workflow	Factors affecting plasmid results	Problems
Culture	Tube/bottle ↓	Tightly closed? Enough space in bottle or tube for oxygen? Appropriate selective antibiotics added to the medium?
Purification plasmid/ miniprep	RNase Alcohol Buffers ↓	RNase added to buffer? RNase not expired? Alcohol added to appropriate solutions and appropriate volume? Check storage conditions and creation date of buffers, especially those containing alcohol (evaporation of alcohol)? Precipitation in the buffers? Sequence of restriction enzyme not in target sequence?
OD measurement	Yield ↓	Good 260/280 ratio? Check OD of reference
Sequencing plasmid	No or bad sequence result	Check amount of plasmid input for sequencing More than 50 ng/μL plasmid used in sequence reaction? Universal M13 or specific primers used?

**Table 3**  
**Potential pitfalls in the construction of a q-PCR**

q-PCR workflow	Factors affecting q-PCR results	Problems
Sample type	Blood/serum/plasma/ tissue ↓	Suitable for assay?
Sample storage	Fresh/frozen/archival ↓	Is target RNA or DNA? Correct storage temperature. Number of freeze/thaw steps?
Extraction	Commercial automatic or by hand Input material Input volume ↓	Extraction method suitable for the used material? Input volume is always dependent on the method and/or material

(continued)

**Table 3**  
(continued)

q-PCR workflow	Factors affecting q-PCR results	Problems
PCR conditions	Target amplification Types of detection chemistry Primer/probe design Primer/probe final concentrations HotStart strategy Thermostable DNA polymerases Buffer conditions Cycling conditions: Ramping rate/annealing temperature and time/ extension time Real-time instrument performance	Amplicon length: 50–210 bp Competition (for ingredients) between target and internal control (IC)? Optimal primer/probe design? Primer <ol style="list-style-type: none"> <li>1. Length 19–23 nucleotides</li> <li>2. GC content: 35–65 %</li> <li>3. <math>T_m</math>: 60–68 °C</li> <li>4. 3' End stability: Composition of last 3 bp</li> <li>5. Complementaries: Avoid primer self- or cross annealing stretches &gt;4 bp</li> <li>6. Specificity: Blast primer sequence</li> </ol> Probe <ol style="list-style-type: none"> <li>1. Length 18–30 nucleotides, optimum = 20</li> <li>2. GC content: 20–80 %</li> <li>3. <math>T_m</math>: 8–10 °C higher than primers <math>T_m</math></li> <li>4. 3' End stability: Composition of last 5 bp</li> </ol> Efficiency of final primer/probe concentrations? PCR mix stability? Free of inhibitors of PCR? Proper PCR cycling conditions? 100 % PCR efficiency? <ol style="list-style-type: none"> <li>1. Slope <math>-3.33</math></li> <li>2. <math>R^2 = 1</math></li> </ol>
Data analysis	Absolute quantitation Relative quantitation: Standard curve Correction of PCR efficiencies	All standards and samples amplified equally?

**Table 4**  
**Potential pitfalls in the validation of a new assay**

<b>Validation of the (duplex) assay workflow in background of clinical material</b>	<b>Factors affecting validation results</b>	<b>Problems</b>
Linearity (minimum of 10–12-fold)	Primer/probe design Extraction method Input volume in extraction and in PCR reaction	Optimal primer/probe design? PCR conditions? 100 % PCR efficiency? Inhibition of clinical material?
	↓	
Intra- and inter-assay (minimum of 12-fold; each 3×4)	No reproducibility	One or more persons? Storage of material between runs?
	↓	
Lower limit of detection (20-fold)	No sensitivity	Competition between target and IC?
	↓	
Specificity	Assay not specific enough	Primer/probe design specific enough? Blast more than 500 target sequences to achieve maximum specificity in silico
	↓	
Testing clinical samples	Storage conditions	-70 °C or -20 °C? How many freeze/thaw steps?

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## Protocol for the Detection of *Mycoplasma genitalium* by PCR from Clinical Specimens and Subsequent Detection of Macrolide Resistance-Mediating Mutations in Region V of the 23S rRNA Gene

Jørgen Skov Jensen

### Abstract

*Mycoplasma genitalium* is an established cause of male nongonococcal urethritis, in particular in cases with recurrent disease and in those negative for *Chlamydia trachomatis*. In women *M. genitalium* causes cervicitis and there is increasing evidence that it is causing pelvic inflammatory disease (PID). Nucleic acid amplification tests are currently the only available methods for detection, but no commercially available tests have been thoroughly evaluated. Here we describe a TaqMan-based PCR test for detection of the infection and a conventional PCR that serves as a confirmatory assay with the possibility of sequencing the product for detection of macrolide resistance-mediating mutations.

**Key words:** *Mycoplasma genitalium*, Macrolide resistance, Sample preparation, Chelex

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### 1. Introduction

*Mycoplasma genitalium* has been established as a cause of STIs in both men and women (1). The bacterium is extremely difficult to isolate by culture (2) and serology is hampered by extensive cross-reactions with the closely related *M. pneumoniae*. Consequently, nucleic acid amplification tests (NAATs) are currently the only tools available for detection of *M. genitalium*. Because of a very low load of mycoplasmas in some patients (3), tests with a very low limit of detection are needed in order to achieve sufficient assay sensitivity. No FDA-approved commercial assays have been made available but promising results with research-use-only kits have been presented (4), and very recently, a CE-marked triplex real-time assay detecting *Chlamydia trachomatis*, *N. gonorrhoeae*, and *M. genitalium* has been introduced on the European market from BioRad.

For all STIs, the clinical specificity of the test is essential. Apart from the use of highly specific primers, and for TaqMan™ assays also a probe, specificity can be increased by applying a confirmatory assay. This assay should detect an unrelated sequence from the same species, preferably with a limit of detection comparable to that of the primary assay.

In this protocol, a confirmatory assay amplifying the 23S rRNA gene is described. This assay allows subsequent sequencing of region V of the 23S rRNA gene to be performed with the aim of demonstrating the most common macrolide resistance-mediating mutations (5). These mutations have been shown to occur very often in patients failing treatment with azithromycin, both when the treatment failure is due to selection of the mutations during treatment with an inappropriate dosage of 1 g azithromycin given as a single dose and in cases where the infection is caused by infection with a resistant strain of *M. genitalium*.

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## 2. Materials

Use only molecular grade water for all reagents for DNA preparation and PCR (see Note 1).

### 2.1. Clinical Specimens

The assay has been validated on male and female first void urines (FVUs) and urethral swabs, and on female cervical and vaginal swab specimens. Swabs can be collected in a range of transport media, but Copan universal transport medium (UTM) is widely available, and the flocced swabs that are part of specimen collection kits release more material than regular swabs. The collection kits Copan 361C for urethral swab and 259 for cervical and vaginal swabs contain only 1 mL of transport medium leading to less dilution of the specimen. This is essential with the simple protocol used for DNA extraction described here.

FVU specimens can be transported in sterile propylene tubes, but transport tubes containing nucleic acid-stabilizing components may be an advantage when sample preparation cannot be performed within a few days. Such transport tubes are available from Sierra Molecular Corp. Sonora, CA, USA, as Genelock™ transport medium.

### 2.2. DNA Preparation

A simple lysis protocol is used where the specimen is boiled in a 20 % slurry of Chelex 100 resin in TE buffer.

1. TE buffer: 10 mM Tris-HCl, pH 8.0, 1 mM EDTA  
Mix 0.50 mL 100× TE buffer concentrate (Sigma molecular grade) with 49.5 mL water (see Note 1).

## 2. 20 % Chelex 100 slurry

In a sterile disposable beaker (see Note 2) with a sterile magnetic stirring bar, weigh out 10 g of Chelex 100 (BioRad). Add TE buffer to 50 mL. Stir on a magnetic stirrer in a clean flow hood until completely suspended. While stirring, take out 300  $\mu$ L of the slurry with a 1 mL pipette tip (see Note 3) and add to 0.5 mL Eppendorf tubes. Store the tubes at +4 °C until use for up to 1 month.

## 3. Plasticware

Extra long 200 and 1 mL pipette tips with filter for aspiration of the transport medium and urine, respectively, without contaminating the barrel of the pipette (see Note 3). Single-use Pasteur pipette for aspiration of urine after centrifugation.

### 2.3. MgPa TaqMan™ Real-Time PCR Assay

## 1. Primers and probes (5'–3')

MgPa-355F: GAGAAATACCTTGATGGTCAGCAA.

MgPa-432R: GTTAATATCATATAAAGCTCTACCGTTGT  
TATC.

MgPa-380-TaqMan MGB: 6FAM-ACCTTGCAATCAGAA  
GGT-MGB (see Note 4).

These primers amplify a 78 bp fragment of the *mgpB* gene (MG191) with a 35 % GC content.

Internal processing control (IPC) probe.

Lambda IPC TaqMan TAMRA-TCCTTCGTGATATC  
GGACGTTGGCTG-BHQ2 (see Note 5).

Primers for producing the IPC (5'–3')

Mg-IPC-f GAGAAATACCTTGATGGTCAGCAAT**TTCCG**  
**GGACGTATCATGCT**.

Mg-IPC-r GTTAATATCATATAAAGCTCTACCGTTGTTA  
**TCACCGCTCAGGCATTTGCT**.

The primer regions underlined and in bold amplify a 146 bp fragment of phage lambda DNA from position 13,915–14,061 (GenBank accession number J02459). With the tail of MgPa primers, the total length of the fragment is 204 bp (see Note 6).

### 2.4. DNA Positive and Negative Controls

1. *M. genitalium* DNA purified and quantified by standard procedures (see Note 7). Standards are diluted tenfold from 100,000 genome equivalents (geq)/ $\mu$ L to 1 geq/ $\mu$ L in TE buffer (pH 8.0) containing 1  $\mu$ g/mL of calf thymus DNA (see Note 8).
2. As negative controls, PCR grade water subjected to the same DNA preparation procedure as described for a swab specimen (extraction control) and untreated PCR grade water (reagent control) are tested in duplicates.

**2.5. Mastermix**

## 1. In-house master mix

All reactions are performed in a 50  $\mu\text{L}$  final volume in an ABI 7500 thermocycler with 5  $\mu\text{L}$  template.

All reagents are mixed in an appropriate, sterile polypropylene container (see Note 9).

5  $\mu\text{L}$  10 $\times$  PCR buffer: 200 mM Tris-HCl, pH 8.4, 500 mM KCl, as supplied with the Platinum *Taq* DNA polymerase (Invitrogen).

5  $\mu\text{L}$  50 mM  $\text{MgCl}_2$ .

2.5  $\mu\text{L}$  20  $\mu\text{M}$  each primer MgPa-355F and MgPa-432R (see Note 10).

0.25  $\mu\text{L}$  15  $\mu\text{M}$  FAM-labeled MgPa TaqMan MGB probe (see Note 10).

0.125  $\mu\text{L}$  15  $\mu\text{M}$  TAMRA-labeled phage lambda IPC-R TaqMan probe (see Note 10).

5  $\mu\text{L}$  dUTP-mix: 1.25 mM each dATP, dCTP, and dGTP, 2.5 mM dUTP (see Note 11).

10  $\mu\text{L}$  of 50 % glycerol in PCR grade water.

1  $\mu\text{L}$  0.83  $\mu\text{M}$  6-carboxy-x-rhodamine (ROX) reference dye (see Note 12).

5  $\mu\text{L}$  of the appropriate dilution of IPC (see Note 6).

0.4  $\mu\text{L}$  (2 U) *Taq* DNA polymerase (Platinum *Taq*; Invitrogen).

8.2  $\mu\text{L}$  PCR grade water.

## 2. Plasticware

96-Well reaction plates (e.g., MicroAmp Optical plates (Applied Biosystems)).

Adhesive covers (e.g., ABI PRISM Optical adhesive covers (Applied Biosystems)).

**2.6. 23S rRNA  
Gene Assay**

## 1. Primers (5'-3')

Mg 23S-1992F: CCATCTCTTGACTGTCTCGGCTAT.

Mg 23S-2138R: CCTACCTATTCTCTACATGGTGGTGTT.

These primers amplify a 147 bp fragment of the 23S rRNA gene.

## 2. DNA positive and negative controls

*M. genitalium* DNA containing 100, 10, and 1 geq/ $\mu\text{L}$  as well as extraction and reagent negative controls (as described in Subheading 2.4).

## 3. Mastermix

All reactions are performed in a 100  $\mu\text{L}$  final volume in an ABI 2720 thermocycler with 10  $\mu\text{L}$  of template.

10  $\mu\text{L}$  10 $\times$  PCR buffer: 200 mM Tris-HCl (pH 8.4), 500 mM KCl as supplied with the Platinum *Taq* DNA polymerase (Invitrogen).

3  $\mu\text{L}$  50 mM  $\text{MgCl}_2$ .

2  $\mu\text{L}$  20  $\mu\text{M}$  each primer Mg 23S-1992F and Mg 23S-2138R (see Note 10).

10  $\mu\text{L}$  dUTP-mix: 1.25 mM each dATP, dCTP, and dGTP, 2.5 mM dUTP (see Note 11).

0.4  $\mu\text{L}$  (2 U) *Taq* DNA polymerase (Platinum *Taq*; Invitrogen).

62.6  $\mu\text{L}$  PCR grade water.

4. Plasticware

MicroAmp 8-tube strips 0.2 mL with 8-cap strips.

### 2.7. Gel Electrophoresis

1. Standard agarose gel electrophoresis in 0.5 $\times$  TBE buffer (see Note 13) on 2 % gels (1 % SeaKem GTG agarose and 1 % NuSieve GTG agarose; both from Lonza). Gels can be cast with 5  $\mu\text{g}$  of ethidium bromide per mL agarose gel. Add 5  $\mu\text{L}$  ethidium bromide per 100 mL agarose from a 10 mg/mL stock solution after cooling the agarose to approx. 45  $^\circ\text{C}$  or subsequently stain the gel after electrophoresis in 0.5 $\times$  TBE with the same concentration for 20–30 min. Subsequent destaining in 0.5 $\times$  TBE for 2 $\times$  20 min will decrease background staining, but is optional.
2. Electrophoresis equipment and power supply for agarose gel electrophoresis.
3. UV transilluminating table with photo-documentation equipment.

### 2.8. Sequencing

Sequencing of the 147 bp amplicon produced with primers Mg 23S-1992F and Mg 23S-2138R can be performed for detection of macrolide resistance-mediating mutations. Most laboratories will find it most cost efficient to send the PCR amplicons to an external commercial facility for purification and sequencing. However, it is advised to check the amplicon for purity by gel electrophoresis before sequencing. Alternatively, sequencing can be performed according to in-house protocols with each of the primers used for amplification.

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## 3. Methods

### 3.1. DNA Preparation

1. Swab specimens: In order to release all of the clinical specimens from the swab the Copan UTM tube is vortexed for 30 s. Transport medium is expressed from the swab and the swab discarded. With an extra long 200  $\mu\text{L}$  filter tip, 100  $\mu\text{L}$  of the resuspended specimen in UTM medium is aspirated taking care not to touch the inside of the transport tube with the outside of the barrel of the pipette. The 100  $\mu\text{L}$  specimen aliquot is carefully added to 300  $\mu\text{L}$  of the Chelex-100 slurry in a

0.5 mL Eppendorf tube (as described in Subheading 2.2). The mixture is vigorously vortexed for 60 s and incubated at 95 °C for 10 min in a heating block. A short centrifugation step to collect condensation droplets in the lid is recommended. The sample is then ready for PCR.

2. Urine should be briefly vortexed and then 1,900  $\mu\text{L}$  ( $2 \times 950 \mu\text{L}$  with a 1 mL filter tip) is transferred to a 2 mL Eppendorf tube (see Note 14). The specimen is concentrated by centrifugation at  $30,000 \times g$  for 15 min (or  $20,000 \times g$  for 20 min) at 4 °C. Take note of the expected position of the pellet by positioning the lid hinges away from the center of the centrifuge rotor. Using a single-use Pasteur pipette the supernatant urine is carefully aspirated and discarded. Take care not to dislodge the pellet which may be barely visible. Leave the tubes in a rack for a couple of minutes and then aspirate the remaining urine that has collected in the bottom with a 200  $\mu\text{L}$  pipette with filter tip. Add 300  $\mu\text{L}$  of Chelex-100 slurry and proceed as for swab specimens.
3. If the tubes with lysed specimen and Chelex-100 have been stored frozen, they should be vortexed after thawing and briefly centrifuged as the freezing tends to cause an irregular distribution of the DNA.

**3.2. Plate Setup  
and Real-Time PCR  
for the MgPa  
TaqMan™ Assay**

1. Prepare the appropriate amount of master mix for the MgPa TaqMan assay and distribute 45  $\mu\text{L}$  to each well needed in a 96-well reaction plate.
2. Add controls to wells A1 through B2 according to the template in Fig. 1.
3. Add 5  $\mu\text{L}$  of patient specimens in duplicate to the remaining wells (see Note 15).
4. Seal the plate with ABI PRISM Optical Adhesive Covers and place it in the ABI 7500 thermocycler programmed to a cycle of 50 °C for 1 s (stage 1), 95 °C for 10 min (stage 2), and 50 cycles of denaturation at 95 °C for 15 s and annealing and extension at 60 °C for 1 min. All wells in use should be marked to collect fluorescence in the FAM and TAMRA channels and the wells with positive control assigned as standards with quantities as labeled in the template.
5. After completion of the run, analyze the plate and check the  $r^2$  of the standard curve determined by the software. Assays with  $r^2 > 0.98$  can be accepted for quantitative purposes, but rerun if not fulfilling this criterion. For qualitative testing, an  $r^2 > 0.95$  can be accepted but usually suggest poor pipetting technique or errors in the preparation of the DNA for the standard curve. Both wells receiving the 1 geq/ $\mu\text{L}$  standard (i.e., five copies of

A1 Pos 100,000	A2 Pos 100,000	A3 Pos 1,000	A4 Pos 1,000	A5 Pos 100	A6 Pos 100	A7 Pos 10	A8 Pos 10	A9 Pos 1	A10 Pos 1	A11 Neg Extract. control	A12 Neg Extract. control
B1 Neg Reagent control	B2 Neg Reagent control	B3 Sample 1	B4 Sample 1	B5 Sample 2	B6 Sample 2	B7 Sample 3	B8 Sample 3	B9 ...	B10 ...	B11	B12
C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12
D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11	D12
E1	E2	E3	E4	E5	E6	E7	E8	E9	E10	E11	E12
F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11	F12
G1	G2	G3	G4	G5	G6	G7	G8	G9	G10	G11	G12
H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11	H12

Fig. 1. Template for loading of controls and specimens for the *M. genitalium* real-time PCR assay.

template) should be positive for assay acceptance. All negative controls should show only signal from the IPC probe as should all patient samples deemed negative (see Note 16). If a sample is negative in both the IPC and the MgPa probe, it can be repeated with 2  $\mu$ L of template. If negative again, the specimen should be reported as inhibitory and a new specimen should be collected. The IPC signal needs not to be detected in samples containing *M. genitalium* DNA.

### 3.3. 23S rRNA Gene Confirmatory Assay

1. Prepare the appropriate amount of master mix for the 23S rRNA gene confirmatory assay and distribute 90  $\mu$ L to the wells of the 8-well strips. Add 10  $\mu$ L of the *M. genitalium* DNA containing 100, 10, and 1 geq/ $\mu$ L as well as the extraction and reagent controls to the respective wells. Add 10  $\mu$ L of the clinical specimens found positive in the MgPa TaqMan assay and seal with 8-cap strips and place in a thermocycler (e.g., the ABI 2720) programmed to run at 95  $^{\circ}$ C for 2 min followed by 50 cycles each consisting of 95  $^{\circ}$ C for 15 s and 60  $^{\circ}$ C for 1 min.
2. Analyze 20  $\mu$ L of the amplified material on a 2 % agarose gel, stain with ethidium bromide, and photograph on a UV

transilluminator. Specimens showing a band of 147 bp (aligning with the positive controls) are considered positive. The 1 geq/ $\mu$ L control should be positive and both of the negative controls should be negative for assay acceptance (see Note 16).

### 3.4. Detection of Macrolide Resistance-Mediating Mutations

In order to detect macrolide resistance-mediating mutations in region V of the 23S rRNA gene, specimens found positive in the confirmatory assay can be analyzed by sequencing. Preferably, both strands should be sequenced, but any of the two primers can be used alone, if the sequence can be evaluated for transitions at position 2,058 and 2,059 (*E. coli* numbering).

1. Check the amplicon on an agarose gel. Only a single band of the expected 147 bp length should be visible (primers and primer-dimers smaller than 50 bp will usually be removed by the purification procedure and is of minor importance).
2. Follow the instructions from the sequencing provider for submission of PCR product for purification and sequencing. Be careful to label tubes or plates in a way so that an unambiguous identification of the patient is possible upon return of the sequence.
3. When sequencing files are received, assemble forward and reverse primer sequences for each amplicon and save the consensus file. This can be done by a range of software packages, some of them available as free trials from the Internet such as DNA Baser Sequence Assembler which will work for this purpose also after the trial period.
4. Align the consensus files of each sequence with the wild-type amplified sequence shown in Fig. 2, and look for mutations in positions 2,058/2,059 (marked in grey and bold).

Mutations at position 2,058/2,059 have been found with varying prevalence and are arbitrarily named as types 0–6 as shown in Table 1. Sequence types 1–3 have been confirmed to cause high-level azithromycin resistance by in vitro MIC determination of strains isolated by culture (5), whereas types 5 and 6 have been detected only by PCR in patients failing azithromycin treatment. Sequence type 4 has been found only once and the relation to resistance is unclear.

```

CCATCTCTTGACTGTCTCGGCTATAGACTCGGTGAAATCCAGGTACGGGTGAAGACACCC
GTTAGGCGCAACGGGACGGAAAGACCCCGTGAAGCTTTACTGTAGCTTAATATTGATCAA
AACACCACCATGTAGAGAATAGGTAGG

```

Fig. 2. Sequence of the 23S rRNA gene amplified in the confirmatory assay. The primer binding sites are underlined and the 2,058 and 2,059 positions marked in *grey* and *bold*.

**Table 1**

**Mutations in the *M. genitalium* 23S rRNA gene positions 2,058 and 2,059 (*E. coli* numbering) associated with macrolide resistance. Sequence types 1, 2, and 3 have been found in *M. genitalium* strains isolated by culture and shown to be in vitro macrolide resistant**

Sequence type	Mutation <i>E. coli</i> numbering	Comment
0	WT	Wild type
1	A2059G	Common
2	A2058C	Rare
3	A2058G	Most common
4	A2059T	Very rare
5	A2058T	Relatively rare
6	A2059C	Rare

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#### 4. Notes

1. Several vendors provide high-quality water. We routinely use Ultrapure DNase/RNase-Free distilled water of Invitrogen. From the 500 mL bottle, aliquots of 50 mL are prepared and stored at +4 °C. These aliquots are used only once and the remainder thrown away.
2. We use a sterile 150 mL urine container from Kartell.
3. All pipetting prior to PCR is performed with micropipettes equipped with filter tips (e.g., ART-tips, Molecular Bio Products).
4. The probe contains a minor groove binder (MGB) quencher that stabilizes the binding between probe and target allowing for a much shorter probe than possible for conventional dual-labeled probes. A probe without the MGB moiety will not work in the assay. At present MGB probes are available only from Applied Biosystems.
5. The IPC probe can be labeled with any fluorescent reporter that can be clearly distinguished from the *M. genitalium* reporter probe. The BHQ-2 quencher can be substituted by any nonfluorescent quencher matching the fluorophore.
6. The primers for construction of the IPC are used only once in a standard PCR with 35 cycles each of 94 °C/30 s; 40 °C/30 s; 72 °C/60 s and 1 ng of purified lambda DNA as template.

After gel purification of the amplicon, a tenfold titration is performed, and the dilution of the IPC producing no increase in the detection limit of purified *M. genitalium* DNA is used in the assay.

7. Purified *M. genitalium* DNA is available from the American Type Culture Collection (ATCC) as ATCC number 33530D.
8. The use of carrier DNA in the dilution buffer stabilizes the standard dilutions allowing them to be stored at  $-20^{\circ}\text{C}$  for  $>1$  year and at  $+4^{\circ}\text{C}$  for a month. The addition of carrier DNA is particularly important for the most dilute controls that tend to lose activity due to passive adsorption to the walls of the Eppendorf tube. The dilution of the standard should be done with a new filter-tipped pipette tip for each tenfold step.
9. The master mix is stable for at least 7 days at  $+4^{\circ}\text{C}$  when stored without Taq polymerase. A large batch can be prepared in 50 mL tubes and an amount taken out according to the number of tests performed. For each 24 wells,  $25 \times 45 \mu\text{L}$  master mix should be prepared to allow for loss during the pipetting step.
10. Primers and probes are stored in stock concentrations of  $100 \mu\text{M}$  in TE buffer, at  $-20^{\circ}\text{C}$ . Primer and probe working dilutions at  $20 \mu\text{M}$  in TE buffer, pH 8.0 are stored at  $-20^{\circ}\text{C}$  in  $100 \mu\text{L}$  aliquots for primers and  $25\text{--}50 \mu\text{L}$  aliquots for probes. Each aliquot is used no more than three times and the remainder discarded.
11. Preparation of dUTP-mix  
Dispense 9.375 mL PCR grade water in a PP-tube.  
Add  $125 \mu\text{L}$   $100 \text{ mM}$  dATP.  
Add  $125 \mu\text{L}$   $100 \text{ mM}$  dCTP.  
Add  $125 \mu\text{L}$   $100 \text{ mM}$  dGTP.  
Add  $250 \mu\text{L}$   $100 \text{ mM}$  dUTP.  
Mix and dispense 1 mL in 1.5 mL Eppendorf tubes. Label with content and lot-no. Store at  $-20^{\circ}\text{C}$  for up to 2 years.
12. Preparation of ROX reference dye for mastermix for ABI 7500  
Dispense  $580 \mu\text{L}$  PCR grade water in an Eppendorf tube.  
Add  $20 \mu\text{L}$   $25 \mu\text{M}$  Rox (e.g., of Invitrogen).  
Mix and dispense  $200 \mu\text{L}$  aliquots in 0.5 mL Eppendorf tubes.  
Label with content and lot number. Store at  $-20^{\circ}\text{C}$ . Use dilution before expiry as noted on the ROX tube.
13. Preparation of TBE stock solution ( $5\times$  concentrated)  
Weigh out 54 g of Tris-base and 27.5 g of boric acid and dissolve in approximately 900 mL of deionized water.  
Add 20 mL of 0.5 M EDTA (pH 8.0) and adjust the solution to a final volume of 1 L.

This solution can be stored in glass bottles at room temperature but a precipitate may form during prolonged storage. Discard if a precipitate has formed.

#### Working solution of TBE

For agarose gel electrophoresis, TBE is used at a concentration of 0.5× (1:10 dilution of the concentrated stock). Dilute the stock solution tenfold in deionized water. Final solute concentrations are 45 mM Tris–borate and 1 mM EDTA.

14. Eppendorf tubes should be qualified for centrifugation at high speed. 2 mL Safe-Lock tubes of Eppendorf are qualified for 25,000 × *g*, but we have used these tubes for years at 30,000 × *g* and never had a broken tube.
15. In order to obtain a high clinical sensitivity, it is important to test specimens in duplicate. When only one well is positive, confirmatory testing with the 23S rRNA gene assay should be performed. As this assay accommodates 10 µL of DNA extract, these low positives are often confirmed.
16. If positive controls are not amplified, positive patient's specimens can be further processed for confirmatory assay or for sequencing, but all negative specimens should be retested in an assay fulfilling the acceptance criteria. If positive signals are observed in the negative controls, the assay should be repeated and all reagents proved to be contamination free. Subsequently, all positive patients' specimens should be retested after repeated sample preparation.

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## Protocols for Detection and Typing of *Treponema pallidum* Using PCR Methods

S.M. Bruisten

### Abstract

Syphilis, caused by *Treponema pallidum*, is a sexually transmitted disease which was epidemic in Europe between the 15<sup>th</sup> and 20<sup>th</sup> century. From 2000 onwards it is worldwide mostly encountered among men who have sex with men but also among women in resource poor setting. Syphilis can easily be treated with penicillin but can become chronic, if left untreated. The disease develops in several stages with ulcerative lesions in the primary stage, and systemic spread to organs via the blood in later stages. Taking swab samples from these ulcerative lesions gives the best options for detection by PCR of *T. pallidum*. Alternatively blood samples can be analyzed in later stages. If tested positive in screening PCR assays, *T. pallidum* can also be typed by molecular methods, enabling molecular epidemiology. Different protocols are discussed in this chapter, that include all steps from sample collection, nucleic acid extraction, PCR, and detection. The combination of all steps together determine the final sensitivity and specificity of *T. pallidum* PCR.

**Key words:** *Treponema pallidum*, Syphilis, Sexual transmission, PCR targets, Nested PCR, Typing, Antibiotic resistance, Genital ulcers

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### 1. Introduction to Syphilis

Syphilis is a sexually transmitted disease which was first reported in Europe in the late 1400s at the time Columbus returned from the new world. It quickly reached epidemic proportions in Europe and spread around the world during the early sixteenth century, being ubiquitous by the nineteenth century (1, 2). In the first half of the twentieth century it became known that syphilis can be easily treated with a single injection of penicillin which can be given intramuscularly or, in case of neurosyphilis, intravenously (3). So at the end of the 1990s there were speculations that syphilis would disappear altogether (4, 5). Since 2001 however there has been a resurgence

among men who have sex with men (MSM). In addition syphilis remains endemic in poor-resource regions, and is a leading cause of miscarriage in pregnant women and infant mortality (6–11).

*Treponema pallidum* subsp. *pallidum*, hereafter referred to as *T. pallidum*, is the causative agent of syphilis. The disease can become chronic, if left untreated, and develops in several stages. A primary infection shows ulcerative lesions that last about 4–6 weeks, which heal spontaneously with resolution of the hard chancre(s). Two to six weeks after the primary phase the secondary phase develops in which systemic manifestations may occur. As the bacterium can disseminate to all organs including the central nervous system (CNS), symptoms recorded are headache, mild fever, typical erythematous skin rashes (in particular on the palms, soles, and trunk), and generalized lymphadenopathy. A variable latent phase may follow in which no clinical symptoms are apparent and infection can only be diagnosed serologically. In the early (<1 year) latent phase recurrent symptoms can occur in about 25 % of the cases possibly accompanied by lesions. If still left untreated the tertiary phase develops in about 30 % of infected adults with very severe symptoms (2). Active replication of *T. pallidum* can be encountered in all stages of syphilis but “infectious syphilis” is mostly referred to as the primary (acute infection) or the secondary phase.

Transmission is usually by sexual contact and also rarely by blood transfusion, but transfer via the placenta can also occur (congenital syphilis). Syphilis can seriously complicate pregnancy if the mother is acutely infected, leading in over 40 % of cases to spontaneous abortion, stillbirth, or serious neurological defects of the newborn (8, 11, 12).

### 1.1. Diagnosis of Syphilis

Because *T. pallidum* cannot be grown on artificial medium, the traditional methods of detection are microscopy and serology. The rabbit infectivity test, in which testicles of rabbits are injected with patient samples such as skin scrapings, cerebrospinal fluid (CSF), or blood, is rarely performed for routine purposes since it is highly impractical. In 1909 dark field microscopy was invented to directly detect the highly motile treponemes. This method was improved over the years and is still in use in the twenty-first century (13). For dark field microscopy highly experienced laboratory workers are needed and false positive results may occur because *T. pallidum* is morphologically indistinguishable from commensal treponemes, which may be present in some body fluids. Other types of microscopic detection are silver staining and fluorescent treponemal antibody staining (FTA). Serological methods are either treponeme specific or nonspecific. Nontreponemal serological tests include the Venereal Disease Research Laboratory (VDRL) and the Rapid Plasma Reagin (RPR) card test (2, 13). Both mainly contain purified cardiolipin, a constituent of mammalian cell membranes, which is present in high titers of sera of syphilis patients (13). Although the VDRL and RPR tests are rather inexpensive, they are

**Table 1**  
**Disease association of different spirochaetal bacterial species**

Disease	Spirochaete species	Disease type
Syphilis	<i>T. pallidum</i> subsp. <i>pallidum</i>	Highly invasive
Yaws	<i>T. pallidum</i> subsp. <i>pertenue</i>	Moderately invasive
Bejel	<i>T. pallidum</i> subsp. <i>endemicum</i>	Moderately invasive
Pinta	<i>T. pallidum</i> subsp. <i>carateum</i>	Noninvasive
Peridontitis	<i>T. denticola</i> <i>T. paraluisuniculi</i>	Noninvasive Infects rabbits
Nonpathogenic	<i>T. phagedensis</i> <i>T. refringens</i>	
Leptospirosis	<i>Leptospira interrogans</i>	Highly invasive (non-STI)
Lyme disease	<i>Borrelia burgdorferi</i>	Highly invasive (non-STI)

not very specific in early and late syphilis. False positive reactions occur for example in cases of pregnancy, drug addiction, malignancy, autoimmune diseases such as SLE and rheumatoid arthritis, and hepatitis or mycoplasma infection. Treponemal-specific tests include the fluorescent treponemal antibody absorption test (FTA-Abs or FTA) and the microhemagglutination tests (MHA-TP) also known as the *T. pallidum* hemagglutination assay (TPHA) or the *T. pallidum* particle agglutination assay (TPPA). The TPHA/TPPA test uses lysates of *T. pallidum* and is more expensive but more specific and highly sensitive (13). In Europe the TPHA/TPPA tests are often used for screening, after which the FTA-Abs and the TP-blot are used as confirmatory assays. In addition the VDRL and RPR tests are performed indicating disease activity. In contrast, in the USA, nonspecific tests such as the VDRL and RPR are frequently done for screening purposes followed by a specific assay for *T. pallidum* as confirmation.

Quite often microscopy or serology alone is not sufficient for reliable syphilis diagnosis in the laboratory. Therefore increasingly amplification assays are used in clinical laboratories and most involve the polymerase chain reaction (PCR). As early as the beginning of the 1990s PCR-based detection methods for TP were described (14, 15). Since then different protocols have been developed using targets that are highly conserved for *T. pallidum* but also very specific in order to discriminate them from other spirochetes, such as commensal treponemes or *Borrelia* species. These bacteria and their related diseases are shown in Table 1. Usually the clinical picture and epidemiological data will be indicative for syphilis or other

spirochaetal diseases. For example yaws, which used to be a common tropical skin disease of people infected with *T. pertenue*, occurred mostly in children and could hardly be mistaken for syphilis (16).

The combination of DNA extraction protocols, the different sample matrices (swab of vesicles, skin scrapings, blood, CSF, or other), and the amplification and detection protocol together determine the success rate of using PCR in the clinical diagnosis of syphilis. The applied PCR protocol varying from nested PCR, single PCR, or multiplex PCR, and the different detection platforms (gel based, real time), all influence the ultimate analytical test performance. Each of these influential factors is discussed in more detail below.

### **1.2. Specimen Collection**

Laboratory diagnosis of syphilis is especially dependent on the stage of disease. For PCR testing in the primary, ulcerative phase *T. pallidum* can best be detected by collecting a swab specimen of the lesion(s), present on male or female genitals. If these are dry swabs they can be stored at ambient temperature (18–25 °C) for days or even weeks. Swabs that are collected in medium or buffer should be stored at 4–8 °C for up to 1 week, but they may be stored for up to several years if frozen at –20 to –80 °C. Since ulcerative lesions are not present in secondary or tertiary phase of syphilis infections, other sampling methods are required. In secondary syphilis a skin rash may be present, which could be sampled using swabs. Alternative specimens such as skin biopsies or blood samples such as whole blood, serum, or EDTA-plasma have been successfully tested using PCR (17, 18). For suspected neurosyphilis and congenital syphilis, cerebrospinal fluid (CSF) samples should be obtained by lumbar puncture (13). Scrapings of ear lobes have been described to be useful since *T. pallidum* is found in capillary beds during the asymptomatic phase of syphilis (19, 20). To this purpose a scalpel may be used to scrape the ear lobe until blood appears, which is then collected on a Dacron swab. Then the swab is vigorously agitated for 30 s in a tube containing 1 mL of sterile PBS (19). Pathological skin samples archived in paraffin blocks need to be pretreated to be useful for PCR amplification; see also Subheading 1.3 (18, 21).

### **1.3. Nucleic Acid/DNA Extraction Procedures**

It is important to perform the nucleic acid extraction steps in a clean room or separate from the amplification area to minimize contamination with previous PCR products. The quickest method to release and extract nucleic acids from fluids containing cells or tissues that have *T. pallidum* in them is to heat the specimen for 15 min at 95 °C. After cooling to room temperature and a short spin, 2–5 µL can be used directly in a PCR reaction (15). Although this method was shown to work for *T. pallidum* detection, it was found to be not a very sensitive method (15) (Bruisten, unpublished results). Therefore a specific DNA isolation method is usually

practiced. An easy-to-perform and cheap method that can be used for many PCR protocols is given below in Subheading 3.1. It may be performed on all liquids such as swab eluate, CSF, serum, or EDTA-plasma that supposedly contain DNA from *T. pallidum*. Genital ulcer specimens that are collected on dry cotton or Dacron swabs thus first need to be eluted either in transport or culture medium or in physiological salt buffer (phosphate-buffered saline—PBS). In case of concomitant herpes PCR, two dry swabs of one lesion can be combined in one 1.7 mL vial with 600  $\mu$ L of PBS or other buffer. Subsequently the sticks of the swabs are broken off below the lid of the vial, which is then closed tightly. For elution, the vials are stirred vigorously on a vortex for 30 min and the suspension is centrifuged for 2 min at 19,000  $\times g$ . The swabs may next be removed from the eluate solution or left in the solution if they are not expected to release inhibiting substances.

Alternative isolation procedures may be performed by using for example mini spin-columns, commercial kits such as the Magna Pure LC DNA isolation kit, or other extraction protocols that run on DNA extractor machines. Especially for blood, transport medium, and urine specimens many extraction kits and protocols can be used. In case a thorough extraction is needed for removing inhibiting substances such as hemoglobin, bilirubin, or other components that may stick to nucleic acids, a phenol/chloroform step can be used. This will produce “pure” nucleic acids but also this extensive extraction procedure will diminish the yield. The balance between purity and sensitivity should be experimentally established.

Skin biopsies or other tissue samples that are formalin fixed and embedded in paraffin first need to be de-paraffinized with xylene and ethanol for nucleic acid extraction. The released tissue blocks or fragments should first be digested by treatment with proteinase K at 56 °C for at least 1.5–16 h (overnight). Thereafter a nucleic acid extraction protocol of choice as mentioned above can be performed. As an example the protocol of Behrhof et al. is given (21). Two to three adjacent 3–5  $\mu$ m slides were cut from a block. Slides were deparaffinized with xylene and ethanol. For DNA isolation the tissue portions were incubated with digestion buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl, 5 mM EDTA) and proteinase K (about 20  $\mu$ g/mL) at 56 °C overnight. Subsequently the DNA was recovered by standard phenol–chloroform–iso-amylalcohol extraction and ethanol precipitation. The DNA quality was assessed by testing for the presence of the cellular human bcl-1 gene (21).

#### **1.4. PCR Targets for *T. pallidum***

The full genome sequence of *T. pallidum* subsp. *pallidum*, strain Nichols, was published in 1998 and contains around 1.1 million base pairs, 1,081 open reading frames (Orfs), and 1,041 genes (TP0001–TP1041) (1). Since then few full genome sequences of other treponemal strains have been published. There are three strains of *T. pallidum* subsp. *pallidum* available and six projects in

progress in GenBank as of April 2011. Those three have accession numbers AE000520 for the Nichols strain (1), CP000805 for strain SS14 (22), and CP001752 for the Chicago strain (23). *T. pallidum* belongs to the bacterial group of the spirochetes, which represent a phylogenetically ancient and distinct group (1). Antibodies detected in serologic tests for syphilis are uniformly reactive with treponemal subspecies causing yaws, pinta, and bejel (13, 16) (see Table 1). Thus the specificity of a *T. pallidum* PCR is important to establish in order to exclude cross-reactions with commensal or other spirochetes. For example *T. denticola*, causing periodontitis in subgingival plaques, could be present on swab samples from tonsils from men having sex with men who are tested for *T. pallidum* (24). Strouhal et al. (25) compared whole genomes between different treponemata and found that membranous components of treponemes were mainly responsible for specificity. However, for clinical diagnostic sensitivity the chosen gene targets should be highly conserved for *T. pallidum* subsp. *pallidum*.

Nucleotide numbering as given in the Nichols strain (GenBank AE000520) is used as reference location for primers and probes in Tables 2 and 3. Many diagnostic *T. pallidum* PCR assays use genes that code for *T. pallidum* membrane lipoproteins. A very immunogenic 47 kDa lipoprotein was used both in immunogenetic assays and its gene, *tpp47*, was described as a sensitive and specific PCR target but at the same time has been shown to cross-react with *T. pallidum* subsp. *pertenue* (14, 15). This *tpp47* gene codes for a carboxy peptidase in the TP0574 Orf and has been used by many other scientists in all possible PCR formats, but with the primers being slightly adjusted in time to obtain more specificity (26–30).

The 39 kDa basic membrane protein is coded by the *bmp* gene (TP1016) and has been used by several authors with varying success. Problems arose with the original primers because they lacked specificity but by using a nested PCR protocol this problem was overcome (31, 32). Also other membrane protein genes, such as the *tmpA* gene (Orf TP0768) coding for a 45 kDa structure (33, 34) and the *Treponema pallidum* repeat (*tpr*) genes A–L, have been described as PCR targets, but these genes are highly polymorphic and therefore not an option for clinical detection (35–37).

A more conserved gene that is very useful for clinical diagnosis is the *pol A* gene, which codes for the polymerase of *T. pallidum* (21, 38–42). Within this gene different regions have been chosen as targets by different authors. One of the first protocols, by Liu et al., used highly specific sequences in a single PCR with gel detection (38). This protocol was used by others who were interested in antibiotic resistance testing and adapted this assay for screening purposes (40, 43) (Table 2). The sensitivity of this assay is 10–25 copies per reaction which is quite acceptable. When developing real-time PCR assays the sensitivity needs to remain comparably high, above 90 %, but also the specificity should not suffer, being above 95 %. This was achieved for assays targeting the *pol A* gene

**Table 2**  
**Targets for specific detection of *T. pallidum* by PCR**

Format PCR and detection	Target gene ORF <sup>a</sup>	5' Location AE000520	Primer and probe sequences (5'–3')	Specificity e-value <sup>b</sup>	Length (bp)	Reference
Nested or single PCR <sup>c</sup> Gel detection	Basic membrane protein <i>bmp</i> gene <i>TP1016</i>	1107.609	Ri: TP3 CAGGTAACGGATGCTGAAGT Fi: TP4 CGTGGCAGTAACCGCAGTCT	0.054 <sup>c</sup>	506	(31)
		1107.103		0.054 <sup>c</sup>		
		1107.693	Ro: TP7 CTCAGCACTGCTGAGCGTAG Fo: TP8 AACGCCCTCCATCGTCAGACC	0.054 <sup>c</sup>	617	(32)
		1107.077		0.054 <sup>c</sup>		
Nested or single PCR <sup>c</sup> Gel detection	47 kDa lipoprotein <i>tp47</i> gene <i>TP0574</i>	622.944	R: 1 F GACAAATGCTCACTGAGGATAGT	0.005 <sup>d</sup>	658	(14)
		622.287	F: 1R ACGCACAGAAACCGAATTCCTTG	0.005 <sup>d</sup>		(26)
		622.879	R: 2F TTGTGTAGACACCGTGGGTAC	0.005 <sup>d</sup>	496	(27)
		622.384	F: 2R TGATCGCTGACAAGCTTAGGCT	0.005 <sup>c</sup>		
Real-time PCR	47 kDa lipoprotein <i>tp47</i> gene <i>TP0574</i>	622.049	R: KO3A GAAGTTTGTCCCAAGTTGGGTT	0.005 <sup>d</sup>	260	(49)
		622.790	F: KO4 CAGAGCCATCAGCCCTTTTA	0.21 <sup>c</sup>		
Real-time multiplex PCR with HSV 1 + 2	47 kDa lipoprotein <i>tp47</i> gene <i>TP0574</i>	622.749	R: SY767: CAACACGGTCCGCTACGACTA	0.021 <sup>c</sup>	67	(28)
		622.682	F: SY834: TGCCATAACTCGCCCATCAGA	0.054 <sup>c</sup>		(29)
		622.725	P: SY791: Rox GGTGATGACGCGGAGCTACACCA-BHQ3 or 5'Fam and Tamra-3'	0.002 <sup>d</sup>		
Single PCR <sup>c</sup> Gel detection	DNA Pol I <i>pol A</i> gene <i>TP0105</i>	622.929	R: GGATAGTTTTTCTGCACGTAAGGTAA	5 × 10 <sup>-5c</sup>	70	(30)
		622.860	F: ACCCACCGTGTCTACCAAAG	0.021 <sup>c</sup>		
		622.902	P: VIC-CAGCATGGAGAGCCCGCACG-TAMRA	0.054 <sup>c</sup>		
Semi-nested PCR <sup>c</sup>	DNA Pol I	116.961	F1: TCGCGGTGTGCGAATGGTGTGGTC	6 × 10 <sup>-4c</sup>	377	(38)
		117.336	R1: CACAGTGTCTAAAACCGCCTGCACG	2 × 10 <sup>-4c</sup>		(40)
		116.740	F2: CGTCTGGTCGATGTGCAAATGAGTIG	2 × 10 <sup>-4c</sup>	395	
		117.134	R2: TGCACATGTACACTGAGTTGACTGGG	5 × 10 <sup>-5c</sup>		
		116.102	F: TP-modF1 GTGTGCACCTGGGCATTACAG	0.054 <sup>c</sup>	164	(21)

(continued)

**Table 2**  
(continued)

Format PCR and detection	Target gene ORF <sup>a</sup>	5' Location AE000520	Primer and probe sequences (5'–3')	Specificity e-value <sup>b</sup>	Length (bp)	Reference
Gel detection	<i>pol A</i> gene <i>TP0105</i>	116.265	R: TP-modRI GTCTGAGCACTTGCACCGTA	0.054 <sup>c</sup>	125	
		116.141	F: TP-modF2 TGAAGCTGACGACCTCAITG	0.054 <sup>c</sup>		
Real-time PCR	<i>pol A</i> gene <i>TP0105</i>	116.777	F: TP <sub>s</sub> -GGTAGAAGGGGAGGGTAGTA	0.054 <sup>c</sup>	105	(44)
		116.881	R: TP <sub>as</sub> -CTAAGATCTATTTCTATAGGTATGG	4 × 10 <sup>-6c</sup>		(45)
		116.825	P: TP-Q-Fam-ACACAGCACTGCTCTTCAACTCC-BHQ1	0.002 <sup>d</sup>		
Real-time PCR	<i>pol A</i> gene <i>TP0105</i>	117.211	F: SyphT AGGATCGCCCAATATGTCCAA	NS <sup>f</sup>	67	(42)
		117.268	R: SyphT GTGAGCGTCTCATCATTCCTCCAAA	0.005 <sup>d</sup>		
		117.228	P: SyphT 6-XX-ATGCACCAGCTTCGA-MGB-NF	NS <sup>f</sup>		
Real-time PCR	<i>pol A</i> gene <i>TP0105</i>	117.201	F: TP1 CAGGATCCGGCATATGTCC	0.21 <sup>c</sup>	71	(28)
		117.266	R: TP2 AAGTGTGAGCGTCTCATCATTC	0.005 <sup>d</sup>		
		117.223	P: TP3 Cy5-CTGTCATGCACCAGCTTCGACGTCTT-BHQ3	5 × 10 <sup>-5c</sup>		

*bp* base pairs, *F* forward primer, *R* reverse primer, *F<sub>0</sub>* outer forward primer, *R<sub>0</sub>* outer reverse primer, *F<sub>i</sub>* inner forward primer, *R<sub>i</sub>* inner reverse primer, *P* probe

NS not specific at all: no 100 % match with *T. pallidum* sequences

*BHQ1* black hole quencher 1, *NF* nonfluorescent quencher, *Fam* fluorescein phosphoramidite, *TET* tetrachlorofluorescein phosphoramidite, *Rox* carboxy-X-rhodamine, *XX* carboxyfluorescein

<sup>a</sup>TP-ORF number in GenBank number AE000520: Nichols strain; Fraser et al. (1)

<sup>b</sup>E-Value: Specificity for *T. pallidum* subsp. *pallidum* is indicated by *e*-value in GenBank after blastn: the lower this value, the more specific. Grading indicates if cross-reactivity is to be expected. In general more specificity means less cross-reactivity

<sup>c</sup>Specific for *T. pallidum* subsp. *pallidum*

<sup>d</sup>Very specific for *T. pallidum* subsp. *pallidum*

<sup>e</sup>Highly specific for *T. pallidum* subsp. *pallidum*

<sup>f</sup>Not specific for *T. pallidum* subsp. *pallidum*

**Table 3**  
**Targets for typing of *T. pallidum***

Format PCR	Target gene ORF <sup>a</sup>	GenBank 5'location AE000520	Primer and probe sequences (5'–3')	Specificity <i>e</i> -value <sup>b</sup>	Fragment length (bp)	Reference
Single PCR	<i>arp</i> TP0470	460.921 461.672	F: ARP-1 Fam-CAAGTCAGGACGGACT GTCC R: ARP-2 Tet-GGTATCACCTGGGGATGC	0.054 <sup>c</sup> 0.85 <sup>d</sup>	Variable length 735– 1,553 bp (7–21 repeats). A 14 repeat is 1,155 bp	(41, 50) (48)
Nested PCR	<i>tpr</i> J, E, G genes	328.078 330.273 328.325 330.172	Fo: B1 ACTGGCTCTGCCACACTTGA Ro: A2 CTACCAGGAGAGGGTGAAGC Fi: IP6 CAGGTTTTGCCGTTAAGC Ri: IP7 AATCAAGGGAGAAATACCGTC	0.054 <sup>c</sup> 0.85 <sup>d</sup> 0.85 <sup>d</sup> 0.054 <sup>c</sup>	2,186 1,836	(41, 47–50) (20)
Azithromycin resistance GB: AF200367	23S rRNA between TP0225, 0226 and TP0266 and 0267	233.613 234.241	F: Azi GTACCGCAAACCGACACAG R: Azi AGTCAAAACCGCCACCTAC	0.21 <sup>d</sup> 0.21 <sup>c</sup>	628	(51, 52) (43, 58)

F Forward primer, R reverse primer, Fo outer forward primer, Ro outer reverse primer, Fi inner forward primer, Ri inner reverse primer

P Probe, BHQ1 black hole quencher 1, NF nonfluorescent quencher, Fam fluorescein phosphoramidite; Fam fluorescein phosphoramidite; TET tetra chloro fluorescein phosphoramidite; Rox carboxy-X-rhodamine, XX carboxyfluorescein

NS not specific at all; no 100 % match with *T. pallidum* sequences

<sup>a</sup>TP-ORF number in AE000520; Nichols strain, Fraser et al. (1) bp base pairs

<sup>b</sup>E-Value: Specificity for *T. pallidum* subsp. *pallidum* is indicated by *e*-value in GenBank after blastn: the lower this value, the more specific. Grading indicates if cross-reactivity is to be expected. In general more specificity means less cross-reactivity

<sup>c</sup>Specific for *T. pallidum* subsp. *pallidum*

<sup>d</sup>Not specific for *T. pallidum* subsp. *pallidum*

by several authors (18, 28, 44, 45). Good agreement was found between the *T. pallidum* real-time PCR and the clinical diagnosis of the general practitioner or dermatologist, using serology (kappa values of 0.7). The sensitivity with respect to clinical diagnosis without serology testing was however only 73 %, probably due to the difficulty to recognize a syphilitic infection based only on appearance of the chancre (overestimation by the physician possible). On the other hand the specificity of TP PCR was 96 % (45). In contrast, the assay developed by Leslie et al. (42) used *pol A* gene primers and probes that are not specific for *T. pallidum* subsp. *pallidum* since they show cross-reactivity (Table 2).

When epidemiological studies need to be performed with the aim to link persons that were infected in a single outbreak or to study transmission chains, it is important to use more variable parts of the genome of *T. pallidum* (Table 3). In the earliest studies, where molecular subtyping of *T. pallidum* was performed, the *arp* (acidic repeat proteins) and *tpr* genes, both coding for repeat sequences, were successfully used in PCR-RFLP techniques (18, 41, 46–50). The *arp* and *tpr* genes are not very specific targets and may also detect *T. pallidum* subsp. *pertenue*, subsp. *endemicum* and *T. paraluisancuniculi*, but this should not be problematic if other *T. pallidum* PCR assays are used for screening.

Resistance to azithromycin, a macrolide antibiotic, used to treat syphilis when penicillin is not tolerated, can be monitored by PCR targeting the 23S rRNA genes (51). A polymorphism in this 23S rRNA gene, in which an A–G mutation at position 2,058 in Nichols, or position 2,059 in the Chicago strain occurs, was found to be associated to macrolide antibiotic resistance (51–53). This 23S rRNA target is present in all bacterial species and it is inherently difficult to select specific primers for *T. pallidum* amplification. Primer sequences that did perform well are shown in Table 3, although BLAST analyses show a high degree of cross-reactivity with other bacterial species. In such cases scientists are advised to shift the sequences and BLAST those to see if it is possible to reduce the possible cross-reactivities.

### **1.5. TP PCR Amplification Protocols with Gel-Based Detection**

All possible PCR protocols that have been devised for clinical laboratory diagnostic purposes are also available presently for diagnosing infectious syphilis targeting known sequences of *T. pallidum*. In the earliest assays either a single or a nested PCR with detection of amplicons on acrylamide or agarose gels was described. Nested PCR provided both more sensitivity, using two amplification rounds each of >30 cycles, as well as improved specificity, since each of the inner primers is specific for the target (31, 32). The buffers needed for an in-house-developed PCR protocol are given below in Subheading 2. The protocol for a nested *T. pallidum* assay is also given in Subheading 3.2, adopted from Bruisten et al. (32). The nested PCR was applied in a multiplex format which

facilitated the diagnosis of genital ulcer disease, detecting simultaneously herpes simplex virus (HSV) types 1 and 2, *T. pallidum*, and *Haemophilus ducreyi* (26, 32, 54).

### **1.6. Real-Time PCR TP Amplification Protocols**

Since the turn of the century the so-called real-time PCR techniques (qPCR) have become available. The main advantage of this type of PCR is that detection occurs in the same tube in which the amplification also occurs, thus minimizing the chance of carryover contamination and, by reducing the number of pipetting steps, lessening technical errors. Of major importance is that the test result is available much sooner. All different fluorescence-based techniques, such as SybrGreen or Eva Green with end point detection by melting curve analysis can be used, but presently real-time PCR with dual-labeled (Taqman) probe assays have become the predominant method for the detection of *T. pallidum* (Table 2). As an example the protocol used by Koek et al. (44) detecting the *pol A* gene is given in Subheading 3.3. As an internal control for inhibition the Rox-labeled Phocine herpes virus (PhHV) DNA was used (55).

Real-time PCR assays that are performed in a multiplex format are also currently available (30, 56). The different pathogens causing genital ulcer disease can be detected in one assay with different fluorophore-tagged probes for each pathogen, *T. pallidum*, HSV-1 or -2, and *H. ducreyi*. An additional fluorophore Rox (“orange”) is usually reserved for an internal control assay, such as PhHV, thus monitoring inhibition of the test (55). A limiting step is the number of different channels that are available in an amplification machine to differentiate between the different fluorescent probes. A plethora of different real-time platforms are available and all can be used successfully after optimization. Examples are the ABI Taqman (Applied Biosystems), the Light cycler (Roche), the RotorGene (Corbett, Qiagen), the iCycler (BioRad), and the SmartCycler (Eurogentec).

### **1.7. PCR Amplification Protocols for Typing and Resistance Monitoring of *T. pallidum***

Specimens positive in a screening assay for *T. pallidum* DNA could be subsequently tested in typing PCRs targeting the *arp* and *tpr* genes. The *arp* gene (TP0470) contains 60 bp repeats that vary in number from 7 to 21 among different strains with over half of the strains having 14 repeats (41, 49, 50). A published protocol from Molepo et al. can be taken as an example for the *arp* gene typing and is given in Subheading 3.4 (48). The other typing method involved nested PCR targeting 3 of the *T. pallidum* repeat (*tpr*) genes from the family of 12 genes, *tprA* to *tprL*, present in each genome. Again the protocol of Molepo et al. is given in Subheading 3.5. After amplification the *tpr* inner amplicon was digested with MseI and the PCR-RFLP pattern was analyzed by gel electrophoresis. The PCR amplicons could alternatively be sized by using labeled primers and estimation of the fragment length on a genetic analyzer

machine that is also used for sequencing analysis (ABI 310 or ABI 3130 for instance) (41, 48, 50). In detail, a 1 µL aliquot of each sample was heated at 95 °C for 4 min, chilled on ice, diluted in formamide (1:50), and injected into the capillary gel matrix (POP4; Applied Biosystems). Samples were run at 60 °C under denaturing conditions, and the results were analyzed by computer using the GeneScan software (version 2.1, Applied Biosystems) and compared with a standard mixture with fragments varying in size from 185 to 2,500 bp. The combination of *arp* and *tpr* types constitutes a specific profile consisting of a number of *arp* repeats and a letter of *tpr* pattern type (41, 50). The 14d and 14f profiles appeared to be present in >50 % of patients in North and South American and African patient populations (18, 41, 48, 49).

### **1.8. Antibiotic Resistance Typing**

Penicillin G is the drug of choice for *T. pallidum* treatment and no resistance to this drug has been reported. However, an increasing number of people developed allergic reactions to penicillin and they were therefore treated with azithromycin or other macrolides. Resistance subsequently developed fast to these macrolides and a specific typing PCR assay to monitor this was developed already in 2004 by targeting the 23 SrRNA genes (51–53). Amplification of these 23S rRNA sequences follows essentially a normal PCR protocol. Afterwards the amplicon needs to be sequenced, or a specific point mutation protocol, detecting the A2058G mutation, could be performed (51, 57).

### **1.9. Clinical Specimen Types Suitable for *T. pallidum* PCR**

Whereas venereal syphilis caused by *T. pallidum* subsp. *pallidum* can affect any organ or system, the other non-venereal treponemes such as *T. pallidum* subsp. *carateum* (causing pinta) or subsp. *pertenue* (causing yaws) (Table 1) are usually transmitted during childhood and clinical manifestations are limited to skin, bones, and cartilage. In contrast, syphilitic skin lesions mostly occur in sexually active people and not in children.

Ulcerative lesions on genitals or other skin lesions appear mostly in primary syphilis but can also be present in later stages. These lesions can be swabbed or scraped and the buffer in which the swab is placed can be used successfully for *T. pallidum* PCR. Although detection of *T. pallidum* in blood or blood fractions such as serum or plasma has been reported, it is dependent on the stage of the disease with the highest chance of success in the secondary stage. A comparison of different specimen types was performed using the *tpp47* gene target in a Swiss study (29) and a Canadian study (58), both assessing different PCR targets, including the *tpp47*, *bmp*, and *pol A* genes. It appeared that not the target gene determined the sensitivity but the stage of disease. PCR was positive in ulcerative disease but not in blood samples in the primary stage, while in secondary stage disease approximately 50 % of the blood samples were positive (58). A case of gastric syphilis in a patient with chronic dyspepsia was described using the *tpp47* and

*pol A* genes as targets in real-time PCRs (28). The diagnosis of syphilis was corroborated by serology and the demonstration of spirochetes in diffusely inflamed gastric mucosa by staining with a fluorescent monoclonal antibody specific for pathogenic treponemes, thus excluding PCR positivity due to detection of oral treponemes (28). In a study dedicated to detecting *T. pallidum* in paraffin-embedded skin biopsy specimens the *pol A* gene was used as a target in a semi-nested PCR (21). From a total of 36 suspected syphilitic skin biopsy specimens 20 were diagnosed as *T. pallidum* positive. From these, 17 were found by immunohistochemistry and 14 by PCR using primers as published by Liu et al. (38) and modified primers in a semi-nested format (21) (see also Table 2). As expected, spirochetes could be detected in this study in biopsy specimens of primary and secondary syphilis (including one specimen sampled more than 50 years ago) but it was not possible to detect treponemal DNA in specimens of tertiary syphilis.

The *pol A* gene target was also used in another recent study in cases with suspected secondary syphilis in whole blood and in biopsy samples (18). In this study whole blood could be used successfully to detect *T. pallidum* but it was essential to process the samples for DNA extraction within a few hours after procurement. In this study approximately 60 % of the samples were PCR positive in the *pol A* screening PCR; however, the load was probably too low to perform successful typing, since all of the 26 whole blood samples were negative by *arp* and *tpr* PCR. For biopsy samples taken from these secondary syphilis patients in Colombia on the other hand, six of eight positives in the screening *pol A* PCR could be typed with the *arp-tpr* RFLP method (18).

Neurosyphilis can occur at any time in the course of syphilis, even in the primary stage. Early forms of neurosyphilis affect the meninges, and cerebral or spinal cord vasculature. Late forms of neurosyphilis primarily affect the brain and spinal cord parenchyma. When neurological symptoms are suspected to be related to syphilis a CSF sample can be used and analyzed by PCR. Uveitis and hearing loss related to syphilis are most common in early syphilis and may be accompanied by early neurosyphilis (3).

Inhibition of *T. pallidum* PCR can occur due to substances in the original sample such as hemoglobin in blood, containing iron molecules, or degrading enzymes in tissue biopsy samples. Another, often unrecognized cause of inhibition is overload with the target. For example a titrated number of treponemes obtained from cultures in rabbit testicles showed that an input of  $>10^4$  targets per vial inhibited the PCR (14). This rarely occurs for *T. pallidum* in patient samples such as CSF but on the other hand it may be an explanation for discrepant diagnostic test results such as a positive dark field result and a negative *T. pallidum* PCR. In such cases tenfold dilutions of either the original sample (preferred) or of the prepared DNA sample should be assayed in the PCR.

### 1.10. Discussion

The overall success of a PCR used for acute infection of syphilis in terms of sensitivity and specificity is dependent on all separate steps of the procedure, such as choice of sample type and collection procedure, nucleic acid extraction, amount and quality of the DNA in the PCR reaction, the amplification protocol itself, and the detection procedure. But even more important is the process in total, in which all steps are like beads on a string that connect to each other. The clinical significance of the results obtained by a chosen PCR protocol for syphilis diagnosis is additionally dependent on the prevalence of the *T. pallidum* bacteria in the patient groups that are to be studied (35). In case an outbreak needs to be studied not only the individual PCR success is of importance but of course it is a prerequisite that the diagnostic PCR is positive before TP strain discriminating PCRs can be used. For example targeting the *arp* and *tpr* genes, these PCRs are not highly sensitive, possibly because of the long PCR products that are produced, nor highly specific for *T. pallidum* as the primers show relatively high cross-reactivity to other treponemes (Tables 2 and 3, legend). Also for resistance typing it is of importance that a sensitive screening PCR assay is used for *T. pallidum*. In many laboratories, nested PCR amplification is generally banned for diagnostic purposes due to the possible risk of environmental contamination by amplified DNA compared to single-round PCR. As expected, a (semi)-nested approach, in comparison to single-round amplification, also enhanced sensitivity (21, 32). According to our experience the higher sensitivity justifies a nested PCR approach for tests with critical sensitivity, as in the case of *T. pallidum* detection and typing. As is detailed in the “rules” in Note 4, at the end of Subheading 3, the logistics are of importance to avoid PCR contamination, so preferably at least three separate laboratory rooms should be available to work in.

Real-time PCR, presently called qPCR, has become the mainstay of clinical diagnostics and research purposes because it is fast, sensitive, specific, and easy to perform if all ingredients are optimized for a robust assay. The fluorescent probes enable direct measurement of the relative amount of amplicon that is formed, but a true quantification of the input amount of the target needs more effort such as using a highly standardized workup procedure of samples and use of controlled methods for sample collection. This is usually only possible for fluids such as blood or CSF, but not for swabs that are eluted in a buffer. In addition, external and internal quantification standards need to be developed, quantified, and validated.

Since *T. pallidum* is most often detected in lesions such as genital ulcers that macroscopically cannot be distinguished from lesions caused by HSV-2 or HSV-1 the advantage of multiplex real-time PCR using simultaneously primers for HSV and *T. pallidum* is obvious. In many studies worldwide the HSVs were found to be the most prevalent cause of ulcerations even when syphilis was the prime suspect (26, 32, 54). Also the bacterium *H. ducreyi*

is a known cause of genital ulcer disease, but in the last decades it has hardly ever been detected in the laboratory. Even in southern African countries where laboratory testing has become increasingly feasible, HSV-2 and *T. pallidum* were found to be the most prevalent causes of ulceration and certainly not *H. ducreyi* (32, 54).

In multiplex PCR, detecting each probe in each channel of the PCR instrument with equal sensitivity and specificity (no “cross talk”) has proven difficult. The maximum number of targets in a real-time multiplex format is probably four and one of the channels is usually taken by the internal control. The “Rox” label is used in some commercial kits, limiting the possibility to use all channels for probe detection. Also for each combination of targets in a multiplex setting the concentrations of primers and probes always need to be optimized by varying their amount in relation to the  $MgCl_2$  concentration and that of the nucleotides. A reaction with a dual-labeled probe requires a higher  $MgCl_2$  concentration than traditional amplifications since the 5′–3′ exonuclease domain of the Taq-polymerase prefers a high  $MgCl_2$  concentration.

Sample type is clearly of importance too in detecting *T. pallidum*, especially in combination with the disease stage of the patient. For example the use of whole blood or blood fractions has been assayed by many authors with varying success (29, 38, 39). It is obvious that in primary disease spirochetes must be circulating in the blood. However, if for a patient the equivalent of 50–100  $\mu$ L blood is subjected to a *T. pallidum* PCR that detects 5–10 target copies per reaction, then at least 100–1,000 treponemal organisms per mL should be present in the blood sample taken. Thus it is inherently difficult to detect syphilis by PCR from blood since the bacterial load is usually lower than this. To circumvent this problem it may be advisable to take ear scrapings since the treponemes seem to get caught in the capillary beds of the skin and thus their concentration may be high enough for detection (19, 20).

Detecting *T. pallidum* by PCR is superior to serological detection in settings where tropical skin diseases occur that are caused by other *T. pallidum* subspecies, such as *pertenue* (yaws), *carateum* (pinta), and *endemicum* (bejel). The specificity of the PCR is dependent on the chosen target, but even more so on the specificity of the selected sequences. In general, the *pol A* gene is a good target of choice because of sequence conservation and thus high sensitivity but at the same time sequences within the *pol A* gene need to be selected that are specific for *T. pallidum* sp. *pallidum*. Target genes that code for surface antigens such as the *tpp47* gene and the *tpr* genes are more subject to variation between strains and therefore these may miss future strains that diverged from the sequences chosen. Thus continuous update of treponemal sequence information in GenBank is needed to ensure that *T. pallidum* PCR protocols will always be able to detect circulating strains.

## 2. Materials

### 2.1. Isolation of DNA from Swab Eluate, Blood (Serum, Plasma), or Other Liquid Sample

#### 2.1.1. Isopropanol Precipitation Procedure

#### Buffers

Lysis buffer (L6): Contains mainly 5.5 M guanidium isothiocyanate (L6, BioMérieux) (59).

Glycogen (10 mg/mL).

100 % Isopropanol (store in small bottle at  $-20^{\circ}\text{C}$ ).

70 % (v/v) ethanol.

10 mM Tris-HCl pH 8 buffer ( $T_{10}$ ).

#### Consumables

Plastic Pasteur pipettes, fine tip.

Eppendorf vials.

Heat block adjustable to  $65^{\circ}\text{C}$ .

Eppendorf centrifuge.

### 2.2. *T. pallidum* PCR (See Note 1)

#### Buffers

PCR reaction buffer (10 $\times$ )

100 mM Tris-HCl = 2.11 g.

500 mM NaCl (58,44 g/mol) = 29.22 g.

0.1 % (w/v) gelatine = 1.0 g.

H<sub>2</sub>O, bidest = 1,000 mL.

pH (titrate with 2 N HCl) = 8.0.

Autoclave.

Aliquot in 2 mL vials (see Note 2).

MgCl<sub>2</sub> solution

20 mM MgCl<sub>2</sub> = 4.07 g.

H<sub>2</sub>O, bidest = 1,000 mL.

Autoclave.

Aliquot in small amounts (see Note 2).

dNTPs (stock solutions are 100 mM)

10  $\mu\text{L}$  each dATP, dTTP, dCTP, dGTP = 40  $\mu\text{L}$ .

H<sub>2</sub>O, bidest = 160  $\mu\text{L}$ .

End concentration 5 mM dNTP.

Aliquot in amounts of 100  $\mu\text{L}$  in Eppendorf vials (see Note 2).

*T. pallidum* primers out of a stock solution of 1  $\mu\text{g}/\mu\text{L}$ :

Dilute each primer to 100 ng/mL in T10 (10 mM Tris-HCl (pH 8.0)) (see Note 3).

### Consumables

Eppendorf tubes (directly from a previously unopened plastic bag) 200  $\mu$ L and 1.7 mL.

Pipette tips with plugs.

Plastic bags, resealable.

### Reagents

Mineral oil.

Negative ( $T_{10}$  buffer or RNase-free  $H_2O$ ) control.

Positive control (DNA from control plasmid or patient) in each PCR-run.

Taq polymerase (5 U/ $\mu$ L).

Platinum qPCR Supermix-UDG (Invitrogen).

PhHV internal control DNA.

Tru9I (Roche Diagnostics), which is an isoschizomer of MseI.

10 $\times$  Expand high fidelity reaction buffer (Roche).

Taq polymerase high fidelity (50 U/ $\mu$ L, Roche).

100 bp DNA ladder (Life Technologies).

Agarose for gel.

Ethidium bromide solution (0.5 mg/mL).

1 % Chlorix solution (household bleach).

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## 3. Methods

### **3.1. Isolation of DNA from Swab Eluate, Blood (Serum, Plasma), or Other Liquid Sample**

#### *3.1.1. Isopropanol Precipitation Procedure*

1. Dispense 600  $\mu$ L lysis buffer in a 1.7 mL vial.
2. Add 1  $\mu$ L of glycogen stock.
3. Add 100  $\mu$ L (or 200  $\mu$ L) of swab eluate, serum, or other liquid sample.
4. Incubate for 10 min at 65  $^{\circ}$ C in heat block.
5. Cool to room temp. for 2 min.
6. Short spin (10 s 19,000  $\times g$ ).
7. Add 1 volume ice-cold isopropanol (=700  $\mu$ L or 800  $\mu$ L).
8. Vortex.
9. Spin in Eppendorf centrifuge, 20 min 19,000  $\times g$ .
10. Remove supernatant with fine-tip plastic Pasteur pipette; discard.
11. Add 500  $\mu$ L 70 % ethanol to wash the pellet.
12. Mix carefully (do not vortex!).
13. Centrifuge for 1 min at 19,000  $\times g$ .

14. Remove supernatant with fine-tip plastic Pasteur pipette; discard.
15. Add 500  $\mu\text{L}$  70 % ethanol for second wash of the pellet.
16. Mix carefully (do not vortex!).
17. Centrifuge for 1 min at  $19,000 \times g$ .
18. Remove supernatant, do not remove exhaustively; discard.
19. Short spin at  $19,000 \times g$ .
20. Remove all supernatant until the pellet is as dry as possible.
21. Dry the pellet: Open vial in cabinet (no longer than 5 min).
22. Resuspend the pellet in 100  $\mu\text{L}$   $T_{10}$ .
23. Dissolve the pellet overnight at 4 °C or by heating at 65 °C for 30 min.
24. Use directly for PCR and/or store at -20 °C.
25. Use 2–10  $\mu\text{L}$  for PCR reaction.

**3.2. Protocol  
for Nested TP PCR,  
Target is *bmp* Gene  
(orf TP1016)**

Adopted from Bruisten et al. (32).

The total nested PCR procedure is performed in three rooms. This is done to minimize the danger of contamination of buffers, primers, patient samples, etc. with PCR products (amplimers) (see also Note 4).

**3.2.1. Preparation  
of the Outer PCR Mixtures**

Work in *room 1*

1. Thaw all PCR solutions at room temperature or on ice (keep enzymes at 4 °C). Short vortex and spin before use.
2. Prepare the number of mixes needed according to mix for outer nested TP PCR.

Mix for the outer nested TP PCR

Solutions for outer TP PCR	End concentration	Per sample ( $\mu\text{L}$ )
10 $\times$ PCR buffer including $\text{MgCl}_2$	1 $\times$ , 2 mM	2.5
dNTP (5 mM)	200 $\mu\text{M}$	1.0
TP 7 (100 ng/ $\mu\text{L}$ )	400 pg/ $\mu\text{L}$	0.1
TP 8 (100 ng/ $\mu\text{L}$ )	400 pg/ $\mu\text{L}$	0.1
$\text{H}_2\text{O}$		16.2
Taq polymerase (5 U/ $\mu\text{L}$ )	0.5 U	0.1
Mix volume		20
DNA sample extract		5
Total volume		25

TP7 and TP8 primer sequences can be found in Table 2.

3. Aliquot the mix solution: 20  $\mu\text{L}$ /200  $\mu\text{L}$  vial; add 1 drop of mineral oil.

- Short spin, 15 s 11,000×g. Store in small plastic bags (seal) and take them to room 2 for direct use or store in clean -20 °C freezer for later use (can be stored for 3 months).

### 3.2.2. Preparation of the Inner TP Nested PCR Mix

Work in *room 1*

- Thaw all PCR solutions at room temperature or on ice (i.e., enzymes: keep at 4 °C).  
Short vortex and spin before use.
- Prepare the number of mixes needed according to mix scheme for inner TP PCR.

Mix for the inner *T. pallidum* PCR

Solutions for inner TP PCR	End concentration	Per sample (μL)
10× PCR buffer	1×, 2 mM	2.5
5 mM dNTP	200 μM	1.0
TP 3 (100 ng/μL)	400 pg/μL	0.15
TP 4 (100 ng/μL)	400 pg/μL	0.15
H <sub>2</sub> O		19.1
Taq polymerase (5 U/μL)	0.5 U	0.1
MIX volume		23
Inner PCR sample		2
Total volume		25

TP3 and TP4 primer sequences can be found in Table 2.

- Aliquot 23 μL per 200 μL Eppendorf vial.
- Short spin, 15 s 11,000×g. Store in small plastic bags (seal) and take them to room 2 for direct use or store in clean -20 °C freezer for later use (can be stored for 3 months).

The PCR cycling program for both the inner and outer PCR:

Program	Cycles	Temperature (°C)	Time
Hold 1		95	5 min
Cycling	30	58	30 s
		58 (outer), 55 (inner)	30 s
		72*	60 s
Hold 2		72	7 min
Hold 3		25	5 min

For the nested step 2 μL of outer PCR product is added to 23 μL of the inner PCR mix.

**3.3. Real-Time qPCR  
on *pol A* Gene (TP0105  
Locus) of *T. pallidum***

Adopted from Koek et al. (44) (see Note 4).

Primers are also described in Table 2:

Primers (TP0105)	TPs	5'-GGT AGA AGG GAG GGC TAG TA-3'
Fragment is 105 bp	TPas	5'-CTA AGA TCT CTA TTT TCT ATA GGT ATG G-3'
Probe	TP-Q	FAM 5'ACA CAG CAC TCG TCT TCA ACT CC 3'-BHQ1

Work in *room 1*

1. Thaw all PCR solutions at room temperature or on ice (keep enzymes at 4 °C).  
Short vortex and spin before use.
2. Prepare the number of mixes needed according to mix for real-time TP PCR.

PCR mixture ingredients	μL per
TP real-time PCR	Sample (1×)
RNase-free H <sub>2</sub> O	4.15
2× Platinum qPCR Supermix-UDG (Invitrogen)	10.0
MgCl <sub>2</sub> is included in Supermix → 3 mM end concentration	
Primer TPs (10 μM) → 380 nM end concentration	0.75
Primer TPas (10 μM) → 380 nM end concentration	0.75
Probe TP-Q (5 μM) → 90 nM end concentration	0.35
Taq polymerase is included in Supermix → 0.6 U per reaction	
Mix volume	16
DNA isolate sample	4
Total volume	20

3. Aliquot the mix to vials with each 16 μL per vial.
4. Short spin, 15 s 11,000×g.
5. Store in small plastic bags (seal) and take them to room 2 for direct use or store in clean -20 °C freezer for later use (can be stored for 3 months).

Work in *room 3* with thermocycler

1. Thaw all PCR mixes at room temperature. Short vortex and spin before use.
2. Number the vials.
3. Add 4 μL isolated nucleic acid per patient sample.  
For negative control add 4 μL of T<sub>10</sub> or RNase-free H<sub>2</sub>O.

For positive control add 4  $\mu\text{L}$  of plasmid or known positive patient DNA.

4. Close all vials well and place in the PCR machine after a short spin.

The real-time PCR cycling program is as follows:

Program	Cycles	Temperature ( $^{\circ}\text{C}$ )	Time
Hold 1		93	5 min
Cycling	45	95	30 s
		55	30 s
		72*	30 s
Hold 2		25	5 min

\*In this step the fluorescence is acquired in the FAM/Sybr (green) channel and in the Rox channel in case of simultaneous testing on the control DNA spike with PhHV.

The program takes about 2 h and 10 min.

### 3.3.1. Interpretation of Results (as Performed on a RotorGene Instrument)

1. Open the raw data file and perform analysis according to instrument software.
2. Set the threshold at 0.04.
3. The negative control should be negative; if not the run may be contaminated in the PCR mix.
4. The positive control should have a Ct value between 25 and 35.
5. A positive sample should have a Ct value below 36 and show a sigmoid curve.
6. If the Ct value is between 36 and 40: repeat the sample.
7. If the Ct value is above 40 or if there is no clear sigmoid curve: the sample is negative.
8. In case an internal control is used: this should be positive (Ct < 36) for the sample. In case the sample is TP positive the status of the PhHV control can be either positive or negative for a valid result.

### 3.4. Amplification and Typing of *T. pallidum* Using the *arp* Genes

Adopted from Molepo et al. (48).

#### 3.4.1. *Arp* PCR and Typing

Primers are also described in Table 3:

Primers <i>arp</i> : 735–1,553 bp	F-ARP-1	Fam-5'CAAGTCAGGACGGAC
		TGTCC
	R-ARP-2	Tet-5'GGTATCACCTGGGGATGC

Work in *room 1* (see Note 4)

1. Thaw all PCR solutions at room temperature or on ice (keep enzymes at 4  $^{\circ}\text{C}$ ).

Short vortex and spin before use.

2. Prepare the number of mixes needed according to mix for arp-PCR.

PCR mixture ingredients	μL per
arp PCR	Sample (1×)
RNase-free H <sub>2</sub> O	31.5
10× Expand high fidelity reaction buffer (Roche)	5.0
MgCl <sub>2</sub> is included in Expand buffer → 1 mM end concentration	
dNTPs (10 mM) → 400 μM end concentration	2.0
Primer ARP-1 (10 nM) → 100 pM end concentration	0.5
Primer ARP-2 (10 nM) → 100 pM end concentration	0.5
Taq polymerase high fidelity (50 U/μL)	0.5
Mix volume	40
DNA isolate sample	10
Total volume (μL)	50

3. Aliquot the mix to vials with each 40 μL per vial.
4. Short spin, 15 s 11,000×g.
5. Store in small plastic bags (seal) and take them to room 2 for direct use or store at -20 °C freezer for later use (can be stored for 3 months).
6. Work in *room 3* with thermocycler.
7. Thaw all PCR mixes at room temperature. Short vortex and spin before use.
8. Number the vials.
9. Add 10 μL isolated nucleic acid per patient sample. For negative control add 10 μL of T<sub>10</sub> or RNase-free H<sub>2</sub>O. For positive control add μL of plasmid or known positive patient DNA.
10. Close all vials well and place in the PCR machine after a short spin.

The *arp* gene PCR cycling program is as follows:

Program	Cycles	Temperature (°C)	Time
Hold 1		94	4 min
Cycling	45	94	60 s
		60	60 s
		68*	5 min
Hold 2		68	15 min

The PCR products are resolved by electrophoresis on a 2 % agarose gel at 100 V for 1 h, together with a 100 bp DNA ladder (Life Technologies). The number of *arp* gene 60 bp tandem repeats was estimated by comparison with the molecular weight marker fragments and the *arp* amplicon from the Nichols strain of *T. pallidum* (14 repeats).

### 3.5. *Tpr* Nested PCR and Typing Targeting the *tpr* E, G, and J Genes

Adopted from Molepo et al. (48).

A two-step nested PCR is performed to amplify the *tpr* E, G, and J genes.

The outer PCR produces a fragment of 2,186 bp and the inner PCR a fragment of 1,836 bp.

Primers are also described in Table 3 and below:

Primers outer <i>tpr</i> : 2,186 bp	Fo-B1 Ro-A2	5'-ACTGGCTCTGCCACACTTGA-3' 5'-CTACCAGGAGAGGGTGAAGC-3'
Primers inner <i>tpr</i> : 1,836 bp	Fi-IP6 Ri-IP7	5'-CAGGTTTTGCCGTTAAGC-3' 5'-AATCAAGGGAGAATACCGTC-3'

Work in *room 1* (see Note 4).

1. Thaw all PCR solutions at room temperature or on ice (keep enzymes at 4 °C).

Short vortex and spin before use.

2. Prepare the number of mixes needed according to mix for *tpr*-genes PCR.

PCR mixture ingredients	μL per
<i>tpr</i> outer or inner PCR	Sample (1×)
RNase-free H <sub>2</sub> O	31.75
10× Expand high fidelity reaction buffer (Roche)	5.0
MgCl <sub>2</sub> is included in expand buffer → 1 mM end concentration	
dNTPs (5 mM) → 200 μM end concentration	2.0
Primer Fo-B1 or Fi-IP6 (10 nM) → 60 pmol per reaction	0.6
Primer Ro-A2 or Ri-IP7 (10 nM) → 60 pmol per reaction	0.6
Taq polymerase high fidelity (50 U/μL) → 2.5 U per reaction	0.05
Mix volume	40
DNA isolate sample	10
Total volume (μL)	50

3. Aliquot the mix to vials with each 40  $\mu\text{L}$  per vial.
4. Short spin, 15 s  $11,000\times g$ .
5. Store in small plastic bags (seal) and take them to room 2 for direct use or store at  $-20\text{ }^{\circ}\text{C}$  freezer for later use (can be stored for 3 months).
6. Work in room 3 with thermocycler.
7. Thaw all PCR mixes at room temperature. Short vortex and spin before use.
8. Number the vials.
9. Add 10  $\mu\text{L}$  isolated nucleic acid per patient sample for the outer PCR or add 5  $\mu\text{L}$  outer PCR product + 5  $\mu\text{L}$   $\text{H}_2\text{O}$  to the inner PCR mix. For negative control add 10  $\mu\text{L}$  of  $T_{10}$  or RNase-free  $\text{H}_2\text{O}$ . For positive control add 5  $\mu\text{L}$  of plasmid or known positive patient DNA.
10. Close all vials well and put in PCR machine after a short spin.

The *tpr* genes PCR cycling program is as follows:

Program	Cycles	Temperature ( $^{\circ}\text{C}$ )	Time
Hold 1		94	5 min
Cycling	40	94	60 s
		60	2 min
		68*	2.5 min
Hold 2		68	15 min

11. Unpurified amplicons from the nested PCR assay are digested with the restriction enzyme Tru9I (Roche Diagnostics), which is an isoschizomer of MseI.
12. The restriction mixture contained 10  $\mu\text{L}$  of PCR amplicon, 2  $\mu\text{L}$  SureCut buffer (10 mM Tris-HCl, 50 mM NaCl, 10 mM  $\text{MgCl}_2$ , and 1 mM dithiothreitol), and 10 U restriction enzyme Tru9I. The reaction mixture is incubated overnight at  $37\text{ }^{\circ}\text{C}$ .
13. Restriction fragments are resolved by electrophoresis on a 2 % agarose gel at 100 V for 1 h, together with a 100 bp DNA ladder (Life Technologies).
14. Agarose gels are stained with a 0.5 mg/mL ethidium bromide (Sigma-Aldrich, Paisley, UK) solution and visualized with an ultraviolet transilluminator.
15. Restriction fragment sizes are estimated by comparison with the 100 bp molecular weight marker fragments and the Tru9I restriction fragments of the *tpr* amplicon of the Nichols strain.

## 4. Notes

1. Prepare all buffers in *a clean room*.  
Never use chemicals, reagents, and/or H<sub>2</sub>O (bidest) that were used before in rooms where amplimers or patient materials have been processed.
2. Add the date of preparation on the label and note that the solution can be stored for 3 years at 4 °C.
3. Add the date of preparation on the label and store for a maximum time of 1 year at -20 °C.
4. Wear clean coats and gloves in each of the separate rooms. Within 1 day follow a strict succession of steps: start in the PCR buffer room (room 1) in the morning. Never return there or never enter in the clean buffer room if you have worked with amplimers on that day. Use clean Eppendorf tubes directly from the factory before the plastic bag was opened. Use pipette tips with plugs. Use negative and positive control samples in each PCR-run. Clean all surfaces and pipettes after use in an experiment series with 1 % Chlorix (bleach) solution and rinse afterwards with water and dry the surfaces to prevent erosion. Use pipettes, racks, etc. belonging to the room.

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# Chapter 10

## Extraction of DNA from Dried Blood in the Diagnosis of Congenital CMV Infection

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### Abstract

Viral DNA detection in dried blood spotted on filter paper, dried blood spots (DBS), is valuable in the diagnosis of viral infections, with at the moment congenital cytomegalovirus (CMV) being the most common application. CMV detection in clinical samples taken within the first 2–3 weeks after birth differentiates congenital CMV infection from the in general harmless postnatal acquired cytomegalovirus infection. DBS render the possibility to diagnose congenital CMV infection retrospectively, e.g., when late-onset hearing loss, the most frequently encountered symptom of congenital CMV infection, becomes manifest. Additionally, CMV DNA detection in DBS can be of usage in recently advocated newborn screening on congenital CMV infection. The procedure of CMV DNA detection in DBS consists of two separate steps: (1) DNA extraction from the DBS, followed by (2) CMV DNA amplification. Here, we describe two efficient methods for the extraction of DNA from DBS. Sensitivity, specificity, and applicability of the methods for high-throughput usage are discussed.

**Key words:** DNA extraction, Dried blood spots, Congenital cytomegalovirus

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### 1. Introduction

Newborn blood taken within a few days after birth and dried on filter paper (dried blood spots, DBS) is widely used for newborn screening on metabolic diseases. Additionally, DBS have been proven valuable and are increasingly used in the diagnosis of viral infections. They are most often used in the diagnosis of congenital cytomegalovirus (CMV) infection, the most common congenital viral infection worldwide (1). Congenital CMV infection can be retrospectively diagnosed using DBS, differentiating congenitally acquired CMV infection from postnatally acquired CMV infection, which is generally much less harmful. DBS render the possibility to diagnose congenital CMV infection when the most common symptom of congenital CMV infection, late-onset hearing loss, becomes manifest. Additionally,

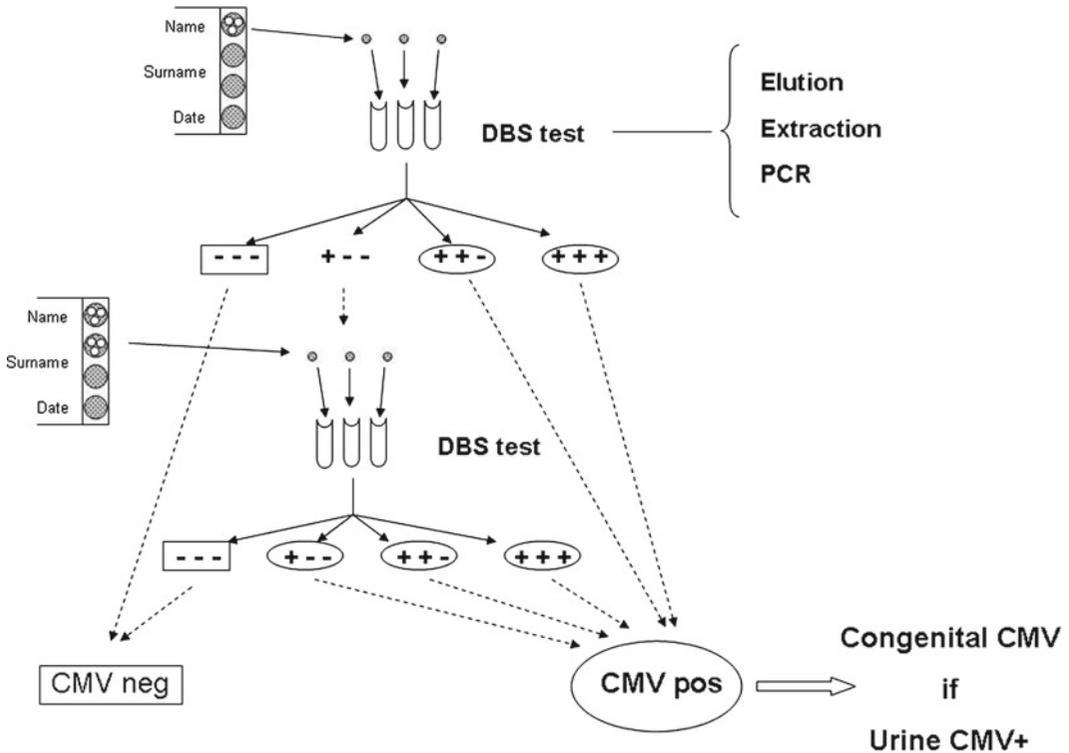


Fig. 1. Flow diagram for interpretation of triplicate testing results as proposed by Barbi et al. (13).

CMV detection in DBS can be of usage in recently advocated newborn screening on congenital CMV infection (2–11).

CMV DNA detection in DBS includes DNA extraction followed by CMV DNA amplification and is increasingly used in clinical virological laboratories worldwide. Whereas detection of CMV DNA in blood and other clinical samples is a routine diagnostic procedure, the extraction of CMV DNA from filter paper is still challenging due to the limited amount of dried blood available; one whole spot of 1 cm in diameter equals approximately 50  $\mu\text{L}$  blood, and one punch of 3 mm in diameter, frequently used for routine metabolic screening, contains as little as 3–5  $\mu\text{L}$  blood. Thus, optimal DNA extraction is crucial in the procedure for CMV DNA detection in DBS.

Currently, several noncommercial and commercial DNA extraction methods for DBS are available. A number of reports evaluating extraction methods for DBS in the diagnosis of congenital CMV infections have been published (3–5, 12–25). Significant differences between extraction methods with respect to the analytical and clinical sensitivity are reported, ranging from 35 to 100 % (3, 14–18, 20, 21, 24–26) depending on the extraction method used and the population tested. Optimizing DNA extraction protocols, PCRs, and algorithms, e.g., by means of performing independent triplicate testing, has been shown to increase analytical sensitivity significantly (15, 16, 18) (Fig. 1). Triplicate

testing (of one punch of 3 mm in diameter per tube) using the heat-shock protocol by Barbi et al. (12), shown to be one of the most sensitive methods (15), results in analytical sensitivities of approximately 100, 86, and 50 % for DBS with CMV DNA loads of 5–4, 4–3, and 3–2  $\log_{10}$  copies/mL, respectively (15). This indicates that limitations in sensitivity apply in the clinically relevant concentration range for congenital CMV disease (reported mean CMV DNA blood loads of 3.4  $\log_{10}$  copies/mL (27), 4.0  $\log_{10}$  copies/mL, and 5.9  $\log_{10}$  copies/mL in asymptomatic newborns with hearing loss at follow-up (28)). In this respect, it is important to note that defined clinically important CMV DNA loads, in the absence of an international CMV DNA quantification standard, are of use only in the laboratory setting where they were obtained. For a general application, standardization of CMV DNA values obtained by different PCR protocols and different quantification standards is essential.

Specificity of CMV DNA detection using DBS has been reported to range between 99.3 and 100 % (12, 14, 25). To our knowledge, transfer of CMV DNA from one DBS to another during storage has been reported once (26). Transfer of CMV DNA during punching can be controlled in the procedure (see below). However, both these potential contaminating events are not likely to be of practical significance given the above-described limited analytical sensitivity.

Above-mentioned advocated newborn screening for congenital CMV can only be achieved using automated, high-throughput DNA extraction methods. Currently, few methods appear suitable for 96-well format high-throughput testing (15).

Here, we describe two methods for efficient extraction of DNA from DBS, used for CMV DNA detection.

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## 2. Materials

### **2.1. DNA Extraction from DBS Using Heat Shock (12, 24)**

1. (Automated) paper puncher.
2. Positive and negative control DBS (or Blanc Guthrie card/Whatman 903 filter paper) (see Note 1).
3. (Eppendorf) tubes or 96-well plate.
4. Minimal Essential Medium (MEM, without additives).
5. Cooler or thermal cycler (4 °C).
6. Heating block or thermal cycler at 55 °C and subsequently 100 °C.
7. (Eppendorf table) centrifuge.
8. Internal control to monitor for PCR inhibition (e.g., phocine herpesvirus (PhHV) DNA).

## **2.2. Column-Based DNA Extraction from DBS**

1. (Automated) paper puncher.
2. Positive and negative control DBS (or Blanc Guthrie card/Whatman 903 filter paper) (see Note 1).
3. Microcentrifuge tube.
4. QIAamp DNA Mini Kit (containing columns, collection tubes, lysis buffer (ATL, AL), proteinase K, wash and elution buffer).
5. Heating block or water bath at 85, 56 °C, and subsequently 70 °C.
6. Eppendorf table centrifuge.
7. Internal control to monitor for PCR inhibition (e.g., PhHV DNA).

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## **3. Methods**

### **3.1. DNA Extraction from DBS Using Heat Shock (12, 24)**

1. For each test DBS (see Notes 1 and 2), punch one disk of 3 mm (1/8 in.) in diameter per tube or well, in triplicate. Punch one disk from a negative control DBS between each test DBS (see Notes 1 and 3).
2. Add 35 µL MEM, including internal control DNA (e.g., PhHV DNA, see Note 4) and spin the punches down (see Note 5).
3. Incubate at 4 °C overnight (see Note 6).
4. Perform heat shock (e.g., in thermal cycler or heating block) according to the following protocol (see Note 7):
  - 55 °C at 60 min.
  - 100 °C at 7 min.
  - Cool rapidly to 4 °C.
5. Centrifugate at  $3,320 \times g$  for 15 min, or at  $8,960 \times g$  for 1–3 min (see Note 8).
6. Transfer the supernatant (approximately 25 µL) to an empty tube or a 96-well plate and freeze at –80 °C for at least 1 h (see Note 9).
7. Thaw; the extract is ready to use for PCR (15).
8. Interpretation of PCR results of triplicates (see Note 10).

### **3.2. Column-Based DNA Extraction from DBS (See Notes 11 and 12)**

1. For each test DBS (see Notes 1 and 2), punch one whole DBS (of approximately 1 cm in diameter, corresponding with approximately 50 µL dried blood) (see Note 12) in a microcentrifuge tube, in triplicates. Punch a negative control DBS between each sample (see Notes 1 and 3).

2. Add 180  $\mu\text{L}$  lysis buffer (ATL), including internal control DNA (e.g., PhHV DNA, see Note 4), to each tube.
3. Incubate at 85  $^{\circ}\text{C}$  for 10 min.
4. Add 20  $\mu\text{L}$  proteinase K, vortex, and incubate at 56  $^{\circ}\text{C}$  for 1 h.
5. Add 200  $\mu\text{L}$  lysis buffer (AL), vortex, and incubate at 70  $^{\circ}\text{C}$  for 10 min.
6. Add 200  $\mu\text{L}$  ethanol 96–100 %, and vortex.
7. Apply the mixture (approximately 600  $\mu\text{L}$ ) to column in a collection tube, centrifuge at  $6,000\times g$  for 1 min, and discard the filtrate.
8. Wash with 500  $\mu\text{L}$  wash buffer (AW1) at  $6,000\times g$  for 1 min, and discard the filtrate.
9. Wash with 500  $\mu\text{L}$  wash buffer (AW2) at  $20,000\times g$  for 3 min, and discard the filtrate.
10. Centrifuge once more at full speed for 1 min, and discard the filtrate.
11. Elute the DNA with 150  $\mu\text{L}$  elution buffer (AE) after incubation for 1 min and centrifugation at  $6,000\times g$  for 1 min.
12. The eluate is ready to use for PCR (15).
13. Interpretation of PCR results of triplicates (see Note 10).

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#### 4. Notes

1. Positive and negative control DBS can be produced by spotting CMV DNA positive and negative (EDTA) blood on Whatman 903 filter paper (approximately 50  $\mu\text{L}$  per spot of 1 cm diameter) followed by air-drying. DBS can be stored at 4  $^{\circ}\text{C}$  or at room temperature.
2. When dried on filter paper, blood spots are considered noninfectious material.
3. DNA contamination from sample to sample during punching is controlled by testing a negative control DBS in between each test DBS.
4. PCR inhibition can be controlled in a simultaneous reaction by adding a fixed amount of internal control (e.g., PhHV DNA) to each sample. Inhibition of internal control amplification is indicative of potential inhibition of amplification of target (CMV) DNA.

## Notes specific for heat-shock DNA extraction

5. Punches must be spun down until the disks are below liquid surface level (15 min at  $3,320 \times g$  may be necessary when using a 96-well plate).
6. Incubation at 4 °C overnight significantly enhances extraction efficiency.
7. During the heat shock, DNA will be extracted from the DBS.
8. Centrifugation yields sufficiently purified DNA.
9. Freezing the supernatant for at least 3 h enhances extraction efficiency (no maximum freezing time implicated).
10. Triplicate testing results in optimal sensitivity (15). Interpretation of triplicate PCR results can be performed using the flow diagram as described by Barbi et al. (13), in which every positive result should be confirmed with at least one additional positive result, in the same run, or, in case of a single positive test result (one of the three replicates), by means of a confirmatory PCR procedure including DNA extraction (second run).

## Notes specific for column-based DNA extraction

11. Detailed protocol is described in the manufacturer's "QIAamp DNA Mini and Blood Mini Handbook," version April 2010.
12. Using one whole DBS (diameter of 1 cm, corresponding with approximately 50  $\mu$ L blood) enhances sensitivity (DNA yield) significantly, when compared to three or six punches of 3 mm in diameter as proposed in the "QIAamp DNA Mini and Blood Mini Handbook" (version April 2010).

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# Chapter 11

## Protocol for the Use of PCR-Denaturing Gradient Gel Electrophoresis and Quantitative PCR to Determine Vaginal Microflora Constitution and Pathogens in Bacterial Vaginosis

Beatrice Vitali, Elena Biagi, and Patrizia Brigidi

### Abstract

In healthy women, the vaginal ecosystem is dominated by *Lactobacillus* spp., but a diverse array of other bacteria can be present in lower amounts. The activity of lactobacilli is essential to protect women from genital infections and to maintain the natural healthy balance of the vaginal microbiota. Bacterial vaginosis (BV) is a complex, polymicrobial disorder characterized by an overgrowth of strict or facultative anaerobic bacteria and a reduction of lactobacilli. Culture-independent techniques based on the analysis of rRNA gene sequences provide powerful tools to reveal the phylogenetic diversity of the vaginal microorganisms in healthy women and patients affected by BV. Polymerase chain reaction (PCR)-denaturing gradient gel electrophoresis conducted with universal primers for eubacteria allows detecting the most abundant bacterial species of an ecosystem. Sequencing of the DNA fragments and comparison with sequences present in publicly available databases allow identifying the corresponding bacterial species. Quantitative PCR is a powerful technique for the quantitative analysis of a selected genus or species.

**Key words:** Bacterial vaginosis, Vaginal microbiota, Vaginal fluids, 16S rRNA gene, PCR-DGGE, qPCR

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### 1. Introduction

The female lower genital tract, consisting of vagina and ectocervix, is an ecological niche where several aerobic and anaerobic microorganisms coexist in a dynamic balance. The homeostasis of the vaginal ecosystem results from complex interactions and synergies among the host and different microorganisms that colonize the vaginal mucosa (1, 2). In healthy women, the vaginal ecosystem is dominated by *Lactobacillus* spp., but a diverse array of other bacteria can be present in lower amounts (3).

Bacterial vaginosis (BV) represents the most common vaginal syndrome afflicting fertile, postmenopausal, and pregnant women, with an incidence rate ranging from 5 to 50 % (2). BV is a complex, polymicrobial disorder characterized by an overgrowth of strict or facultative anaerobic bacteria and a reduction of lactobacilli (4, 5). Although BV is an important medical condition itself, it is associated with serious adverse outcomes, including preterm birth, pelvic inflammatory disease, and acquisition of HIV infection (6). Women with BV may have malodorous vaginal discharge or local irritation, but about 50 % of the women with diagnosable BV have no clear symptoms (7). Amsel clinical criteria (7) and the Nugent method (8) are usually employed for the diagnosis of BV.

In recent years, culture-independent techniques based on the analysis of rRNA gene sequences have been developed, providing powerful tools to reveal the phylogenetic diversity of the microorganisms found within vaginal ecosystem and understand community dynamics (9–12). These molecular studies indicate that the vaginal bacterial communities are dramatically different between women with and without BV. BV is associated with increased taxonomic richness and diversity. The microbiota composition is highly variable among subjects at a fine taxonomic scale (species or genus level), but at phylum level, *Actinobacteria* and *Bacteroidetes* are strongly associated with BV, while higher proportions of *Firmicutes* are found in healthy subjects.

Polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) represents a rapid and reliable technique successfully used to identify the bacterial compositions of different ecological niches, including the vaginal ecosystem (13–15). PCR-DGGE conducted with universal primers for eubacteria allows detecting the most abundant bacterial species of an ecosystem by separation in a denaturing gradient polyacrylamide gel of the hypervariable 16S rRNA gene regions on the basis of the specific nucleotide sequences. Sequencing of the DNA fragments and comparison with sequences present in publicly available databases allow identifying the corresponding bacterial species. Quantitative PCR (qPCR) is a powerful technique for the quantitative analysis of specific microbial populations belonging to complex ecosystems (15–17). Specific primers can be used to focus the quantitative analysis to a selected genus, species, or strain.

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## 2. Materials

### 2.1. Collection of Vaginal Fluids and Extraction of DNA

1. Microcentrifuge with rotor for 1.5 or 2 mL tubes.
2. Vortexer.
3. Thermomixer, shaking water bath, or rocking platform for heating.

4. Saline buffer: 0.9 % NaCl, pH 7.0. Sterilize in autoclave (121 °C for 15 min). Store at 4 °C.
5. Enzymatic lysis buffer: 20 mM Tris-HCl, pH 8, 2 mM EDTA, 1.2 % Triton X-100. Store at room temperature (RT) (see Note 1). Add lysozyme (powder) to 20 mg/mL only prior to use.
6. Glass beads (150–212 µm, Sigma-Aldrich).
7. DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany). Store at RT, with the exception of Proteinase K that needs to be stored at 4 °C.

## **2.2. Polymerase Chain Reaction**

1. DNA quantification device, e.g., NanoDrop 1000 (Thermo Scientific, Wilmington, DE).
2. Thermal Cycler for PCR, e.g., Biometra Thermal Cycler (Biometra, Göttingen, Germany).
3. Thermostable polymerase: GoTaq Flexi DNA Polymerase (Promega, Madison, WI). Store at -20 °C.
4. 5× Green GoTaq Flexi buffer supplied with GoTaq Flexi DNA Polymerase. Store 100 µL aliquots at -20 °C.
5. 5× Colorless GoTaq Flexi buffer supplied with GoTaq Flexi DNA Polymerase. Store 100 µL aliquots at -20 °C.
6. MgCl<sub>2</sub> solution, 25 mM supplied with GoTaq Flexi DNA Polymerase. Store 100 µL aliquots at -20 °C.
7. HDA1-GC (5'-CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAC TCC TAC GGG AGG CAG CAG T-3') and HDA2 (5'-GTA TTA CCG CGG CTG CTG GCA C-3') primers (18) (see Note 2). Lyophilized primers are stored at RT.
8. Primer solutions: 50 pmol/µL in 10 mM Tris-HCl, pH 7.0. Store 100 µL aliquots at -20 °C.
9. Deoxynucleoside triphosphate (dNTP): DNA Polymerization mix, 20 mM each dNTP (e.g., GE Healthcare). Store at -20 °C.
10. Working solution of 2.5 mM dNTP is prepared by diluting 20 mM dNTP in sterilized bi-distilled water. Store 100 µL aliquots at -20 °C.

## **2.3. Pre-stained DNA Agarose Gel Electrophoresis**

1. Electrophoresis chamber and power supply.
2. Gel casting tray and combs, properly cleaned with 70 % (v/v) ethanol solution to avoid DNA contamination.
3. Agarose powder for molecular biology.
4. Electrophoresis buffer, usually 0.5× Tris-borate-EDTA (TBE).
5. Ethidium bromide solution 10 mg/mL in water (Sigma) or other non-mutagen gel stain product, e.g., GelRed 10,000× reagent in water (Biotium, Hayward, CA).

6. Sample loading buffer, unless PCR buffer already contains it, e.g., 5× Green GoTaq Flexi buffer (Promega).
7. 100 bp DNA ladder.
8. Ultraviolet transilluminator, e.g., GelDoc XR (BioRad).

**2.4. Denaturing  
Gradient Gel  
Electrophoresis**

1. D-Code Universal Mutation System Apparatus (Bio-Rad, Hercules, CA).
2. Model 485 Gradient Former (Bio-Rad).
3. 20-cm by 20-cm glass plates for vertical electrophoresis (Bio-Rad) (see Note 3).
4. Gel spacers (0.75 mm).
5. Acrylamide-*N,N'*bisacrylamide, 40:3 w/v (GE Healthcare). Store at 4 °C.
6. TAE buffer 50×: 2 M Tris, 1 M glacial acetic acid, and 50 mM EDTA, pH 8. Store at RT.
7. Low-density (LO) solution containing 8 % w/v polyacrylamide and 30 % gradient of urea and formamide (volume: 15 mL) for the preparation of the denaturing gel: 3 mL Acrylamide-*N,N'*bisacrylamide, 40:3 (w/v), 300 μL TAE buffer 50×, 1.8 mL formamide, 1.89 g urea, 135 μL ammonium persulfate (APS) 10 % (w/v) (see Note 4), 13.5 μL TEMED, sterilized bi-distilled water up to 15 mL (see Note 5).
8. High-density (HI) solution containing 8 % (w/v) polyacrylamide and 50 % gradient of urea and formamide (volume: 15 mL) for the preparation of the denaturing gel: 3 mL Acrylamide-*N,N'*bisacrylamide, 40:3 (w/v), 300 μL TAE buffer 50×, 3 mL formamide, 3.15 g urea, 135 μL 10 % APS (see Note 4), 13.5 μL TEMED, sterilized bi-distilled water up to 15 mL (see Note 5).
9. Stacking gel containing 8 % (w/v) polyacrylamide (volume: 5 mL): 1 mL Acrylamide-*N,N'*bisacrylamide, 40:3 (w/v), 100 μL TAE buffer 50×, 45 μL APS 10 % (see Note 4), 4.5 μL TEMED, sterilized bi-distilled water up to 5 mL.
10. Running buffer: TAE 1×. Prepare from stock solution of TAE 50× and immediately use.
11. Solutions for silver staining:
  - (a) Fixing solution: 10 % (v/v) ethanol/0.5 % (v/v) glacial acetic acid.
  - (b) Silver solution: 0.15 % (w/v) silver nitrate, 0.15 % (v/v) formaldehyde.
  - (c) Developing solution: 3 % (w/v) sodium carbonate, 0.08 % (w/v) sodium thiosulfate, 0.15 % (v/v) formaldehyde.
  - (d) Stop solution: 7.5 % (v/v) glacial acetic acid.

All these solutions must be immediately used after preparation.

### 2.5. Sequencing of the V2–V3 Region of the 16S rRNA Gene

1. 1× Colorless GoTaq reaction buffer: Prepare from 5× Colorless GoTaq reaction buffer.
2. Materials for PCR: The same as described in Subheading 2.2 (see Note 6).
3. QIAquick PCR purification kit (Qiagen). Store at RT (see Note 7).
4. Sequencing service.

### 2.6. qPCR

1. Capillary-based real-time PCR apparatus (e.g., Light Cycler system 2.0, Roche, Mannheim, Germany) or Multiwell plate-based real-time PCR apparatus.
2. Benchtop microcentrifuge. If a multiwell plate-based system is used, a multiwell plate centrifuge is necessary.
3. Centrifuge adapters for capillaries with a dedicated aluminum cooling block. Cool at 4 °C prior to use.
4. 20 µL glass capillaries for real-time PCR (e.g., Light Cycler Capillaries, Roche). Store at room temperature, do not touch the surface of the capillaries.
5. SYBR Green-based qPCR kit, e.g., Light Cycler Fast Start DNA Master<sup>PLUS</sup> SYBR Green I kit (Roche). All reagents needed for the qPCR reaction (Light Cycler FastStart Enzyme, Light Cycler FastStart DNA Master PLUS Reaction Mix, PCR-grade H<sub>2</sub>O) are provided with the kit. Store at –20 °C, avoid repeating freezing and thawing, and protect the Light Cycler FastStart Enzyme vial from light. Any other SYBR Green-based qPCR kit can be used, depending on the available real-time PCR machine.
6. qPCR dedicated software, e.g., Light Cycler Software 4.0, installed on a personal computer connected to the real-time PCR available apparatus.
7. DNA quantification device, e.g., NanoDrop 1000 (Thermo Scientific).
8. Standard DNA of known concentration. Genomic DNA can be purchased from DSMZ, or other culture collections (see Note 8). Genomic DNA of the target organism or of a representative organism belonging to the targeted bacterial group (i.e., *Gardnerella vaginalis* ATCC14018 for *G. vaginalis*-specific PCR; *Prevotella bivia* ATCC29303 for *Prevotella* genus-specific PCR; *Veillonella parvula* ATCC10790 for *Veillonella* genus-specific PCR; *Atopobium vaginae* ATCC BAA-55 for *Atopobium* genus-specific PCR; *Lactobacillus acidophilus* NCFM for *Lactobacillus* genus-specific PCR) is used. Standards are prepared by serial dilutions of genomic DNA (start concentration around 10–20 ng/µL), using PCR-grade H<sub>2</sub>O, as described in Subheading 3.

**Table 1**  
**Primer sets used for quantification of relevant target bacterial groups for the characterization of the BV-related vaginal microbiota**

Target	Primer	Sequence	Position (5'–3') <sup>a</sup>	Amplicon length (bp)	References
<i>Gardnerella vaginalis</i>	F-GV1	5'-TTACTGGTGTATCACT GTAAGG-3'	16S–23S spacer	330	(19)
	R-GV3	5'-CCGTCACAGGCTGAACAG-3'			
<i>Atopobium</i> cluster	c-Atopo-F	5'-GGGTTGAGAGACCGACC-3'	285–301	195	(20)
	c-Atopo-R	5'-CGGRGCTTCTTCTGCAGG-3'	498–481		
<i>Prevotella</i> spp.	g-Prevo-F	5'-CACRGTAACGATGGATGCC-3'	800–819	513	(21)
	g-Prevo-R	5'-GGTCGGGTTGCAGACC-3'	1,319– 1,304		
<i>Veillonella</i> spp.	Veillo-F	5'-AYCAACCTGCCCTCAGA-3'	120–136	342	(22)
	Veillo-R	5'-CGTCCCGATTAACAGAGCTT-3'	443–424		
<i>Lactobacillus</i> spp.	Bact-0011f	5'-AGAGTTTGATCATGGCTCAG-3'	1–20	700–710	(23)
	Lab-0677r	5'-CACCGCTACACATGGAG-3'	686–670		

<sup>a</sup>Positions are relative to the sequence of the 16S rRNA gene of *Escherichia coli* ATCC8739, sequenced genome (accession number CP000946)

- Species- or genus-specific 16S rRNA gene-targeted primer sets as described in Table 1 (see Note 9). Lyophilized primers are stored at RT. Primer stock solutions 50 pmol/μL are prepared in sterile 10 mM Tris–HCl, pH 7. Store 100 μL aliquots at –20 °C.

### 3. Methods

#### 3.1. Collection of Vaginal Fluids and Extraction of DNA

- Standardized vaginal rinsings with 2 mL of saline are collected by flushing and re-aspirating the fluid through a needle in the left, central, and right upper vaginal vaults.
- The vaginal rinsings are stored at –80 °C until use.
- One milliliter of the vaginal rinsing is centrifuged at 7,500 × *g* for 15 min, and the pellets washed three times in saline at 40 °C.
- The pellet is resuspended in 180 μL of Enzymatic Lysis Buffer and incubated at 37 °C for 30 min.
- 200 mg glass beads are added and the sample is mixed by vortexing for 1 min.
- Total bacterial DNA is extracted by using DNeasy Blood & Tissue Kit (see Note 10). The sample is incubated for at least

30 min at 37 °C. Proteinase K (25 µL) and Buffer AL (200 µL) are added and the sample is mixed by vortexing. The sample is incubated at 56 °C for 30 min and centrifuged at 5,500×*g* for 5 min to remove glass beads. 200 µL ethanol (96–100 % (v/v)) are added to the supernatant and the sample is mixed by vortexing. The mixture is pipetted into the DNeasy Mini spin column placed in a 2 mL collection tube and centrifuged at 6,000×*g* for 1 min. Flow-through and collection tube are discarded. The DNeasy Mini spin column is placed in a new 2 mL collection tube and 500 µL Buffer AW1 are added. Centrifugation at 6,000×*g* for 1 min is performed. Flow-through and collection tube are discarded. The DNeasy Mini spin column is placed in a new 2 mL collection tube and 500 µL Buffer AW2 are added. Centrifugation at 20,000×*g* for 1 min is performed to dry the DNeasy membrane. Flow-through and collection tube are discarded. The DNeasy Mini spin column is placed in a clean 1.5 mL microcentrifuge tube and 200 µL Buffer AE are pipetted onto the DNeasy membrane. After incubation at room temperature for 1 min, a centrifugation for 1 min at 6,000×*g* was performed to elute DNA.

### **3.2. PCR**

1. Amplification of the V2–V3 region of the bacterial 16S rRNA gene is carried out using the universal eubacterial primers GCclamp-HDA1-GC and HDA2 (18). The amplification reactions are performed in a Biometra Thermal Cycler. GoTaq Flexi DNA Polymerase is used as a thermostable polymerase under conditions suggested by the supplier.
2. Concentration of DNA sample is measured by NanoDrop 1000 or any other DNA quantification device. DNA sample is diluted to 15–20 ng/µL in sterilized bi-distilled water and used as template for PCR reaction.
3. The PCR reaction mixture contains 0.5 µM of each primer, 200 µM of each dNTP, 2 mM MgCl<sub>2</sub> solution, 1.25 U of GoTaq Flexi DNA Polymerase, 5 µL of Green GoTaq Flexi buffer 5× (see Note 11), and 2 µL of the diluted DNA template in a final volume of 25 µL.
4. The thermocycle program consists of the following time and temperature profile: 95 °C for 5 min; 30 cycles of 95 °C for 30 s, 56 °C for 30 s, 72 °C for 60 s; and 72 °C for 8 min.

### **3.3. Pre-stained DNA Agarose Gel Electrophoresis**

1. Prepare gel casting tray and combs. Properly seal the edges of the gel tray with tape, or use provided clips.
2. Mix appropriate amounts of 0.5× TBE buffer and agarose powder in a glass flask. For small PCR amplicons (200–500 bp) prepare 1.2–1.5 % agarose gel (1.2–1.5 g agarose powder for each 100 mL buffer). The buffer should not occupy more than 50 % of the flask volume.

3. Loosely plug the flask with kimwipes and heat the slurry in a boiling water bath or microwave until the agarose dissolves.
4. Cool the solution at 60 °C and add ethidium bromide to a final concentration of 0.5 µg/mL. Alternatively, add 10 µL of GelRed 10,000× for each 100 mL gel solution. Mix thoroughly.
5. Pour the warm agarose solution into the gel casting tray.
6. After the gel is completely set (35–40 min RT), remove the combs and the strips of tape.
7. Mount the gel in the electrophoresis chamber and add enough 0.5× TBE buffer to cover the gel.
8. Mix 5 µL of PCR reaction with the appropriate amount of gel loading buffer if necessary.
9. Load each sample in a separate well. The first and/or last well of the gel are usually used for DNA ladder.
10. Close the lid of the electrophoresis chamber and connect the electrical leads so that the DNA will migrate toward the anode (red lead). Apply a voltage 1–5 V/cm. Run the gel until the colored loading dye have migrated the appropriate distance through the gel.
11. Examine the gel by ultraviolet light and check for the cleanness of the PCR amplification and the approximate amount of PCR amplicons by comparison with the DNA ladder bands.

### 3.4. DGGE

DGGE analysis is performed using the D-Code Universal Mutation System Apparatus with 20-cm by 20-cm by 0.75-mm gels. Sequence-specific separation of the PCR fragments is obtained in 8 % (w/v) polyacrylamide gels in 0.5× TAE buffer. The denaturing gel contains a 30–50 % gradient of both urea and formamide increasing in the direction of electrophoresis. An example of DGGE profiles of vaginal fluids is shown in Fig. 1.

1. Clean the glass plates with water and 70 % (v/v) ethanol and assemble the parallel gradient gel sandwich, using the gel spacers.
2. Prepare the LO and HI solutions of 8 % (w/v) polyacrylamide. Do not add 10 % APS and TEMED at this step.
3. Connect the Gradient Former to the gel sandwich by a tube with a needle. Place the needle at the top-center of the gel sandwich. Turn on the pump and turn off the two taps in the Gradient Former.
4. Add 135 µL 10 % (w/v) APS and 13.5 µL TEMED to the LO and HI polyacrylamide solutions. Insert 11.5 mL of LO solution in the left chamber and 11.5 mL of HI solution in the right chamber of the Gradient Former.

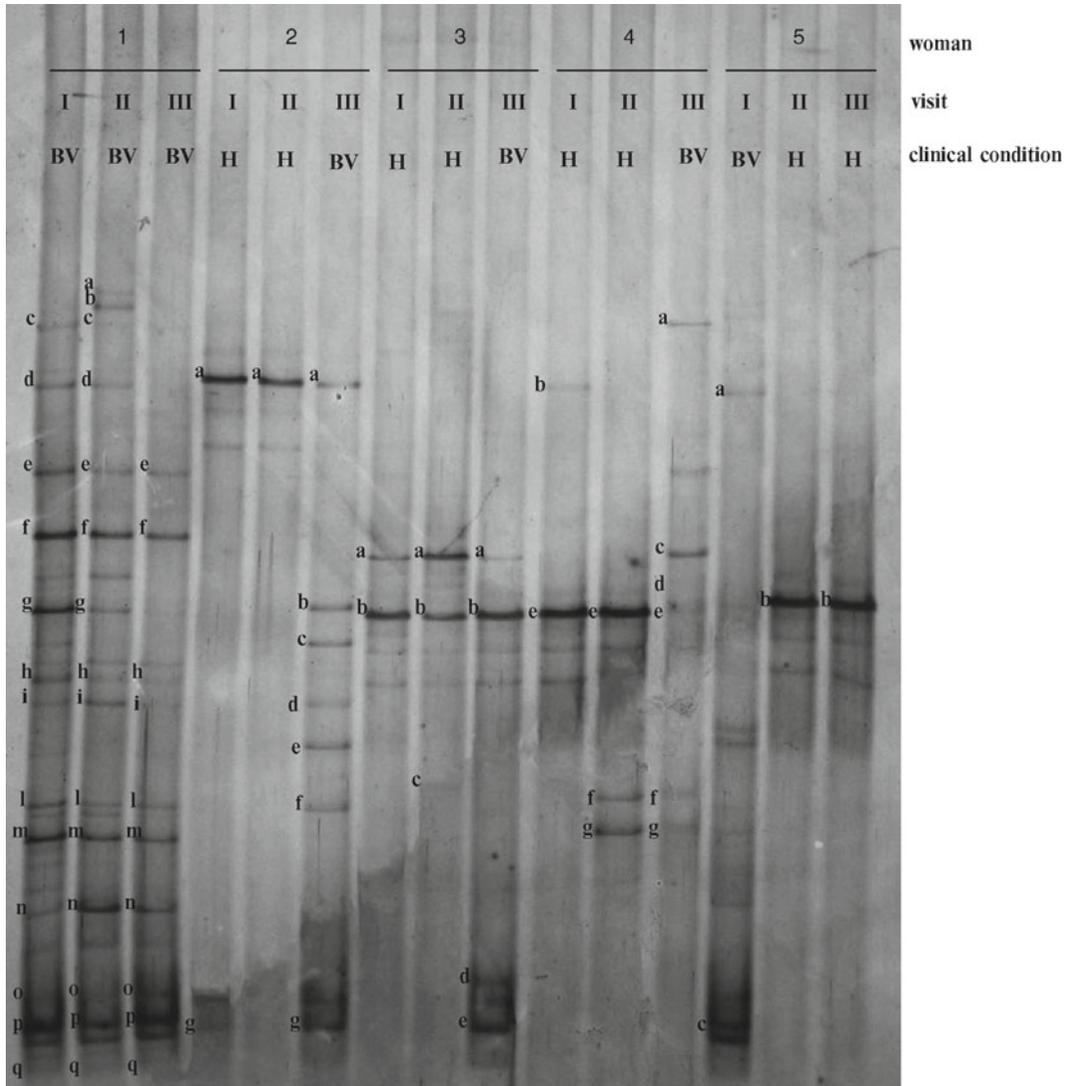


Fig. 1. DGGE profiles of vaginal fluids collected from five women developing bacterial vaginosis. Three DGGE patterns correspond to the three monthly gynecologic visits. The clinical condition at each visit, healthy (H) or bacterial vaginosis (BV), is reported. The bands correspond to the following bacterial taxa: *Lactobacillus iners*: 3b, 4e, 5b; *Lactobacillus acidophilus*: 3a, 4d; *Lactobacillus plantarum*: 4a; *Lactobacillus gasseri*: 2a, 4b; *Lactobacillus vaginalis*: 3c; *Atopobium vaginae*: 1q, 2g; *Gardnerella vaginalis*: 1m, 1o, 1p, 3d, 3e, 5c; *Leptotrichia amnionii*: 1a, 1b, 1d; *Leptotrichia sanguinegens*: 5a; uncultured *Prevotella* sp.: 1c, 1f, 1g, 1h; *Prevotella* sp.: 2b, 2e; uncultured *Megasphaera* sp.: 1l, 4f, 4g; uncultured Chloroflexi bacterium: 1i; uncultured *Clostridium* sp.: 1n; *Streptococcus* sp.: 2c; *Staphylococcus* sp.: 1e, 4c; *Veillonella montpellierensis*: 2d, 2f.

5. Turn on simultaneously the two taps of the Gradient Former and start up the pump.
6. After finishing the filling of the gel sandwich, overlay the gel with water and let the gel polymerize for about 60 min at RT.
7. Remove the water from the polymerized gel and prepare 5 mL of stacking gel. Load the stacking gel solution on the separating gel and insert the comb to form the wells. The stacking gel

should polymerize within 30 min at RT. Remove the comb by pulling it straight up slowly and gently (see Note 12).

8. Prepare 7 L of the running buffer (1× TAE) by diluting the stock solution of 50× TAE buffer. Fill the electrophoresis tank to the “Fill” line with 1× TAE buffer (see Note 13).
9. Place the temperature control module on the top of the electrophoresis tank and connect to a power supply. Preheat the buffer to the temperature of 60 °C for 1–1.5 h.
10. When the running buffer has reached the desired temperature, place the core and the attached gel assemblies into the buffer chamber. Add the running buffer between the glass plates.
11. A volume of 8–16 µL of PCR sample is loaded per each well, depending on the concentration of the sample (see Note 14). Include at least three wells for the molecular weight markers.
12. Electrophoresis is carried on at 150 V for the first 5 min and then at 90 V for 16 h at constant temperature of 60 °C.
13. After electrophoresis, the gel is silver stained based on the protocol of Bassam et al. (24).
  - (a) Put the gel in fixing solution for 20–30 min.
  - (b) Rinse the gel in sterilized bi-distilled water three times (2 min for each washing).
  - (c) Stain the gel with silver solution for 30 min.
  - (d) Wash with sterilized bi-distilled water for 20 s.
  - (e) Put the gel in developing solution until the appearance of the bands.
  - (f) Add stop solution and remove it after 5 min. Soak the gel in sterilized bi-distilled water.

### **3.5. Sequencing of the V2–V3 Region of the 16S rRNA Gene**

1. DNA fragments of interest are excised from the silver stained, denaturing gels with a sterile scalpel, washed once in 1× Colorless GoTaq reaction buffer, and incubated in 20 µL of the same buffer overnight at 4 °C.
2. Two microliter of the buffer solution are used as template for PCR reaction. Re-amplification of the V2–V3 regions is conducted as described above by employing the primers HDA1 and HDA2 (see Note 6).
3. The re-amplified fragments are purified using the QIAquick PCR Purification Kit (see Note 15). Five volumes of Buffer PB are added to one volume of the PCR sample and the sample is mixed. QIAquick spin column is placed in a 2 mL collection tube. To bind DNA, the sample is applied to the QIAquick column and centrifuged for 60 s. Flow-through is discarded and the QIAquick column is placed into the same collection

tube. To wash, 0.75 mL Buffer PE are added to the QIAquick column and the column is centrifuged for 60 s. Flow-through is discarded and the column is centrifuged for an additional 1 min. The QIAquick column is placed in a clean 1.5 mL microcentrifuge tube. To elute DNA, 50  $\mu$ L Buffer EB or water is added to the center of the QIAquick membrane and the column is centrifuged for 1 min (see Note 16).

- Both DNA strands of the purified PCR products are subjected to automated sequence analysis by an external service with HDA1 and HDA2 primers.
- The sequence identities are determined by comparison with the rRNA gene sequences deposited in GenBank database using BLAST algorithm. The microorganisms corresponding to each sequence are determined by similarity search using the blastn tool of BLAST algorithm ([www.ncbi.nlm.nih.gov/BLAST](http://www.ncbi.nlm.nih.gov/BLAST), (25)). Select the appropriate reference database (“Nucleotide collection” among “Other databases,” see Note 17).

### 3.6. qPCR

- qPCR reactions for each different bacterial group (*Lactobacillus* spp., *G. vaginalis*, *Prevotella* spp., *Veillonella* spp., and *Atopobium* cluster) are carried out separately.
- Quantify DNA amounts in the DNA samples extracted from the vaginal fluids by using NanoDrop 1000 (or any other quantification device). Optimal DNA concentration for bacterial DNA samples should be 20–25 ng/ $\mu$ L. Samples having higher DNA concentration are diluted accordingly, using PCR-grade water, and re-quantified. Do not use EDTA-containing buffers to dilute DNA samples.
- Prepare DNA dilutions to use as quantification standards. Start from 10 to 20 ng/ $\mu$ L of concentrated DNA solution and make serial dilutions using PCR-grade water. Dilutions 1:5, 1:10, 1:50, 1:100, 1:500, 1:1,000, and 1:5,000 can be used as standards for quantification of *Lactobacillus* spp., *G. vaginalis*, *Prevotella* spp., *Veillonella* spp., and *Atopobium* cluster. Use NanoDrop 1000 (or any other quantification device) to obtain the exact concentration of each dilution. To store dilutions at  $-20^{\circ}\text{C}$  it is recommended to prepare small aliquots in order to avoid repeated freezing and thawing.
- Program the qPCR instrument with the aid of the dedicated software following one of the qPCR protocols reported in Table 2, depending on the bacterial groups which are being quantified. Briefly, an initial denaturation of the template DNA is required ( $95^{\circ}\text{C}$  for 10 min); this step is also required when a thermostable heat-activated DNA polymerase is being used (e.g., FastStart Taq DNA polymerase provided with the LightCycler FastStart DNA Master PLUS SYBR Green I kit).

**Table 2**  
**PCR parameters for a Light Cycler Carousel-based System PCR run with Light Cycler Start DNA Master<sup>PLUS</sup> SYBR Green I kit**

Target	Gardnerella vaginalis		Atopobium cluster		Prevotella spp.		Veillonella spp.		Lactobacillus spp.									
	F-GV1-R-GV3	Incubation T (°C)	Incubation	T (°C)	cAtopoF-cAtopoR	Incubation	T (°C)	gPrevoF-gPrevoR	Incubation	T (°C)	VeilloF-VeilloR	Incubation	T (°C)	Incubation	T (°C)	Incubation	Transition rate (°C/s)	Cycles
Denaturation	95	10 min	10 min	95	10 min	10 min	95	10 min	10 min	95	10 min	10 min	95	10 min	20	1	None	
Amplification	95	15 s	15 s	95	15 s	15 s	95	15 s	15 s	95	15 s	15 s	95	15 s	20	30	None	
	60	20 s	20 s	60	20 s	20 s	62	20 s	20 s	63	20 s	20 s	60	20 s	20	None		
	72	45 s	45 s	72	45 s	45 s	72	45 s	45 s	72	45 s	45 s	72	45 s	20	None		
	85	5 s	5 s	85	5 s	5 s	85	5 s	5 s	85	5 s	5 s	87	5 s	20	Single		
Melting curve	95	0 s	0 s	95	0 s	0 s	95	0 s	0 s	95	0 s	0 s	95	0 s	20	1	None	
	60	15 s	15 s	60	15 s	15 s	60	15 s	15 s	60	15 s	15 s	60	15 s	20	None		
	99	0 s	0 s	99	0 s	0 s	99	0 s	0 s	99	0 s	0 s	99	0 s	0,1	Continue		
Cooling	40	20 s	20 s	40	20 s	40	20 s	20 s	40	20 s	20 s	40	20 s	20	1	None		

Protocols for *Gardnerella vaginalis*, *Atopobium* cluster, *Prevotella* spp., *Veillonella* spp., and *Lactobacillus* spp. Specific qPCRs are indicated.

Amplification is composed by 30–35 cycles (see Note 18) of denaturation (95 °C for 15 s), annealing (20 s, the temperature varies depending on the primer set), elongation (72 °C for 45 s), and fluorescence acquisition (5 s, the temperature varies depending on the amplicon length). Melting curve is produced at the end of the amplification step by increasing the temperature up to 99 °C with a low transition rate (0.1 °C/s), with a continued fluorescence acquisition. These protocols were developed using capillary-based real-time PCR system; if a multiwell plate-based system is used temperatures and incubation times indicated in the protocols may need some optimization.

5. Program the qPCR run using the dedicated software. Insert samples and standards names in the appropriate order, corresponding to the order in which capillary will be placed in the carousel (or corresponding to the order in which standards and samples will be placed in the multiwell plate), and define them as “standard,” “negative control,” or “unknown.” For each standard calculate the amount of DNA (ng) per reaction, considering that 2 µL of each dilutions will be used as template, and insert these data in the appropriate field. It is recommended to do this programming step before preparing PCR mix and samples in order to keep them in the light and at RT as shortly as possible.
6. If LightCycler FastStart DNA Master PLUS SYBR Green I kit is being used, prepare Light Cycler 5× MasterMix by pipetting the appropriate amount of Light Cycler FastStart Enzyme in the pre-thawed Light Cycler FastStart DNA Master PLUS Reaction Mix vial. Mix gently by pipetting (avoid vortexing) and store in ice or in the precooled cooling block until ready to use, keeping the vial away from light. Prepared 5× Master Mix can be aliquoted and stored at –20 °C up to 3 months. Each vial of prepared 5× Master Mix is sufficient for 32 qPCR reactions. These instructions are easily adaptable to any other qPCR commercial kit.
7. Prepare qPCR Mix for the required number of reactions, based on the qPCR Mix composition reported in Table 3. Primers are used at a final concentration of 0.5 µM. The total number of reactions is given by the number of samples to analyze, plus the number of required standards, plus a negative control. It is recommended to keep the qPCR mix in ice and away from light until ready to use.
8. Place the required number of capillaries in precooled centrifuge adapters, placed on the cooling clock. It is recommended to work at low temperature also for multiwell plate, i.e., use a dedicated cooling block or work in ice.

**Table 3**  
**PCR mix composition for a 20  $\mu\text{L}$  qPCR reaction**

Components	One reaction ( $\mu\text{L}$ )
5 $\times$ Master Mix	4
Primer F 50 pmol/ $\mu\text{L}$	0.2
Primer R 50 pmol/ $\mu\text{L}$	0.2
PCR grade water	13.6
Total volume	18

9. Pipette 18  $\mu\text{L}$  of qPCR mix in each capillary or well in the multiwell plate.
10. Add 2  $\mu\text{L}$  of DNA sample or standard to each capillary/well. To prepare a negative control replace the DNA template with PCR-grade water.
11. Seal each capillary with the provided stopper or seal the multiwell plate with plastic film.
12. Place the centrifuge adapters into a standard benchtop microcentrifuge and spin at  $700\times g$  for 5 s.
13. Transfer the capillaries in the sample carousel and then into the qPCR instrument. If a multiwell plate is being used simply insert it in the appropriate place in the qPCR machine.
14. Cycle the samples as programmed in step 4.
15. When the PCR reaction is finished melting curve analysis is performed with the qPCR dedicated software. Positive amplification product is indicated by a sharp peak at a temperature higher than 87  $^{\circ}\text{C}$  (for *Prevotella* amplification) or 85  $^{\circ}\text{C}$  (for all the other bacterial groups analyzed in this protocol). Check the melting curve for the absence of peaks after the temperature of fluorescence acquisition in the negative control; a peak around 75  $^{\circ}\text{C}$  can be visible due to the primer dimers.
16. Perform linear regression of the crossing points obtained for the standards. Check that the  $r$  correlation coefficient obtained by the linear regression is as close as possible to 1 (see Note 19). Hence, the software will automatically quantify the target DNA in “unknown” samples as ng of target DNA per reaction (20  $\mu\text{L}$ ). Calculate the concentration of the target DNA in the original samples as ng of target DNA/ $\mu\text{g}$  of total bacterial DNA.

## 4. Notes

1. Calculate the volume of enzymatic lysis buffer on the basis of the number (No.) of the vaginal fluids from which DNA will be extracted (Volume = No.  $\times$  180  $\mu$ L).
2. An example of a supplier of PCR primers is M-Medical, Milan, Italy.
3. Glass plates must be cleaned with 96–100 % (v/v) ethanol before use.
4. Solution of 10 % (w/v) APS should be prepared and immediately used in order to avoid loss of catalytic activity in the polymerization reaction.
5. Volumes of 10 % APS and TEMED must be decreased for temperatures  $>25$   $^{\circ}$ C (10 % APS: 100  $\mu$ L, TEMED: 10  $\mu$ L) because of the stimulation effect of high temperatures on acrylamide polymerization.
6. The primer HDA1 was used without the 5' GC clamp (5'-ACG GGG GGA CTC CTA CGG G AG GCA GCA GT-3').
7. Any other PCR purification kit may be suitable, according to the manufacturer's instructions.
8. Bacterial DNA can also be extracted and purified from a pure bacterial culture, e.g., using DNeasy Blood & Tissue kit (Qiagen). If lyophilized genomic DNA is purchased, dissolve lyophilized material in PCR-grade water. Do not use EDTA-containing buffers. Overnight incubation at 4  $^{\circ}$ C should ensure optimal suspension of the DNA material.
9. More bacterial groups may be of interest in the study of BV (e.g., *Mobiluncus* and *Mycoplasma*). In this perspective, species- or genus-specific primer sets and amplification conditions will need to be set on the different targeted microorganisms, following the examples here reported.
10. Equipment and reagents are provided in the DNeasy Blood & Tissue Kit. 96–100 % (v/v) ethanol and microcentrifuge tubes must be supplied by user.
11. The 5 $\times$  Green GoTaq Flexi Buffer contains two dyes (a blue dye and a yellow dye) that separate during electrophoresis to monitor migration progress.
12. Invert the gel and dry the wells with blotting paper.
13. It is recommended that the running buffer shall not be reused. Reusing the running buffer may affect the migration rate and band resolution.
14. An approximate estimation based on the agarose gel electrophoretic bands is sufficient at this step.

15. Equipment and reagents are provided in the QIAquick PCR Purification Kit. Add 96–100 % (v/v) ethanol to buffer PE before use. Ethanol and microcentrifuge tubes must be supplied by user. Any other PCR purification kit can be used.
16. Alternatively, for increased DNA concentration, add 30  $\mu$ L elution buffer to the center of the QIAquick membrane, let the column stand for 1 min, and then centrifuge.
17. Homology >95 % was used as a criterion for the identification of a species.
18. Depending on the health status of the enrolled subjects (i.e., healthy or BV) it may be necessary to increase the number of amplification cycles in order to obtain a more accurate quantification of low-abundant species. For instance, it may be necessary to increase the number of cycle from 30 to 35 to quantify *Prevotella* spp. in healthy women.
19. Linear regression analyzes the relationship between two variables. The  $r$  correlation coefficient indicates the slope of the straight line that most closely relates two correlated variables.

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## Protocol for the Use of a Bead Array for the Multiple Detection of Genotype of *Chlamydia trachomatis*

Chung-Te Huang and Shu-Ying Li

### Abstract

The identification of *Chlamydia trachomatis* genotypes is important for both molecular epidemiology and infection control such as contact tracing and identification of high-risk groups. Currently, at least 19 human serovars have been recognized by using polyclonal and monoclonal antibodies against the major outer membrane protein. In sexually transmitted diseases, multiple pathogens or genotype infections are not uncommon. Hence, detection of multiple gene targets in one reaction is becoming increasingly important. Here, we describe the multiplex detection of eight genotypes of *C. trachomatis* by a combination of a PCR amplification with a multiplex bead array detection. The bead array system comprises distinct bead sets, which are color coded by different fluorescent intensities and a dual-laser flow cytometer analyzer to identify the identity of the bead and the intensity of the reporter dye that binds to the target molecules. The DNA sequences of the variable segments (VS2 or VS1–VS2) in outer membrane protein (*omp1*) gene are PCR amplified and biotin labeled and used as a gene target for the genotyping of *C. trachomatis*. Genotype-specific probes coupled to beads are used for capturing the labeled target amplicons through specific hybridization. Thus, multiple genotypes are detected and differentiated simultaneously by yielding quantitative data.

**Key words:** *Chlamydia trachomatis*, Genotype, Multiplex detection, Bead array

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### 1. Introduction

The *Chlamydia trachomatis* infection is the most prevalent sexually transmitted bacterial disease. It is estimated that 89 million cases occur annually worldwide (1). Because 50 % of infected men and 80 % of infected women are asymptomatic, the actual number of reported cases represents only a fraction of the infected population (1). Currently, 19 human serovars have been recognized by using polyclonal and monoclonal antibodies against the major outer membrane protein (MOMP) (1–5). However, the serological

**Table 1**  
**Probes used for multiplex *Chlamydia trachomatis* genotyping of VS1–2 PCR**

Probe	Gen Bank accession no.	Probe sequence (5'–3')	Probe signal range (MFI) <sup>a</sup>		
			Negative	Positive	Min ratio <sup>b</sup>
Ba <sup>c</sup>	AY950630	GAGAACCAGACTAAAGTTTCAA	0–32	136	3.8
D <sup>d</sup>	X62918	AAAAAACGGTCAAAGCGGAGTC	0–45	225–851	5.0
E <sup>d</sup>	X52557	ACAGATACTGCCTTCTCTTGG	0–31	225–977	7.3
F	X52080	ATCTGCAGCAGGTTTCGTGG	0–25	105–311	4.2
G	AF063199	CAGGCTGCGTGCGCTTTT	0–31	602–791	19.4
H <sup>d</sup>	X16007	ACAAAATCTTCTGATTTTAATACAGC	0–24	163–426	6.8
J	AF063202	TCTTTTTCCTAACACCGCTTTGAA	0–28	68–102	2.4
K <sup>d</sup>	AF063204	AACACTGCTTTGGATCGAGCTGTG	0–41	171–271	4.2

<sup>a</sup>The negative range is the range of the median fluorescence intensity (MFI) for all negative strains after the subtraction of the background for the given probe. The positive range is the range of the MFI for all positive strains after the subtraction of the background for the given probe

<sup>b</sup>The minimum ratio is the lowest recorded positive value divided by the highest negative value. The minimum ratio of >2 is used as the threshold for defining positive events

<sup>c</sup>The *C. trachomatis* type-specific probe Ba has previously been published (6)

<sup>d</sup>These *C. trachomatis* type-specific probes, D, E, H, and K, have previously been published (8)

typing method usually requires the culturing of clinical isolates and a large pool of monoclonal antibodies, which makes application in a clinical laboratory difficult (6). The outer membrane protein (*omp1*) gene encoding MOMP exhibits extensive DNA sequence variation in four discrete regions, termed variable segments (VS1–4). Using molecular methods such as gene sequencing and restriction fragment length polymorphism (RFLP), which target the VS2 or VS1–VS2 region of *omp1* gene, the genotypes of *C. trachomatis* could also be identified and differentiated (6–9). However, these methods face limitations in typing multiple infections with different *C. trachomatis* genotypes in one specimen. Molecular cloning increases the rate of detecting multiple infections (10). Nevertheless, it is laborious to screen and sequence different clones. Herein we describe a molecular genotyping method by using a bead array platform which can accurately discriminate eight clinically important *C. trachomatis* genotypes in one clinical specimen (see Table 1). Compared to gene sequencing or RFLP, this assay is economic and time-saving, and is able to identify multiple infections in one tube (see Table 2) like other array-based methods (6, 8, 9).

In comparison to southern blot or line blot analyses in which sample DNA or oligonucleotide probes are coated on a solid

**Table 2**  
**Representative examples for multiplex**  
***Chlamydia trachomatis* genotyping of VS1–2**  
**PCR products from clinical specimens**

Serovars	Clinical specimens	
	Urine	Endocervical swab
D/Da	9	5
E	5	6
F	3	11
G	5	3
J	1	1
K	5	5
Ba	0	1
H	0	1
D/K	0	1
K/E	0	1
Total	28	35

membrane, this bead array method is liquid based beads coupled with probes and subject to a flow cytometrical analysis. Probes with a 5'-end amino C-12 modification directed to the VS2 region of *C. trachomatis* are covalently coupled to 1 of 100 fluorescence differentially labeled bead sets with surface carboxyl groups. Forward and reverse primers modified by 5' biotinylation are used to amplify VS1–2 of *omp1* of specimens. The biotinylated PCR products are captured by specific probes coupled to bead sets and incubated with fluorescent reporter molecule (streptavidin–R-phycoerythrin). The bead mixture is then identified in a dual-laser cytometer detecting the bead identity via its unique internal color code and the signal and quantity of the reporter dye tagged to PCR amplicon bound to the bead.

In this chapter, we provide a detailed protocol for the bead array and add troubleshooting information experienced in the process. In general, the process can be divided into five steps: sample preparation, amplification of *omp1* gene regions, coupling of C12-amine-modified oligonucleotides to carboxylated beads, oligonucleotide hybridization to the *omp1* amplicons of a specimen, and detection.

## 2. Materials

### 2.1. Sample Collection, Storage, and Genomic DNA Extraction

1. 1.5 mL microcentrifuge tubes.
2. QIAamp Viral RNA mini Kit (QIAGEN). This Kit contains QIAamp Mini Spin Columns, Collection Tubes (2 mL), Buffer AVL, Buffer AW1, Buffer AW2, Buffer and AVE, Carrier RNA.
3. 100, 200, and 1,000  $\mu$ L micropipettes and tips are used for this assay.
4. Ethanol (96–100 %).
5. Freezer ( $-20$  °C and  $-80$  °C).
6. Microcentrifuge.

### 2.2. Amplification of *omp1* Gene Regions by Specific 5'-Biotin-Labeled Primers

1. Extracted DNA (see Subheading 2.1).
2. The outer primer pair, NLO: 5'-ATGAAAAAACTCTTGA AATCG-3' and NRO: 5'-CTCAACTGTAAGTGCCTATTT-3', is used to amplify VS1–4 fragment (1,130 bp) of *omp1*.
3. The inner primer pair, MOMP87:TGAACCAAGCCTTATGATCGACGGA and C214:TCTTCGAYTTTATAGTTTATAGAT TGA, with a 5'-biotin is used to amplify VS1–2 fragment (584 bp) of *omp1*.
4. 2 $\times$  PCR master mix which contains 0.05 U/ $\mu$ L Taq DNA Polymerase, reaction buffer, 4 mM  $MgCl_2$ , 0.4 mM of each dNTP (dATP, dCTP, dGTP, dTTP).
5. PCR machine.
6. 2, 10, 20, 100, and 200  $\mu$ L micropipettes and tips are used for this assay.
7. 200  $\mu$ L microcentrifuge tubes, eight tubes/strip.

### 2.3. Coupling of C12-Amine-Modified Oligonucleotides to Carboxylated Beads

1. 1-Ethyl-3(2-dimethylaminopropyl) carbodiimide hydrochloride (EDC) (Pierce).
2. 2-(*N*-morpholino) ethanesulfonic acid (MES): 0.1 M, pH 4.5 (Sigma).
3. Eight different carboxylated beads ( $1.25 \times 10^7$ /mL): numbers 26, 30, 34, 38, 42, 46, 54, and 56 (Bio-Rad, Hercules, CA, USA) (see Note 1).
4. Eight kinds of 5'-C12 amine-modified oligonucleotide probes for eight *C. trachomatis* genotypes as shown in Table 1 (see Note 2). These probes are coupled to carboxylated beads by a carbodiimide-based coupling mechanism. The concentration of probe used for coupling is 1 mM (1 nmol/ $\mu$ L) in ddH<sub>2</sub>O.

5. 1.5 mL microcentrifuge tubes.
6. 2, 10, 20, 100, and 200  $\mu$ L micropipettes and tips are used for this assay.
7. Microcentrifuge.
8. Vortexer.
9. Sonicator.
10. ddH<sub>2</sub>O.
11. 0.02 % Tween-20.
12. 0.1 % SDS.
13. TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.4).
14. Shaker.
15. Cooler (2–8 °C).

#### **2.4. Direct Oligonucleotide Hybridization**

1. 1.5 $\times$  TMAC (for 250 mL): 225 mL of 5 M TMAC, 1.88 mL of 20 % *N*-lauryl sarkosyl (Sigma), 18.75 mL of 1.0 M Tris-HCl, pH 8.0, 3.0 mL of 0.5 M EDTA pH 8.0, 1.37 mL of ddH<sub>2</sub>O.
2. 1 $\times$  TMAC (for 250 mL): 150 mL of 5 M TMAC, 1.25 mL of 20 % *N*-lauryl sarkosyl (Sigma), 12.5 mL of 1.0 M Tris-HCl pH 8.0, 2 mL of 0.5 M EDTA pH 8.0, 84.25 mL of ddH<sub>2</sub>O.
3. Streptavidin-R-phycoerythrin (as reporter molecule), 4  $\mu$ g/mL.
4. Eight kinds of coupled beads stock ( $5 \times 10^4/\mu$ L).
5. 2, 10, 20, 100, and 200  $\mu$ L micropipettes and tips are used for this assay.
6. 200  $\mu$ L microcentrifuge tubes, eight tubes/strip.
7. PCR machine.
8. Microcentrifuge.
9. ELISA plate.
10. Vortexer.
11. Sonicator.

#### **2.5. Analytical Techniques**

1. Bio-plex 200 Bio-Rad (Hercules, CA, USA), integrated with array reader system, microplate platform, and high-throughput fluid (HTF) system.
2. Sheath fluid (Bio-Rad), used as the delivery medium by HTF to push the samples into the optics component of the Bio-plex 200.
3. 100  $\mu$ L micropipettes and tips are used for this assay.
4. Bio-Plex Manager 4.1 software.

### 3. Methods

#### **3.1. Sample Collection, Storage, and Genomic DNA Extraction**

1. Original urine or endocervical swabs extracted in transport medium can be frozen at  $-80^{\circ}\text{C}$  until further analysis.
2. DNA was extracted using the QIAamp viral RNA minikit (Qiagen) from urine and endocervical samples according to the manufacturer's instructions.
3. Pipet 560  $\mu\text{L}$  of prepared Buffer AVL containing carrier RNA into a 1.5 mL microcentrifuge tube.
4. Add 140  $\mu\text{L}$  urine or endocervical samples to the Buffer AVL-carrier RNA into the microcentrifuge tube. Mix by pulse-vortexing for 15 s.
5. Incubate at room temperature ( $15\text{--}25^{\circ}\text{C}$ ) for 10 min.
6. Briefly centrifuge the tube to remove drops from the inside of the lid.
7. Add 560  $\mu\text{L}$  of ethanol (96–100 %) to the sample, and mix by pulse-vortexing for 15 s.
8. After mixing, briefly centrifuge the tube to remove drops from inside the lid.
9. Carefully apply 630  $\mu\text{L}$  of the solution from step 8 to the QIAamp Mini column (that is positioned in a 2 mL collection tube) without wetting the rim. Close the cap, and centrifuge at  $6,000\times g$  for 1 min. Place the QIAamp Mini column into a clean 2 mL collection tube, and discard the tube containing the filtrate.
10. Carefully open the QIAamp Mini column, and repeat step 9.
11. Carefully open the QIAamp Mini column, and add 500  $\mu\text{L}$  of Buffer AW1. Close the cap, and centrifuge at  $6,000\times g$  for 1 min. Place the QIAamp Mini column in a clean 2 mL collection tube (provided), and discard the tube containing the filtrate.
12. Carefully open the QIAamp Mini column, and add 500  $\mu\text{L}$  of Buffer AW2. Close the cap and centrifuge at  $20,000\times g$  for 3 min.
13. Place the QIAamp Mini column in a new 2 mL collection tube, and discard the old collection tube with the filtrate. Centrifuge at full speed for 1 min.
14. Place the QIAamp Mini column in a clean 1.5 mL microcentrifuge tube. Discard the old collection tube containing the filtrate. Carefully open the QIAamp Mini column and add 60  $\mu\text{L}$  of Buffer AVE equilibrated to room temperature. Close the cap, and incubate at room temperature for 1 min. Centrifuge at  $6,000\times g$  for 1 min.
15. The extracted DNA (1–3  $\mu\text{g}$ ) can be frozen at  $-20^{\circ}\text{C}$  until used in the nested PCR.

**3.2. Amplification  
of MOMP Gene by  
Specific Primer  
Labeled with 5'-Biotin**

1. The first PCR step is carried out in a final reaction volume of 25  $\mu\text{L}$ , containing 0.4 mM each NLO/NRO outer primer pair, 5  $\mu\text{L}$  (50 ng) extracted DNA and 12.5  $\mu\text{L}$  2 $\times$  PCR master mix. The amplification condition: 95  $^{\circ}\text{C}$  for 5 min, 35 cycles of 94  $^{\circ}\text{C}$  for 60 s, 54  $^{\circ}\text{C}$  for 60 s, and 72  $^{\circ}\text{C}$  for 80 s, and a final elongation step at 72  $^{\circ}\text{C}$  for 10 min.
2. In the nested PCR step, 3  $\mu\text{L}$  of PCR products from the reactions of step 1, 0.4 mM each MOMP87/C214 inner primer pair, and 12.5  $\mu\text{L}$  2 $\times$  PCR master mix are added to a final volume of 25  $\mu\text{L}$ . The PCR conditions are as follows: 95  $^{\circ}\text{C}$  for 5 min, 35 cycles of 94  $^{\circ}\text{C}$  for 50 s, 56  $^{\circ}\text{C}$  for 50 s, and 72  $^{\circ}\text{C}$  for 50 s; and a final elongation step at 72  $^{\circ}\text{C}$  for 10 min. The length of the amplicon is 584 bp.

**3.3. Coupling  
of Amine-Modified  
Oligonucleotides  
to Carboxylated Beads**

1. Bring a fresh aliquot of desiccated Pierce EDC powder from  $-20^{\circ}\text{C}$  to room temperature.
2. Resuspend 5  $\mu\text{L}$  of the 1 mL 5' amino-modifier C-12-linked oligonucleotide probes into 1.5 mL microcentrifuge tubes.
3. Resuspend the carboxylated beads ( $1.25 \times 10^7/\text{mL}$ ) gently by vortexer and sonicator with an energy of 100 W and 40 kHz for approximately 20 s.
4. Transfer 200  $\mu\text{L}$  ( $2.5 \times 10^6$ ) of the stock beads to a 1.5 mL microcentrifuge tubes (see Note 3).
5. Pellet the stock beads by centrifugation at  $20,000 \times g$  for 1–2 min.
6. Remove the supernatant and resuspend the beads in 200  $\mu\text{L}$  of 0.1 M MES, pH 4.5 by vortexer and sonicator for approximately 20 s and then centrifuge at  $20,000 \times g$  for 4 min. Remove 175  $\mu\text{L}$  of the supernatant leaving 25  $\mu\text{L}$  of the mixture in the tube (see Note 4).
7. Repeat step 6.
8. Add 1  $\mu\text{L}$  of 5' amino-modifier C-12-linked oligonucleotide probes to the 25  $\mu\text{L}$  resuspended beads and mix by a vortexer.
9. Prepare a fresh solution of 30 mg/mL EDC in ddH<sub>2</sub>O (see Note 5).
10. Add 1  $\mu\text{L}$  of fresh 30 mg/mL EDC to the beads and mix by vortexing.
11. Incubate for 30 min at room temperature with shaking. EDC reacts with a carboxyl group first and forms an amine-reactive O-acylisourea intermediate that quickly reacts with an amino group to form an amide bond between probes and beads.
12. Prepare a second fresh solution of 30 mg/mL EDC in ddH<sub>2</sub>O (see Note 5).

13. Add 1  $\mu\text{L}$  of fresh 30 mg/mL EDC to the beads and mix by vortexing.
14. Incubate for 30 min at room temperature with shaking. This can increase the bond formation.
15. Add 1.0 mL of 0.02 % Tween-20 to the coupled beads.
16. Pellet the coupled beads by centrifugation at  $20,000\times g$  for 1 min.
17. Remove the supernatant and resuspend the coupled beads in 1.0 mL of 0.1 % SDS by vortexing.
18. Pellet the coupled beads by centrifugation at  $20,000\times g$  for 1 min.
19. Remove the supernatant and resuspend the coupled beads in 50  $\mu\text{L}$  TE, pH 8.0 ( $5\times 10^4$  beads/ $\mu\text{L}$ ) and store at 2–8 °C in the dark.

#### **3.4. Direct Oligonucleotide Hybridization**

1. Prepare a hybridization working mixture by adding 3  $\mu\text{L}$  ( $1.5\times 10^5$ ) of each coupled beads stock (here eight bead sets were added) and diluting in 1 mL of  $1.5\times$  TMAC hybridization buffer.
2. Resuspend mixture by vortexer and sonicator for approximately 20 s.
3. Add 33  $\mu\text{L}$  of working mixture to 200  $\mu\text{L}$  microcentrifuge tube.
4. 17  $\mu\text{L}$  of the 25  $\mu\text{L}$  amplified 5' biotinylated PCR products are added to reaction tube of step 3.
5. Homogenize reaction mixtures gently by pipetting up and down several times.
6. Cover the reaction tubes to prevent evaporation and incubate at 95 °C for 5 min to denature any secondary structure in the sample oligonucleotides.
7. Incubate the reaction tubes in PCR machine at hybridization temperature of 45 °C for 35 min.
8. Centrifuge the reaction tubes at  $1,000\times g$  for 3 min to pellet the beads.
9. After centrifugation, carefully remove the supernatant.
10. During centrifugation, prepare fresh reporter mixture by diluting 1:250 streptavidin–R-phycoerythrin in  $1\times$  TMAC hybridization buffer (see Note 6).
11. Add 75  $\mu\text{L}$  of reporter mixture to each tube and mix gently by pipetting up and down several times.
12. Incubate the reaction tubes in PCR machine at hybridization temperature of 45 °C for 10 min.
13. Transfer the entire reaction mixer (75  $\mu\text{L}$ ) from the reaction tubes into the ELISA plate.

### **3.5. Analytical Techniques**

1. Create an experimental panel from the menu of the Bio-Plex Manager 4.1 software by keying in the numbers of bead sets used and the bead number (here 26, 30, 34, 38, 42, 46, 54, and 56) and the probe it carry (here Ba for bead 26, D for bead 30, E for bead 34, F for bead 38, G for bead 42, H for bead 46, J for bead 54, and K for bead 56) (see Note 7).
2. Set the minimal number of beads to be analyzed and calculated per bead set (default 100) per well.
3. Put the ELISA plate into the Bio-plex 200 Analyzer. A total of 50  $\mu$ L of reaction mixture will be drawn and analyzed. This analyzer can analyze the fluorescence intensity of each bead set and display the median fluorescence intensity (MFI) of each bead set in each well.
4. MFIs for all negative samples are calculated after subtraction of background for the given probe.
5. The MFIs for all positive samples are calculated after subtraction of background for the given probe.
6. The “min ratio” (minimal ratio) is the lowest recorded positive MFI value divided by the highest recorded negative MFI value (see Table 1). The “min ratio”  $>2$  is used as a threshold for defining positive events (see Note 8).

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## **4. Notes**

1. This suspension array assay is based on polystyrene beads that are internally dyed with various ratios of two spectrally distinct fluorophores. A total of 100 beads with specific spectra are available. Thus, the analytical machine can differentiate up to 100 kinds of beads in one reaction. The choice of bead set combination is up to the users.
2. Probe sequences of Ba, D, E, H, and K were adopted from previous publications (6, 8). For genotypes F and G, antisense probes were used to improve the signal intensity. Probe J with one nucleotide mismatch was designed to increase specificity and to prevent cross-reaction.
3. One reaction would need 5,000 beads for each specific probe in one tube. After beads coupling, these beads could be used for 500 reactions.
4. These beads may not be completely sedimented; therefore care should be taken removing the supernatant or alternatively centrifuge at a higher revolution to save the beads in pallet completely.
5. It is recommended to use a fresh aliquot of EDC powder for each coupling test.

6. Each test sample should be resuspended with 75  $\mu\text{L}$  of TMAC hybridization buffer. For example, reporter mixture for ten tests could be prepared by adding 3  $\mu\text{L}$  of streptavidin-R-phycoerythrin stock in 750  $\mu\text{L}$  of  $1\times$  TMAC hybridization buffer.
7. The software will automatically recognize the bead identity and create a panel to differentiate the number and kinds of beads added.
8. For example, the MFI of Probe D for unknown sample X is 700 after the subtraction of the background. And the MIF values of Probe D for other samples are 0–45 after the subtraction of the background. The sample-to-negative control (S/N) ratio is the recorded positive value divided by the highest negative value. Therefore the ratio (S/N) of unknown sample X is 15.6 (700/45) which is  $>2$  and is identified as type D of *C. trachomatis*.

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# Chapter 13

## Protocol for the Detection and Genotyping of Human Papillomaviruses Using a Liquid Bead Microarray Assay

Stephen Cherne, Viorica Popov, and Qinghua Feng

### Abstract

More than 100 human papillomavirus (HPV) types have been identified, and over 40 of them infect the anogenital epithelium. Because each HPV type is associated with different risks for the development of cervical cancer, detecting and genotyping HPVs has increasingly become an integral part of cervical cancer control. Here, we describe a Luminex assay-based liquid bead microarray assay for genotyping 37 HPV types, which is objective, scalable, amenable to a high-throughput configuration, and has the potential to be automated.

**Key words:** Human papillomavirus, HPV genotyping, PCR, Luminex, Liquid bead microarray assay

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### 1. Introduction

Human papillomavirus (HPV) is the causal agent for cervical cancers (1). Of the over 40 genital HPV types, 15 of them (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68, and 73) (1, 2) are currently confirmed as high-risk types for the development of cervical cancer. Conventional HPV detection and genotyping are achieved through consensus PCR amplification of the L1 region, which is conserved among the different HPV types, coupled with reverse line blot assays (3–5). However, most reverse line blot assays require subjective visual readout, are difficult to scale up, and are not amenable for automation.

The liquid bead microarray assay (LBMA) HPV genotyping assay described here utilizes Luminex's xMAP technology, which combines flow cytometry with color-coded microspheres. Briefly, each color-coded bead set is coated with an HPV type-specific probe, allowing the capture and detection of that specific HPV type from a sample, while HPV DNA present in the sample is PCR

amplified and detected by a reporter dye. Within the Luminex 100/200 system (Luminex Corporation, Austin, TX), both the internal dyes that identify each microsphere particle and the reporter dye captured during the assay are detected and measured. Because of the unique fluorescent dye signature carried by each set of beads, HPV genotyping can be unambiguously determined. Specifically, type-specific probes for each HPV are conjugated to each bead set. HPV DNA is amplified using biotin-labeled consensus PCR primers. The PCR products are then hybridized to sets of beads coupled with HPV type-specific probes. Beads that have captured the biotin-labeled HPV DNA are subsequently hybridized to phycoerythrin (PE)-conjugated streptavidin (SA-PE). Both bead intrinsic fluorescent dye signature and PE dye are detected by the Luminex 100/200 analyzer.

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## 2. Materials

### 2.1. Bead Conjugation

1. Carboxylated microspheres (Luminex Corp, Austin, TX): Remove bead vials from 2 to 8 °C and allow equilibration to room temperature. For 2 mL vials, centrifuge for 30 s at 10,000×*g* to minimize cap retention, sonicate in a water bath sonicator to disperse the microsphere pellet, and mix by gentle vortexing immediately prior to dispensing. For larger volume bottles, place on rotator and gently rotate for 1–2 min, mix five to ten times by gentle inversion, and gently tap sample container bottom on bench top to minimize sample retention in the cap immediately prior to dispensing (see Note 1).
2. Oligonucleotides with 5' Amino Modifier C12 (Integrated DNA Technologies, Coralville, IA): Resuspend the oligos in dH<sub>2</sub>O to make 1 mM (1 nM/μL) solutions. Probe sequences for 37 HPV types are listed in Table 1.
3. EDC (1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride): Store 10 mg aliquots in a desiccator at –20 °C.
4. 0.1 M MES–NaOH, pH 4.5: Dissolve 4.88 g MES in 250 mL dH<sub>2</sub>O, add five drops of 5 N NaOH, filter sterilize, and store at 4 °C for up to 6 months.
5. 0.02 % (v/v) Tween-20: Dilute 50 μL Tween-20 in 250 mL dH<sub>2</sub>O, filter sterilize, and store at room temperature for up to 6 months.
6. 0.1 % (w/v) SDS: Dilute 2.5 mL 10 % SDS into 250 mL dH<sub>2</sub>O, filter sterilize, and store at room temperature for up to 6 months.
7. TE-buffer, pH 8.0: Dilute 2.5 mL 100× Tris–EDTA buffer (1 M Tris–Cl, 100 mM EDTA, pH 8.0) into 250 mL dH<sub>2</sub>O,

**Table 1**  
**Probe sequences for 37 HPV types**

HPV type	Probe 1 (5'–3')	Probe 2 (5'–3')
6	TGGAAGATGTAGTTACGGATG	
11	TGTAGCAGATTTAGACACAGA	
16	GATATGGCAGCACATAATGAC	CAATTGCCTGGGATGTTAC
18	CTTAAATTTGGTAGCATCATATTG	TCAGCCGGTGCAGCATCC
26	GCTGACAGGTAGTAGCAGAGTT	GCCATAACATCTGTTGTAAGTG
31	GATCTTCCTTGGGCTTTTGG	AATTGCAGCACAAACAGACA
33	CTGTCACTAGTTACTTGTGTGCAT	TTTGGAGGTACTGTTTTTTGA
35	ATCATCTTTAGGTTTTGGTGC	CTGTCACTAGAAGACACAGCAG
39	GTAGAAGGTATGGAAGACTCTA	AGATGGTGGAGGAGCTACAG
40	TGGGGTTGGTGTGGGGACTGT GTGGCAGC	
42	GCGTTGTTACCTTAGCCTGA	ATCACCAGATGTTGCAGTG
45	GCACAGGATTTTGTGTAGAG	GCTTTTCTGGAGGTGTAGTAT
51	CATCCTCCAACTAGCAGAC	CAGCAGTGGCAGTGCTAATA
52	ACAAGTTATAGCAGTAGAAGTG	CCTTTCTTTAGGTGGTGTGT
53	CCAGTCTTCCAGTAAGGTAGAA	GACATAGACTGTGTGGTTGC
54	TTATTAAAGCTATCCTGCGTGG	TCCTCCAACTACTTGTAGCTG
55	CGCATGTATTGTTTATATTCTGTA	AGACTGAGTTGTAGCAGCAC
56	CGTGCATCATATTTACTTAACTG	CGTTGACATGTTATAGCTGTGC
58	TCCTTTTCTTTAGGGGGTGCT	CTTAGTTACTTCAGTGCATAATGTC
59	GGGTCTCTGTTTAACTGGC	CTGGTAGGTGTGTATWCATTAGG
61	TTCCCTAAAGCTTGTGGCTT	GCATAGCGATCCTCCTTGG
62	CAAATTCCTCCGTGTGTGCGC	TGTCATTTGCGCATAACGGG
64	GCATATGGATCCTCAGATTCC	
66	AATGTGCTTTTAGCTGCATTAAT	GGCTGTTCCCTCTGACATG
67	TCATGGTGTGTATGTATTGCA	CCTTTGCTGTTGGAGGGGATG
68	CTGATTGCAGATAGCGGTATG	GGTACAGCTGATTCAGTAGTAG
69	GCAGATTGTGCAGATACAGTAC	GCTGTGCAGGGGCATCGC
70	GGCCGTTTCGGTGCAGGC	
71	GTTTTGGTAGCACAGATGGAC	CTCCAAAATGTAAGATCTGC
72	ATACAGAGGACGCTGTGGC	GTGTGGCGAAGATACTCACG

(continued)

**Table 1**  
**(continued)**

HPV type	Probe 1 (5'–3')	Probe 2 (5'–3')
73	GAGCTACTAGCCTGTGTACCTAC	GTTGAGGACGTTGGCAACT
81	GCAGCAGATGTAGCTGTGC	TGTCCAAAATGACATGTTCGGC
82	TGTTTGTGCAACAGATTGAG	CTGAGGGGGGCAAGGTTA
83	GAGGCTGTGTATTCATTAGCC	ATCAAGGCTGGTGGGAAGGA
84	TTTATATTCTGATTCCGGTGTTGG	TGGAGGGAGGGGGCACAAC
is39	CGTTGACAAGAGGTTGCTGC	GTTGGAGTGAATGTCTGTGC
cp6108	GGCAGACTGGGAAGCAGCA	GGTTCTGGGGCAGCAGTGC

filter sterilize, and store at room temperature for up to 6 months.

### **2.2. Genomic DNA Isolation**

1. QIAamp DNA Mini Kit: QIAamp mini spin columns, Buffer AL, Buffer ATL, Proteinase K, Buffer AW1, Buffer AW2, Buffer AE.
2. K562 cell pellet: Culture K562 cells (ATCC CCL-243) in RPMI1640 media at 37 °C. Harvest cells by trypsin digestion, wash in PBS, and count using hemacytometer. Aliquot about  $5 \times 10^6$  cells into each 4.5 mL Nunc cryotube and store cell pellets at –80 °C.
3. Phosphate-buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.76 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.4.

### **2.3. PCR Amplification (First and Second Runs) and PCR Product Cleanup**

1. Thermocycler.
2. AmpliTaq Gold polymerase (e.g., Applied Biosystems).
3. Primer mix 1: 50 mM MY09, 50 mM MY11, 10 mM HMB01, 10 mM HPRT(F), 10 mM HPRT(R) (see Table 2).
4. Primer mix 2: 50 mM biotinylated MY11 and 10 mM biotinylated HPRT(R) (see Table 2).
5. 96-Well PCR plate and strip caps.
6. QIAquick 96 PCR Purification kit: QIAquick 96 plates, Buffer PM, Buffer PE, Buffer EB.

### **2.4. HPV Hybridization and Signal Detection**

1. Qiagen LiquiChip Workstation.
2. Qiagen LiquiChip Filter microplate.
3. Streptavidin–Phycoerythrin (SA–PE) (e.g., BD Pharmingen).
4. Qiagen LiquiChip System Fluid 10×.

**Table 2**  
**Primers used for PCR amplification**

Name	Sequence (5'–3')
MY09	CGTCCMARRGGAWACTGATC
MY11	GCMCAGGGWCAYAAAYAATGG
HMB01	GCGACCCAATGCAAAATGGT
HPRT(F)	CTTGCCACAAGTTATGGGCT
HPRT(R)	GGCACAAAAGATCCACTGGT
Bio-MY11	Biotin-GCMCAGGGWCAYAAAYAATGG
Bio-HPRT(R)	Biotin-GGCACAAAAGATCCACTGGT

5. 70 % isopropyl alcohol.
6. Deionized or distilled water.
7. Tetramethyl Ammonium Chloride (TMAC).

### 3. Methods

#### **3.1. Bead Conjugation with Oligonucleotide Probe (Optimized for 12–16 Bead Sets) (See Note 2)**

*Purpose.* Label each bead set with the respective HPV-specific probe(s).

1. Bring two aliquots of EDC powder to room temperature.
2. Sonicate stock microspheres for 2 min, vortex, and then transfer  $5.0 \times 10^6$  (400  $\mu\text{L}$ ) of each stock of microspheres to a 1.7 mL microfuge tube.
3. Spin the stock microspheres at  $\geq 8,000 \times g$  for 1–2 min.
4. Remove the supernatant and resuspend the microspheres in 50  $\mu\text{L}$  of 0.1 M MES, pH 4.5 by vortexing for approximately 20 s (see Note 3).
5. Prepare 100  $\mu\text{M}$  oligo probe in  $\text{dH}_2\text{O}$  (0.1 nM/ $\mu\text{L}$ ) (see Note 4).
6. Add 5  $\mu\text{L}$  of 100  $\mu\text{M}$  oligo probe to the resuspended microspheres and mix by vortex (see Note 5).
7. Prepare a fresh solution of 10 mg/mL EDC in  $\text{dH}_2\text{O}$ .
8. Add 5  $\mu\text{L}$  of freshly prepared 10 mg/mL EDC to each bead coupling reaction (50  $\mu\text{g}$  in each reaction or a final concentration of  $\sim 1 \mu\text{g}/\mu\text{L}$ ) and mix by vortex.
9. Incubate for 30 min at room temperature in the dark.
10. Prepare a second fresh solution of 10 mg/mL EDC in  $\text{dH}_2\text{O}$  (see Note 6).

11. Add 5  $\mu\text{L}$  of fresh 10 mg/mL EDC to each coupling reaction and mix by vortex.
12. Incubate for another 30 min at room temperature in the dark.
13. Add 1.0 mL of 0.02 % Tween-20 to the coupled microspheres.
14. Pellet the coupled microspheres by microcentrifugation at  $\geq 8,000 \times g$  for 1–2 min.
15. Remove the supernatant and resuspend the coupled microspheres in 1.0 mL of 0.1 % SDS by vortex (see Note 3).
16. Pellet the coupled microspheres by microcentrifugation at  $\geq 8,000 \times g$  for 1–2 min.
17. Remove the supernatant and resuspend the coupled microspheres in 100  $\mu\text{L}$  of TE buffer, pH 8.0 by vortex for approximately 20 s (see Note 3).
18. Enumerate the coupled microspheres by hemacytometer.
  - (a) Dilute the resuspended and coupled microspheres 1:100 in  $\text{dH}_2\text{O}$ .
  - (b) Mix thoroughly by vortex.
  - (c) Transfer 10  $\mu\text{L}$  to the hemacytometer.
  - (d) Count the microspheres within the four large corners of the hemacytometer grid.
  - (e)  $\text{Microspheres}/\mu\text{L} = (\text{sum of microspheres in four large corners}) \times 2.5 \times 100$  (dilution factor) (see Note 7).
19. Store coupled microspheres refrigerated at 2–8  $^\circ\text{C}$  in the dark (see Note 8).
20. Prepare a suspension of 1,000 beads/type/ $\mu\text{L}$ , vortex, and aliquot into multiple tubes (see Note 9). An example of bead number calculation, dilution, and mixing is illustrated in Table 3.

**3.2. Genomic DNA Isolation from PreservCyt Cytology Specimens (See Note 10)**

*Purpose.* Extract DNA to be used for PCR amplification.

1. Using a 5 mL pipette, transfer 4 mL of well-mixed PreservCyt cytology specimens into pre-labeled 15 mL conical tubes.
2. Spin the conical tubes at  $2900 \times g$  for 15 min.
3. Carefully pour off as much of the supernatant as possible for all samples centrifuged, and then with a pipette remove as much residual fluid as possible without disturbing the pellet.
4. Dry the pellet under the hood for 30 min with the caps open, making sure that the caps do not touch one another.
5. Add 200  $\mu\text{L}$  ATL buffer and 20  $\mu\text{L}$  Proteinase K into each tube, as well as one tube of K562 cell pellet ( $5 \times 10^6$  cells) as a “negative control” (see Note 11). Vortex.
6. Incubate at 56  $^\circ\text{C}$  for 1–2 h. Vortex every 30 min (see Note 12).
7. Add 200  $\mu\text{L}$  AL buffer to each tube, vortex, and incubate at 70  $^\circ\text{C}$  for 10 min in a water bath.

**Table 3**  
**Bead enumeration and mixing**

Bead Set	HPV	Bead enumeration		Bead dilution		Bead mixing (1 mL)
		Bead Count	Concentration (beads/ $\mu$ L)	TE ( $\mu$ L)	Final concentration (beads/ $\mu$ L)	( $\mu$ L)
Region: 33, 1 mL	6	226	56,500	13	50,000	20
Region: 45, 1 mL	11	257	64,250	29	50,000	20
Region: 35, 1 mL	16	267	66,750	34	50,000	20
Region: 36, 1 mL	18	216	54,000	8	50,000	20
Region: 37, 1 mL	26	243	60,750	22	50,000	20
Region: 38, 1 mL	31	237	59,250	19	50,000	20
Region: 42, 1 mL	33	259	64,750	30	50,000	20
Region: 43, 1 mL	35	176	44,000	0	44,000	23
Region: 44, 1 mL	39	229	57,250	15	50,000	20
Region: 46, 1 mL	40	237	59,250	19	50,000	20
Region: 47, 1 mL	42	220	55,000	10	50,000	20
Region: 51, 1 mL	45	233	58,250	17	50,000	20
Region: 52, 1 mL	51	283	70,750	42	50,000	20
Region: 53, 1 mL	52	278	69,500	39	50,000	20
Region: 54, 1 mL	53	272	68,000	36	50,000	20
Region: 55, 1 mL	54	215	53,750	8	50,000	20
Region: 56, 1 mL	55	285	71,250	43	50,000	20
Region: 61, 1 mL	56	152	38,000	0	38,000	26
Region: 62, 1 mL	58	250	62,500	25	50,000	20
Region: 63, 1 mL	59	188	47,000	0	47,000	21
Region: 64, 1 mL	61	220	55,000	10	50,000	20
Region: 65, 1 mL	62	272	68,000	36	50,000	20
Region: 66, 1 mL	64	206	51,500	3	50,000	20
Region: 72, 1 mL	66	284	71,000	42	50,000	20
Region: 73, 1 mL	67	271	67,750	36	50,000	20
Region: 74, 1 mL	68	239	59,750	20	50,000	20
Region: 75, 1 mL	69	253	63,250	27	50,000	20

(continued)

**Table 3**  
**(continued)**

Bead Set	HPV	Bead enumeration		Bead dilution		Bead mixing (1 mL)
		Bead Count	Concentration (beads/ $\mu$ L)	TE ( $\mu$ L)	Final concentration (beads/ $\mu$ L)	( $\mu$ L)
Region: 76, 1 mL	70	287	71,750	44	50,000	20
Region: 77, 1 mL	71	291	72,750	46	50,000	20
Region: 17, 1 mL	72	252	63,000	26	50,000	20
Region: 18, 1 mL	73	189	47,250	0	47,250	21
Region: 34, 1 m	81	202	50,500	1	50,000	20
Region: 19, 1 mL	82	186	45,500	0	45,500	22
Region: 20, 1 mL	83	227	56,750	14	50,000	20
Region: 21, 1 mL	84	209	52,250	5	50,000	20
Region: 24, 1 mL	is39	267	66,750	34	50,000	20
Region: 25, 1 mL	cp6108	182	45,500	0	45,500	22
Region: 26, 1 mL	HPRT	239	54,000	8	50,000	20
TE						225

8. Add 200  $\mu$ L 100 % ethanol. Vortex.
9. Load each specimen into a Qiagen column. Connect to vacuum and suction solution through the column. If fluid does not vacuum through, then spin for 1 min at 6,000 $\times g$ .
10. Add 750  $\mu$ L of AW1 buffer to columns. Apply vacuum to draw fluid through the column (see Note 13).
11. Add 750  $\mu$ L of AW2 buffer. Apply vacuum once again to draw fluid through the column (see Note 14).
12. Release vacuum and close column lids. Place columns into clean collection tubes and spin at 16,000 $\times g$  for 1 min to dry the membrane completely. Discard the collection tube and fluid, and place a labeled 1.5 mL Eppendorf tube under the column.
13. Open column lids, one at a time. Add 50  $\mu$ L of 1 $\times$  AE buffer directly onto the center of the filter area of each column. Cap column, incubate at room temperature for 5 min, and then spin at 6,000 $\times g$  for 1 min.
14. Discard columns and store the isolated DNA at  $-20\text{ }^{\circ}\text{C}$  (see Note 15).

**Table 4**  
**Reaction mix for first run PCR**

	1× (μL)	100× (μL)
10× Master mix buffer	5	500
MgCl <sub>2</sub> (25 mM)	12	1,200
dNTPs (10 mM)	1	100
Primer mix 1	0.5	50
dH <sub>2</sub> O	25.75	2,575
TaqGold (5 U/μL)	0.75	75

**3.3. PCR Amplification:  
First Run**

*Purpose.* Amplify a 450 bp HPV amplicon and a 359 bp HPRT amplicon.

1. Prepare first run Master Mix in 4 mL sterile tube as in Table 4 under the PCR hood. Invert tube several times to mix the Master Mix well.
2. Aliquot 45 μL of Master Mix into each well of a 96-well plate.
3. Using plugged pipette tips, add 5 μL of isolated DNA sample into each well.
4. Add 5 μL of positive control to the second to last well on the plate (see Note 16).
5. Perform PCR reaction using the following program: 95 °C, 9 min; 95 °C, 30 s, 55 °C, 1 min, 72 °C, 1 min for 40 cycles; 72 °C, 5 min; 15 °C hold (see Note 17).

**3.4. PCR Product  
Cleanup**

*Purpose.* Remove excess primers so that Round 2 PCR will generate only single-stranded product.

1. Add 150 μL of Buffer PM to each well.
2. Set up QIAvac apparatus including the waste container, base, plate holder, and QIAquick plate.
3. Transfer the sample (about 200 μL) to the QIAquick plate. Once all samples have been added, apply vacuum at 200 mbar until all liquid has passed through the wells.
4. Add 900 μL Buffer PE to each well. Apply vacuum at 200 mbar until all liquid has passed through.
5. Repeat step 4.
6. Increase vacuum to 600 mbar for 10 min to completely dry the plate.
7. Remove QIAquick plate from QIAvac and place on a stack of paper towels. Lift the plate and paper towels as one and strike on counter several times to blot droplets from plate.

**Table 5**  
**Reaction mix for second run PCR**

	1× (μL)	100× (μL)
10× Master mix buffer	5	500
MgCl <sub>2</sub> (25 mM)	12	1,200
dNTPs (10 mM)	1	100
Primer mix 2	0.5	50
dH <sub>2</sub> O	10.75	1,075
TaqGold (5 U/μL)	0.75	75

8. Replace waste container from QIAvac with collection tubes and place plate holder and QIAquick plate on top.
9. Add 60 μL RNase-free water to each well.
10. Tap down droplets and incubate for 1 min.
11. Apply vacuum at 600 mbar for 5 min.
12. Carefully remove the QIAquick plate.
13. Store cleaned Round 1 PCR product in plate at -20 °C until used in Round 2 PCR (see Note 17).

### **3.5. Second Run PCR Reaction**

*Purpose.* Generate single-stranded biotinylated HPV (450 bp) and HPRT (359 bp) product.

1. Prepare second run PCR master mix in a 4 mL sterile tube (Table 5).
2. Mix well by vortexing, and aliquot 30 μL to each well of a 96-well PCR plate.
3. Add 20 μL of cleaned Round 1 PCR product to the PCR plate (see Note 18).
4. In the last well, add 20 μL of a cleaned Round 1 PCR product negative control from previous run. This control will be used for the cutoff calculation and troubleshooting.
5. Perform PCR reaction using the following program: 95 °C, 9 min; 95 °C, 30 s, 55 °C, 1 min, 72 °C, 1 min for 20 cycles; 72 °C, 5 min; 15 °C hold. The PCR plate can be capped and stored at -20 °C up to 5 days before being detected.

### **3.6. HPV Hybridization**

*Purpose.* Capture HPV and HPRT product on Luminex microspheres and detect Median Fluorescent Intensity from reporter dye.

1. Prepare the hybridization mixture by diluting the appropriate volume of vortexed Bead Mixture with 1.5× TMAC solution.

**Table 6**  
**Preparation of hybridization mix**

	1 rxn	12 rxns	24 rxns	48 rxns	96 rxns
<i>1,500 beads/type/rxn</i>					
Beads (μL)	1.5	18	36	72	144
1.5× TMAC (μL)	31.5	378	756	1,512	3,024
<i>1,750 beads/type/rxn</i>					
Beads (μL)	1.75	21	42	84	168
1.5× TMAC (μL)	31.25	375	750	1,500	3,000

See Table 6 for examples of hybridization mixtures for various numbers of reactions and bead concentrations (see Note 19).

2. Vortex hybridization mixture, and aliquot 33 μL to each well.
3. Transfer 20 μL of the second run PCR product to each well.
4. Run hybridization program on thermocycler: 95 °C, 9 min, 55 °C, 30 min.
5. When hybridization is almost complete, prepare the LiquiChip filter microplate by adding 30 μL 1× TMAC to each well and apply vacuum. Add another 150 μL 1× TMAC without applying vacuum.
6. Dilute 36 μL SA-PE (0.5 mg/mL) in 9 mL of 1× TMAC. This should be sufficient for a 96-well plate (see Note 20).
7. Immediately take out the hybridization plate from the thermocycler after completion and place on ice tray (see Note 21).
8. Transfer hybridized beads to 1× TMAC in the filter plate, and apply vacuum. Remove vacuum hose from QIAvac after each vacuum step.
9. Wash beads with an additional 150 μL 1× TMAC and apply vacuum.
10. Add 75 μL of diluted SA-PE to the filter plate. Cover wells with plate tape and blot bottom of plate with paper towels.
11. Shake at 580 rpm in a 55 °C incubator for 30 min either in the dark or covered in foil.
12. Turn on the Luminex Instrument, the Luminex XY plate handling platform, and the Luminex SD sheath fluid delivery system to warm up at least 30 min before use (see Note 22).
13. During the last 15 min of incubation, open the Luminex xPO-NENT software and prepare Luminex 100/200 for analysis as described below under Subheadings 3.8–3.11.
14. After a 30-min incubation, remove film and apply vacuum to the plate.

15. Add 75  $\mu$ L 1 $\times$  TMAC and blot bottom of plate as before.
16. Samples are now ready to be read on the Luminex machine. Select “Eject” to open the machine and insert filter plate and then select “Retract.” Under *Batches*, pick the appropriate saved batch in the Pending Batches list and click “Run Batch.”
17. When the detection is complete, go to *Maintenance* and perform two “Wash” and three “Soak” commands using deionized water. Click “Eject” to remove the plate. Keep the plate protected from light until analysis of the run is completed (see Note 23).
18. Turn off the three components of the Luminex platform, “Log off,” and “Exit” the Luminex xPONENT software.

### **3.7. Interpretation of Specimen Results**

1. For each run, the background Median Fluorescent Intensity (MFI) for each HPV type will be determined as the average MFI of the five to nine negative controls included in the run.
2. For each HPV type, a specimen is considered positive for that HPV type if the MFI is equal or greater than 10 $\times$  the background MFI for that HPV type.
3. For each HPV type, a specimen is considered weak positive for that HPV type if the MFI is equal or greater than 7 $\times$  but less than 10 $\times$  the background MFI for that HPV type.
4. For each HPV type, a specimen is considered negative for that HPV type if the MFI is less than 7 $\times$  the background MFI for that HPV type.
5. A run is considered valid only if all negative controls are negative for any HPV types and all positive controls are positive for the specific HPV types.
6. Specimens with bead count <70 are considered “invalid”, need to be repeated.
7. Specimens with HPRT MFI equal or less than 100 are considered “insufficient”, unless positive for any one of the 37 HPV types.
8. Specimens weak positive for any HPV need to be repeated. If the repeat testing is weak positive or positive, the specimen will be reported positive for that type. If repeat testing is negative, the specimen will be reported negative for that type.

### **3.8. Signal Detection on Luminex 100/200 Analyzer (See Note 24): Luminex Maintenance**

1. Choose the *Maintenance* page prior to starting up a run and after a run is complete.
2. The following commands should be completed prior to calibrating or running samples and can be performed while the laser is warming up (30 min): “Prime,” “BackFlush” (2 $\times$ ), “Drain” (2 $\times$ ), “Alcohol Flush,” and “Wash” (3 $\times$ ). The commands “Prime,” “BackFlush,” and “Drain” all use the internal sheath fluid while the “Alcohol Flush” and “Wash” require external fluids to be added to the reservoir or a specified well

on a plate. For the “Alcohol Flush,” 70 % isopropyl alcohol is used and for the “Wash,” deionized or distilled water is used.

3. After a run, the following commands should be run prior to shutting down: Sanitize (10 % bleach if proteins are analyzed; this is not necessary for DNA/RNA assays), “Wash” (2×), and “Soak” (deionized or distilled water).
4. These commands can all be found and run individually in the *Commands* tab, or can be run automatically in the *Routines* tab.
  - (a) To set up a routine, go to the *Routines* tab and click on “Create New Routine.”
  - (b) Assign a Routine Name and choose the commands from the list (see Note 25). For commands that require external fluids or that result in an outflow of fluid from the probe (“Drain”), the location of the external fluid or receptacle of outflow will need to be indicated (see Note 26).
  - (c) Click “Save” once routine is complete.
  - (d) To run a routine, insert external fluids in appropriate wells or reservoir in XY platform and close door. Under *Routines* tab, use drop-down box under Routines to indicate routine of interest and click “Run.”

### **3.9. Luminex Calibration** (See Note 27)

1. After the Luminex 100/200 has warmed up and the start-up commands have been performed, the appropriate calibrator and control commands can be found in the *Commands* tab of the *Maintenance* page.
2. Resuspend calibrators (CAL1 and CAL2) and controls (CON1 and CON2) (L100-CAL1, L100-CAL2, L100-CON1, and L100-CON2, Luminex Corporation, Austin, TX) by shaking vigorously.
3. Add five drops of each calibrator and control in adjacent wells in a 96-well filter plate. To test each individually, choose the appropriate command: “CAL1,” “CAL2,” “CON1,” “CON2,” “MagCAL1,” or “MagCON1” (see Note 28).
4. Click “Run” and repeat steps 2 and 3 until all calibrators and controls are complete.
5. To verify that all calibrators and controls have passed, go to *Analytix* page and choose *Reports* tab.
6. In the drop-down box next to Report: choose Calibration Summary and in the drop-down box next to Type: choose a calibrator or control. Indicate the date range, or specific date, and click “Generate Report.”
7. Each report, specific for the calibrator or control chosen, will indicate whether all tested data ranges fall into accepted parameters and will show either Pass or Fail. Check all calibrators and controls and print.

**3.10. Luminex Protocol**

1. Choose *Protocol* page and then *Protocol* tab (should be default) and click on “New Protocol” which will open step 1: Protocol Settings.
2. Assign Name in the box following Name.
3. Choose Bead Type: Standard or Magnetic.
4. Enter volume (50  $\mu$ L if beads are resuspended in 75  $\mu$ L 1 $\times$  TMAC).
5. Additional options: Time-out enabled (will stop reading sample after designated time, i.e., 200 s), XY Heater enabled (for protocols which require maintaining sample temperature higher than room temp during reading), DD Gating (default 7,500–15,000), and Reporter Gain (Default or High PMT).
6. Choose Analysis Settings Type: None (used for raw data output only), Qualitative or Quantitative (both Qual. and Quant. will require entering additional parameters for the analysis, usually defined in the manufacturer’s protocol).
7. Click on “Next” which will open step 2: Select Analytes.
8. Select Analytes (bead regions) that will be used in assay.
9. Edit Analytes (designate name or code of each bead region, units (if applicable), and minimum number to be analyzed (default 100)).
10. Click “Next” which will open step 3: Plate Layout.
11. Plate layout is optional. If a protocol includes controls and standards that are always placed in the same positions with specific values, this is where it could be entered to avoid having to enter those values for each run.
12. Click “Save Protocol.”

**3.11. Create Luminex Batches**

1. Choose *Batch* page to create “New Batch” (use existing protocol), “New Custom Batch” (new batch without existing protocol), or “New Multi-Batch” (new multi-batch from pending batches), or a previously saved batch will be listed under Pending Batches.
2. To create a “New Batch,” click “New Batch” which will open Create New Batch step 1: Select Protocol.
3. Assign Batch Name and fill in Description (optional).
4. Select Protocol from listed protocols created previously.
5. If specific Lots and/or Kits are being used there is the option to identify those here by clicking on “Select Lots & Kits.”
6. Click on “Next” which opens Create New Batch step 2: Plate Layout.
7. Select wells and assign a task for each (unknown, standard, control, background, or wash), including Replicate Count and

Grouping. Some of the wells may have already been assigned when the protocol is created.

8. Additional options include Maintenance Commands and Add & Change Plate.
9. Once complete, choose either “Save Batch for Later” or “Run Batch.”
10. If “Save Batch for Later” is used, the batch information and protocol will be stored in the Pending Batches. To use a pending batch, click on the batch of interest and then click either “Edit Batch” or “Run Batch.”

### **3.12. Quality Control**

Quality control (QC) is an integral part for the success of any laboratory assay, especially for the LBMA assay. QC is necessary to ensure comparable sensitivity and specificity of the assay from time to time, thus generating reproducible results from batch to batch. In addition, well-designed QC steps will facilitate the identification and correction of laboratory problems in a timely fashion. Below are a few QC guidelines for the LBMA assay.

#### **3.12.1. Test for Assay Sensitivity and Specificity**

Because many steps are involved in the LBMA assay, it is important to determine that each batch of conjugated beads has similar sensitivity and specificity for HPV genotyping. Either the full-length or L1 fragment plasmid of each HPV type can be used for this purpose, although L1 fragment plasmids are advantageous due to their small size and ease to propagate in *Escherichia coli*. The quantity of each HPV plasmid should be determined using UV spectrometer and converted to copies/ $\mu\text{L}$ . The plasmids should be diluted into 30 ng of purified K562 genomic DNA before being tested in the LBMA assay. For sensitivity, the LBMA assay should be able to detect 50–500 copies of HPV plasmid in each reaction; for specificity, the LBMA assay should not detect any cross hybridization with  $10^5$ – $10^6$  HPV plasmid copies in each reaction.

#### **3.12.2. Test for Assay Reproducibility**

In order to determine the reproducibility of the LBMA assay, at least 10 % of all samples as well as 10 % of positive samples should be randomly selected and retested every month. The results of two assays should be compared and any discordant result should be analyzed to determine the reason for discrepancy. The most common reason could be low viral load. If this is the case, viral load should be determined using a different method, such as quantitative PCR assay.

#### **3.12.3. Identification of Contamination and Cross Hybridization Problems**

The incorporation of negative controls in each step will facilitate the identification of steps of potential contamination. It is important to include more than one negative control if the whole 96-well plate is used. It is ideal that at least one control is included in each row. When multiple HPV types are detected in samples, it is important

to perform statistical analysis and rule out the possibility of cross hybridization among different HPV types.

In addition, an Alu-based DNA quantitation assay can be used to monitor potential DNA contamination in the laboratory. Alu elements are repetitive sequences in the human genome, usually present at  $10^5$ – $10^6$  copies per cell. Because of the high sensitivity of Alu-based DNA quantitation assay, Alu-based quantitative PCR assay has the ability to detect the very low amount of ambient background DNA ( $\sim 10$  pg/mL), which is unavoidable in routine laboratories (6, 7). The detection of more than 1 pg/ $\mu$ L in no template control (NTC) samples indicates the presence of contamination (7). Weekly testing for background DNA level NTC samples is recommended.

*3.12.4. Participation  
in WHO HPV Genotyping  
Proficiency Study*

The WHO Global HPV LabNet is a WHO initiative established to improve quality of laboratory HPV testing for HPV surveillance and monitoring of HPV vaccination programs (<http://www.who.int/biologicals/vaccines/hpv/en/index.html>). The WHO HPV LabNet has generated a panel of samples for HPV genotyping proficiency test. Although the proficiency panel is not distributed at will, any laboratory can participate in the proficiency study which is offered every other year through the WHO HPV LabNet.

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## 4. Notes

1. Failure to perform these steps can result in decreased amounts of microspheres recovered in future sample aliquots. Over time, it is normal to observe the presence of beads on the surface of the liquid in the stock vials. It is recommended to limit the number of times a stock vial is opened in order to reduce the risk of photo bleaching and possible bacterial contamination.
2. Microspheres should be protected from prolonged exposure to light throughout the bead coupling procedure.
3. The following strategy is employed to minimize bead loss. No more than eight bead sets should be processed simultaneously to minimize bead loss. Spin all bead tubes for 2 min and remove as much supernatant as possible without disturbing the beads. Spin for an additional 2 min and remove the remaining supernatant using a 200  $\mu$ L pipette. This process is performed in steps 4, 15, and 17.
4. Dilute 1 mM oligo stock 1:10 in a small volume to avoid more than three freeze–thaw cycles.
5. Probe input should be titrated to optimize coupling for each reaction. Bead conjugation can be scaled up or down based

**Table 7**  
**Bead conjugation scaling**

Microspheres	Reaction ( $\mu\text{L}$ )	Probe (nmol)	EDC (mg/mL)	Tween-20 (mL)	SDS (mL)	Final ( $\mu\text{L}$ )
$1 \times 10^6$	10	0.04–0.1	0.5–2.5	0.5	0.5	20
$2.5 \times 10^6$	25	0.1–0.2	0.5–1	0.5	0.5	50
$5 \times 10^6$	50	0.2–1	0.5–1	1.0	1.0	100
$10 \times 10^6$	50	0.5–1	0.5–1	1.0	1.0	200
$50 \times 10^6$	50–100	1–4	0.5–1	1.0	1.0	1,000
$100 \times 10^6$	100	1–4	0.5–1	1.0	1.0	2,000

on manufacturer's recommendations (see Table 7). If two type-specific probes are conjugated to the same bead set, the probes are first combined in  $\text{dH}_2\text{O}$  with a concentration of 100  $\mu\text{M}$  each.

6. Always use a fresh aliquot of EDC powder for each coupling reaction.
7. The maximum concentration should be 50,000 microspheres/ $\mu\text{L}$ . Dilute with TE-buffer if needed.
8. The conjugated beads are stable for at least 2 years when stored in TE-buffer, pH 8.0 in the dark. Avoid using translucent storage containers.
9. Use parafilm to seal the bead mixture tubes that are not currently in use.
10. Other clinical samples can be used, including cervical swab samples collected in specimen transport media (STM) as well as other DNA isolation kits if clean PCR-ready DNA can be obtained. DNA isolation should be performed in a fume hood. To decrease the possibility of contamination, the hood should be UV irradiated for 15–30 min before the start of preparation. Samples are processed in batches. Each batch contains 22 clinical samples, as well as a negative control (K562 cell pellet).
11. K562 cells can be cultured and aliquoted into  $5 \times 10^6$  cell pellets. If K562 cells are not available, they can be replaced with 200  $\mu\text{L}$  ATL as "blank."
12. This is the optional stop point. Samples can be put in the refrigerator for later processing. After this point, the process must continue to completion.
13. If using a centrifuge, add 500  $\mu\text{L}$  AW1 buffer, and spin at  $6,000 \times g$  for 1 min.

14. If using a centrifuge, add 500  $\mu\text{L}$  AW2 buffer, and spin at  $16,000\times g$  for 3 min.
15. Isolated DNA can be stored for up to 1 year.
16. The positive control can be either genomic DNA from cervical cancer cell lines (Siha for HPV16 or HeLa for HPV18), a mixture of HPV plasmids DNA, or PCR product positive for specific HPV type produced in large volume and aliquoted for repeated usage. In addition, HPV plasmids or HPV PCR products are HPRT negative and can be used as a contamination control.
17. Products can be stored at  $-20\text{ }^{\circ}\text{C}$  for up to 1 week before proceeding to the next step.
18. To avoid contamination, set up second run PCR in a separate area. It is recommended to include a negative control from a previously tested cleaned first run PCR reaction.
19. Always make extra hybridization mixture to compensate for loss during pipetting.
20. The SA-PE solution is light sensitive, so it should be kept covered until ready to use.
21. The thermocycler will hold at  $55\text{ }^{\circ}\text{C}$  indefinitely. However, it is better to remove the hybridization plate immediately upon completion of hybridization.
22. Make sure that the waste bottle is empty or below the maximum mark on larger 10 L carboys.
23. After hybridization, the bead plate can be stored overnight in the dark and analyzed the following day without loss of signal. If necessary, the plate can be vacuumed and 75  $\mu\text{L}$  fresh  $1\times$  TMAC added before signal detection.
24. We use Luminex xPONENT version 3.0.380.0 to run the Luminex 100/200 analyzer. The exact commands may vary due to different software versions. See the specific user's manual for assistance. *Pages* (listed horizontally across the screen) and *Tabs* (listed vertically on each *Page*) will be *underlined* while "Commands" will be written in "quotations."
25. If a command is to be executed more than once consecutively, the command needs to be listed repeatedly. For example, "Wash"  $3\times$  would be entered in a routine as "Wash," "Wash," "Wash."
26. Do not include two commands that will require different fluids being drawn from the same location within a routine as the machine will not stop to allow the change of fluids.
27. Depending on the requirements of the assay, calibrations should be performed weekly, monthly, or prior to each run.
28. If the default well differs from the actual location of the calibrator or the control of interest, click "Change Location" and indicate the actual well location.

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# Chapter 14

## Protocol for the Use of Enzyme-Linked Hybridization Assays for Genital Ulcer Disease

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### Abstract

Etiologic agents of genital ulcer disease include herpes simplex 1 and 2, *Treponema pallidum pallidum*, *Haemophilus ducreyi*, and *Klebsiella granulomatis*. The advent of PCR has allowed for more rapid and sensitive detection of microbial pathogens. In this protocol, we describe the simultaneous detection of these five pathogens and an internal control using a single-tube multiplex PCR and colorimetric enzyme-linked amplicon hybridization assay.

**Key words:** Herpes simplex, *Haemophilus ducreyi*, *Treponema pallidum*, *Klebsiella granulomatis*, Genital ulcer, Enzyme-linked hybridization, Colorimetric, Multiplex PCR, Detection

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### 1. Introduction

Common etiological agents of genital ulcer disease (GUD) include herpes simplex viruses 1 and 2 (HSV1 and 2), *Treponema pallidum* subspecies *pallidum* (syphilis), and *Haemophilus ducreyi* (chancroid). A less frequent GUD pathogen, albeit one which is common in certain regions of the world, is *Klebsiella granulomatis*, the causative organism in donovanosis (more commonly known as granuloma inguinale) (1). *K. granulomatis* was originally known as *Calymmatobacterium granulomatis*, but has recently been reclassified due to phylogenetic analyses which showed the organism's close relation to other *Klebsiella* species (2, 3). Effective therapies exist for most of these GUD pathogens, but their diagnosis, particularly in busy laboratories or those without experienced microscopists, can be problematic.

The use of PCR has revolutionized diagnostic testing, with the method facilitating more rapid and specific microbial diagnosis

when compared to traditional culture and microscopy techniques. In addition to its high sensitivity, PCR testing does not rely on the pathogen to be viable, therefore allowing for a wider range of less invasive samples to be used (4, 5). Several PCR assays can be combined into a multiplex PCR which can detect multiple targets or organisms in one reaction, thereby maximizing limited clinical sample material, as well as saving time, labor, and reagent costs.

In this protocol, we describe a method to simultaneously detect HSV 1 and 2, *T. pallidum*, *H. ducreyi*, and *K. granulomatis*, using a multiplex PCR and an enzyme-linked amplicon hybridization assay (ELAHA) (6). Briefly, the nucleic acid extract from the clinical sample is initially run through the single-tube multiplex PCR. The PCR also includes a primer set targeting the human endogenous retrovirus 3 (ERV3) (7), which acts as an internal control, and can indicate inherent problems with the nucleic acid extract, such as template degradation, or presence of PCR inhibitors. The PCR products (amplicons) are then individually bound to pathogen-specific, biotinylated probes. These amplicon/probe hybrids are then bound to streptavidin-coated microtiter plates. During the initial amplification process digoxigenin (DIG)-labeled uracil bases were incorporated into each amplicon. Anti-DIG horseradish peroxidase-conjugated antibodies are then bound to these DIG-labeled amplicon/probes, and a substrate is added which is catalyzed by the conjugated enzyme to produce a yellow color change. This reaction can subsequently be detected using a colorimetric plate reader. Using synthetic controls, we found that this assay had a detection limit of  $3 \times 10^0$  copies/reaction when each control template was present individually, or  $3 \times 10^1$  copies/reaction when all six control templates were present concurrently (6).

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## 2. Materials

All reagents, water, pipette tips, and plasticware need to be PCR-grade, i.e. sterile, and DNase and RNase free. A proper PCR unidirectional workflow through dedicated rooms for PCR reagent storage/preparation, sample extraction/input, and amplification/detection should be observed in order to avoid PCR product contamination. For example, once the amplification/detection room has been entered, it would be highly inadvisable to reenter the reagent storage/preparation room for the remainder of the day.

The ideal specimen type to use is a lesion swab, although other sample types may be adapted to this method with proper evaluation.

### 2.1. Nucleic Acid Extraction

1. Roche High Pure™ kit (Roche Diagnostics) or similar.
2. 100 % Ethanol.

**Table 1**  
**Multiplex PCR primer sequences**

Target	Primer name	Primer sequence (5'–3')
HSV 1 and 2	HSV01.16a HSV02.16	GCCGTAACCGGGGACATGTACACAAAGT TTCAAGGCCACCATGTACTACAAAGACGT
<i>H. ducreyi</i>	HD01.1 HD02.1	CAAGTCGAACGGTAGCACGAAG TTCTGTGACTAACGTCAATCAATTTTG
<i>T. pallidum</i>	TPAL01.3 TPAL02.3	CAGAGCCATCAGCCCTTTTCA GAAGTTTGTCCCAGTTGCGGTT
<i>K. granulomatis</i>	CG01.1 CG02.1	TCCTCTGCCAGACCGATAACTTTATG CCAGGTAGATATTGTTGGCGTCA
ERV3 internal control	ERV01.1 ERV02.1	CATGGGAAGCAAGGGAATAATG CCCCAGCGAGCAATACAGAATT

3. 1.5 mL conical flip-top tubes, DNase and RNase free.
4. Heating block capable of holding 1.5 mL conical tubes.
5. Vortex.
6. Tabletop centrifuge.
7. Freezer (–20 °C or –80 °C).

**2.2. PCR (Note: All Reagents Stored at –20 °C)**

1. Platinum Taq® DNA Polymerase kit (e.g., Invitrogen), including 10× PCR Buffer: 200 mM Tris–HCl, pH 8.4, 500 mM KCl, and 50 mM MgCl<sub>2</sub>.
2. dNTPs: 100 mM dATP, dCTP, dGTP, and dUTP.
3. 10 mM digoxigenin-11-dUTP (DIG-11dUTP) (Roche Diagnostics).
4. PCR primers (Table 1); rehydrated with PCR-grade water to 200 μM stock (see Note 1).
5. Conventional PCR machine.
6. Thin-walled 0.2 mL PCR tubes.
7. PCR-grade water.
8. Freezer (–20 °C).

**2.3. ELAHA**

1. 5' biotin-labeled probes (Table 2); rehydrated with PCR-grade water to 200 μM stock (see Note 2).
2. 1× SSC buffer: NaCl (0.03 M), sodium citrate (0.019 M) in 1 L double-distilled water, pH 7.0 (using HCl). Store at room temperature.
3. Conventional PCR machine.
4. Thin-walled 0.2 mL PCR tubes.

**Table 2**  
**Biotinylated probe sequences**

Target	Probe name	Probe sequence (5'–3')
HSV 1	HSV1_P01.162	Biotin-GCGTTGGCCGGTTTCAGCTCC
HSV 2	HSV2_P02.16	Biotin-GACCTTCGCCGGCTTGAGCTC
<i>H. Ducreyi</i>	HD_P02.1	Biotin-CCGAAGGTCCCACCCTTTAATCCGA
<i>T. pallidum</i>	TPAL_P01.3	Biotin-CGGGCTCTCCATGCTGCTTACCTTA
<i>K. granulomatis</i>	CG_P01.1	Biotin-GCCGTCAGCGCAGCCTACACCAGC
ERV3 internal control	ERV_P01.3	Biotin-TCTTCCCTCGAACCTGCACCATCAAGTCA

5. Streptavidin-coated microtiter strips or plates (e.g., Thermo Scientific).
6. 750 mU/mL Anti-DIG, horseradish peroxidase-conjugated antibody (100 µL/well) (Roche Diagnostics). Store at 4 °C protected from light.
7. Incubator or warm room capable of a steady 37 °C temperature.
8. Soluble tetramethylbenzidine (TMB) substrate (100 µL/well) (Roche Diagnostics). Store at 4 °C protected from light.
9. Hydrochloric acid (1 M; 100 µL/well).
10. A plate spectrophotometer capable of reading at the 450 nm wavelength with a reference filter at 690 nm.

### 3. Methods

#### 3.1. Sample Extraction

1. With dry swabs, vigorously swirl the swab in 400 µL of the extraction kit's initial buffer (this typically is either a binding or lysis buffer). In case of swabs which have been transported in liquid buffers/media, swirl vigorously prior to removal of the swab. In both cases, try and scrape off excess liquid trapped in the swab on the inside edge of the swab collection tube.
2. Extract the swab liquid as per kit instructions (see Note 3). See steps 3–18 for guide on how to extract samples specifically using the Roche High Pure™ kit.
3. Prior to using the Roche High Pure™ kit, several of the reagents need to be prepared:
  - (a) Add 5 mL of the Binding Buffer to the lyophilized Proteinase K. Mix thoroughly and aliquot into 1.5 mL tubes. Store unused portions at –20 °C.

- (b) Add 20 mL of 100 % Ethanol to the Inhibitor Removal Buffer and mix well. This can be stored at room temperature.
  - (c) Add 40 mL of 100 % Ethanol to the Wash Buffer and mix well. This can be stored at room temperature.
4. Preheat the heating block to 72 °C.
  5. Using 1.5 mL tubes, combine the sample, Binding Buffer, and Proteinase K. If the swab is supplied in a liquid media, add 200  $\mu$ L of the sample to 200  $\mu$ L of Binding Buffer and 50  $\mu$ L of Proteinase K. In the case of dry swabs, use the entire 400  $\mu$ L of sample containing buffer from step 1, and add 50  $\mu$ L of Proteinase K.
  6. Mix well by vortexing, followed by brief centrifugation to bring down all liquid off the top of the tubes.
  7. Incubate at 72 °C for 10 min.
  8. Add 100  $\mu$ L of Binding Buffer, and mix well.
  9. Assemble an extraction column, and add the sample mixture to the top reservoir of the filter basket, taking care not to touch the filter with the pipette tip.
  10. Centrifuge in a tabletop microfuge for 1 min at 8,000  $\times g$ .
  11. Discard the bottom tube which holds the filter basket, and replace with a new tube.
  12. Add 500  $\mu$ L of Inhibition Removal buffer to the filter basket, and centrifuge at 8,000  $\times g$  for 1 min.
  13. Discard the bottom tube which holds the filter basket, and replace with a new tube.
  14. Add 450  $\mu$ L of Wash Buffer, and centrifuge at 8,000  $\times g$  for 1 min.
  15. Discard the bottom tube which holds the filter basket, and replace with a new tube.
  16. Centrifuge at maximum speed for 30 s (see Note 4).
  17. Discard the bottom tube which holds the filter basket, and replace with a labeled 1.5 mL tube.
  18. Add 50  $\mu$ L of Elution buffer to the upper reservoir of the basket, and centrifuge at 8,000  $\times g$  for 1 min.
  19. Nucleic acid extracts can now be used directly in the PCR, temporarily stored at 4 °C if used within a few days, or can be stored at -20 °C (short term) or -80 °C (long term) until needed (see Note 5).

### 3.2. PCR

1. Make up primers and probes to 2  $\mu$ M stock concentration with PCR-grade water.
2. Dilute dATP, dCTP, dGTP, and dUTP to 10 mM working stocks with PCR-grade water.

3. Working on ice or a 4 °C cooling block, prepare the multiplex PCR master mix using the following formula:

	Volume (μL/reaction)	
10× PCR Buffer	5	
MgCl <sub>2</sub> (50 mM)	4	
dATP (10 mM)	1	
dCTP (10 mM)	1	
dGTP (10 mM)	1	
dUTP (10 mM)	2	
DIG-11-dUTP (10 mM)	15	
Primers (2 μM), each	1 each (10 total)	
Platinum Taq® Polymerase (5 U/μL)	0.4 (2 Units)	
PCR-grade water	5.6	
	45	Subtotal volume
Nucleic acid extract	5	
	50	Total volume

4. Aliquot the PCR master mix into individual 0.2 mL PCR tubes (see Note 6). After lightly capping the tubes to prevent contamination, transfer them to the sample input room, and add 5 μL of sample nucleic acid extract per PCR mix aliquot (see Note 7).
5. Cap the tubes, ensuring a firm seal, and place into a conventional thermal cycler (PCR machine) (see Note 8). Run the PCR using the following cycling parameters:  
2-min incubation at 95 °C followed by 45 cycles of 20 s at 94 °C, 20 s at 62 °C, and 30 s at 72 °C, and a final 7-min extension at 72 °C.
6. Cool reaction to 4 °C until ready to perform the ELAHA.

### 3.3. ELAHA

1. Aliquot 5 μL of each PCR reaction into six 0.2 mL PCR tubes containing 85 μL of 1× SSC.
2. Add 10 μL of the respective biotinylated probe (0.2 μM) to the six aliquots.
3. Incubate reactions at 94 °C for 5 min, followed by rapid cooling to 4 °C using a thermal cycler.
4. Add the entire volume of each tube into a well in streptavidin-coated microtiter strips or plates, carefully noting which well is associated with which sample and specific probe.
5. Incubate wells at 37 °C for 20 min.
6. Remove the supernatant with a flick of the wrist into a sink.

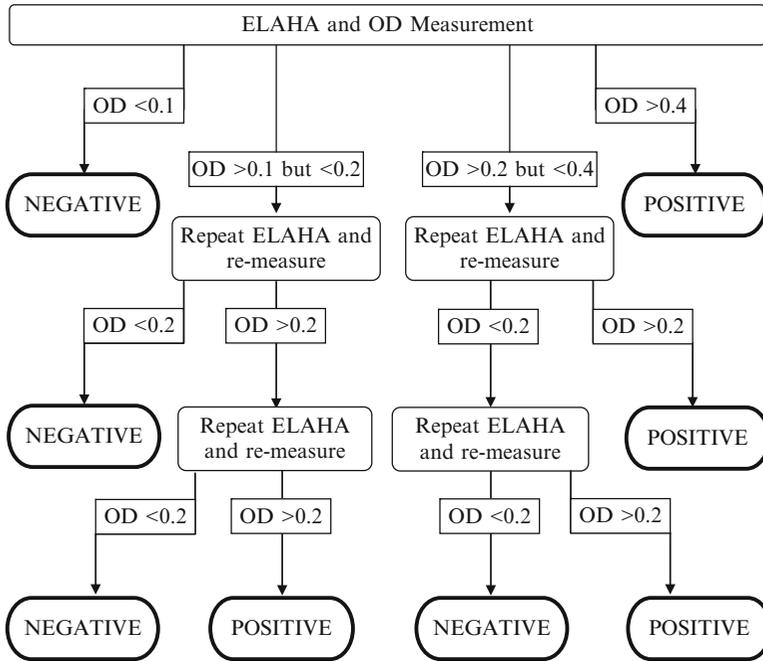


Fig. 1. Testing algorithm for the GUD ELAHA.

7. Wash three times with  $1\times$  SSC at room temperature, each time ensuring that all wells are completely covered (see Note 9).
8. Ensure that the majority of SSC is removed from all wells.
9. Add  $100\ \mu\text{L}$  of  $1:1,000$  anti-DIG, horseradish peroxidase-conjugated antibodies to each well and incubate at  $37\ ^\circ\text{C}$  for 20 min.
10. Remove liquid from wells and wash three times with SSC, as described in steps 7 and 8.
11. Add  $100\ \mu\text{L}$  of TMB substrate into each well and incubate at room temperature in the dark for 10 min.
12. Stop reaction with  $100\ \mu\text{L}/\text{well}$  of  $1\ \text{M}$  HCl.
13. Read strip or plate promptly on a plate spectrophotometer at a wavelength of 450 nm.
14. (See Fig. 1) Samples that have an absorbance  $>0.4$  are considered POSITIVE. This is indicative of the presence of the relevant target DNA.

Samples that have an absorbance  $<0.1$  are considered NEGATIVE. This is indicative of a negative PCR result for the relevant target DNA.

Specimens which produce an initial O.D.  $>0.2$  but  $<0.4$  and which on repeat produce an O.D.  $>0.2$  are considered POSITIVE. Specimens which produce an initial O.D.  $>0.2$  but  $<0.4$  and which on repeat produce an O.D.  $<0.2$  are inconclusive. These samples should be tested a third time. If this third test produces an O.D.  $>0.2$  the specimen is considered

POSITIVE. If the third test is  $<0.2$  then the specimen is considered NEGATIVE.

Specimens which produce an initial O.D.  $>0.1$  but  $<0.2$  and which on repeat produce an O.D.  $<0.2$  are considered NEGATIVE. Specimens which produce an initial O.D.  $>0.1$  but  $<0.2$  and which on repeat produce an O.D.  $>0.2$  are inconclusive. These samples should be tested a third time. If this third test produces an O.D.  $>0.2$  the specimen is considered POSITIVE. If the third test is  $<0.2$  then the specimen is considered NEGATIVE.

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## 4. Notes

1. Primers used in PCR do not normally need the highest grade of purity, thus we typically use desalted (or sometimes called PCR/sequencing grade) primers. Unless a large quantity of PCR reactions are expected to be run using the primers, a smaller quantity can be ordered (40 nmol synthesis quantity). Rehydrating primers to a high stock concentration is advisable, as this helps to slow the primer degradation and binding to the plastic of the storage tube. We typically make up our stock primer solutions to 200  $\mu\text{M}$ , after which we dilute an aliquot to prepare the working stock, which is typically 2–10  $\mu\text{M}$ . The working stock can be stored at  $-20\text{ }^{\circ}\text{C}$  for several months.
2. Probes labeled with biotin used in the ELAHA can also be of desalted purity grade. Rehydration and storage protocols are similar to those of the primers.
3. Ensure that the final elution buffer does not contain EDTA, as this has the capacity to inhibit PCR through the chelation of the necessary magnesium ions within the reaction. If the elution buffer does contain EDTA, PCR-grade water at a neutral pH can be used instead.
4. The second centrifugation step is to ensure that all ethanol from the buffers is removed from the filter and upper basket. Avoid reintroducing ethanol into the basket as the bottom collection tube is removed. Failure to remove all ethanol from the purified nucleic acids will inhibit the subsequent PCR.
5. Multiple freeze/thaw cycles should be avoided, as this will gradually degrade the DNA.
6. Gently mix the PCR master mix prior to aliquoting to ensure even distribution of all components.
7. Use the pipette to mix nucleic acid extract into the master mix to ensure sample/PCR mix homogenization.

8. Ensure that there are no bubbles at the bottom of each tube. A quick flick with the wrist may dislodge any such bubbles.
9. Surface tension can prevent some liquid in the wells from being removed during the flicking motion. Tapping the microtiter strips or wells against the edge of the sink can help release any remaining supernatant or SSC.

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# Chapter 15

## Protocol for the Rapid Detection of the Urogenital Tract Mollicutes and Chlamydia with Concomitant LGV-(sub)typing

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### Abstract

Urogenital tract infections can be caused by a number of pathogens, some of which, like the obligate intracellular *Chlamydia trachomatis*, are difficult to culture, or the cell wall-less mollicutes, like *M. hominis* or *Ureaplasma* spp. Real-time PCR (qPCR) has become an important diagnostic tool as it enables not only the species-specific detection of the organism but also the quantification essential to define the etiological relevance of a facultative pathogenic bacterium. We developed a set of TaqMan qPCRs for the detection of the species *M. genitalium* and *M. hominis* (Mh/Mg-duplex qPCR), *U. parvum* and *U. urealyticum* (Uu/Up duplex-PCR), and *C. trachomatis* (CT-qPCR), and for typing of lymphogranuloma venereum-associated L-serovars of *C. trachomatis* (LGV-qPCR) as well as a sub-typing of L1, L2, and L3. In addition, the human *gap*-gene was amplified as quality control of the specimen, and a cryptic plasmid co-amplified in CT-qPCR as an inhibition control. The present protocol focuses on the step-by-step description for the establishment of these TaqMan multiplex qPCRs.

**Key words:** LGV, Lymphogranuloma venereum, STD, qPCR, Urogenital mollicutes

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### 1. Introduction

The diagnosis of sexually transmitted diseases has significantly improved since molecular biological methods enabled the detection of pathogens that are difficult to culture or time-consuming. Among the organisms for which these methods have made the greatest impact are the cell wall-less mollicutes *Mycoplasma hominis*, *M. genitalium*, *Ureaplasma parvum*, and *U. urealyticum* as well as the obligate intracellular *Chlamydia trachomatis*. Both the mollicutes and Chlamydia are associated with urogenital tract infections which persist, in many cases symptomless, over years (1). Based on the fact that non-gonococcal urethritis is mainly caused by one of these pathogens we decided to establish *in-house* TaqMan-qPCR assays for the detection of these *Mollicutes* (2) and *C. trachomatis*.

An increasing number of infections with *Lymphogranuloma venereum*-associated L2-serotype in men who have sex with men (MSM) has been observed in Europe (3) and thus we expanded this panel of qPCRs to detect all L-serotypes (LGV-PCR) including the subtyping of L1, L2, and L3 (4).

Design, evaluation, and establishment of in-house qPCRs is generally an ambitious project beginning with the choice of target sequences and the design of primers and probes, in silico and experimental verification of sensitivity, specificity, and efficiency, and the choice of both reference genes and internal amplification controls. A set of TaqMan PCR assays with detection limits of 50 genome copies per reaction were established in 96-well plate format which had already proved itself in the routine diagnostic molecular biological laboratory.

Following the step-by-step protocol given below this TaqMan PCR format can be established in toto or as single reactions in every laboratory. In addition, several tips are given that facilitate development, implementation, and documentation of TaqMan qPCRs for further pathogens.

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## 2. Materials

### 2.1. Primers and Probes

1. Pipettes (0.5–10  $\mu$ L; 10–100  $\mu$ L; 100–1,000  $\mu$ L).
2. Sterile, nuclease-free filter-pipette tips.
3. Sterile, nuclease-free 1.5 mL microtubes.
4. 10 mM Tris-HCl, pH 7.5.
5. Primers and probes (see Note 1).
6. Freezer ( $-20$  °C).

### 2.2. Generation of (Internal) PCR Control Plasmids

1. Pipettes (0.5–10  $\mu$ L; 10–100  $\mu$ L; 100–1,000  $\mu$ L).
2. Sterile, nuclease-free filter-pipette tips.
3. Sterile, nuclease-free 1.5 mL microtubes.
4. Human DNA (e.g., of HeLa cells or mucosal swab specimen) (see Note 2).
5. Genomic DNA of the respective pathogens: *C. trachomatis* serovars L1, L2, and L3, *Ureaplasma parvum*, *U. urealyticum*, *Mycoplasma genitalium*, and *M. hominis*.
6. DNA of *Drosophila simulans* or ninja-transposon carrying plasmids (5).
7. Primers and probes (see Table 1).
8. Sterile, nuclease-free water.
9. Ready-to-use 2 $\times$  PCR buffer (e.g., iQ-Supermix (Biorad Laboratories, Munich): 100 mM KCl, 40 mM Tris-HCl, pH

**Table 1**  
**Primers and probes**

(Sub-)species and targets	Primers and probes	Sequence (5'–3')
<i>Homo sapiens</i> ( <i>gap</i> )	Gap-F Gap-R Gap-P	CCA CCC ATG GCA AAT TCC ATG GGA TTT CCA TTG ATG ACA AG <b>FAM-TGG CAC CGT CAA GGC TGA GAA CG-BHQ1</b>
<i>C. trachomatis</i> ( <i>omp-1</i> )	CT_F CT_R <sup>a</sup> CT-P	GGT TTC GGC GGA GAT CCT AGT AAC CMA YAC GCA TGC TGA T <b>FAM-CTT GCA CCA CTT GGT GTG ACG C-BHQ1</b>
<i>U. parvum</i> ( <i>rpoB</i> )	Up-F Up-R Up-P	AGG AAA TGA AGA TAA AGA ACG CAA A AAC GAA TAG CAG TAC CTG ATG GAA T <b>HEX-TTG CTT ATG GAC GAC GTT TCG A-BHQ1</b>
<i>U. urealyticum</i> ( <i>rpoB</i> )	Uu-F Uu-R Uu-P	TGG CAC AGA TTG CGC ATA AT TGG AAC GAT CAT TTG TGT TTC AG <b>FAM-TAG GTA ATC GTC ATA TGC CTG CTC A-BHQ1</b>
<i>M. genitalium</i> (16S rDNA)	Mg-F Mg-R Mg-P	AAA CGT CAC TAT GCC CAT GTT G TGC AGC ACC TGT GAT CAT ATT TT <b>FAM-CTG TCC TGG ACA TGC TGA CTA CAT T-BHQ1</b>
<i>M. hominis</i> ( <i>hitA</i> )	Mh-F Mh-R Mh-P	TGC TTT TTG AGG CAC AGC AA TGC TAA CGC AAC GCC AAT TC <b>HEX-TAG CAA CC5 CA5T T5CT5CA5-BHQ1</b> (Biospring)
LGV ( <i>pmp-H</i> )	LGV-F LGV-R LGV-P	CTG TGC CAA CCT CAT CAT CAA AGA CCC TTT CCG AGC ATC ACT <b>HEX-CCG CCT GCT CCA ACA GTT AGT GAT G-BHQ1</b>
L1 ( <i>omp-1</i> )	L1-F L1-R L1-P	CAG CAT CTT TCA ACT TAG TTG GGT TA AGC TCA TAT TTG GTA CAG CAT CCT T <b>HEX-TCGGAGATAATGAAAATCAAAGCACGGTCA-BHQ1</b>
L2 ( <i>omp-1</i> )	L2-F L2-R L2-P	CAG CAT CTT TCA ACT TAG TTG GGT TAT TGA TCT AAG CTC ATA TTT GGT ACA AGC TTA <b>FAM-CGGAGATAATGAGAACCATGCTACAGTTTCAGA-BHQ1</b>
L3 ( <i>omp-1</i> )	L3-F L3-R L3-P	CGC TTC CTT CAA CTT AGT TGG ATT TCA AAG CAG TGT TAG GAA CAA GCT <b>FAM-TTCGGAACAAAAACACAATCTACTAACTTTAATACAGC G-BHQ1</b>
<i>Drosophila</i> ( <i>ninja</i> )	IK-P	<b>HEX-ATG CCT CTT CAC ATT GCT CCA CCT TTC CT-BHQ1</b>
Internal control	IKC-F IKC-R	<i>GGT TTC GGC GGA GAT CCT</i> ACA GGA AAG GTG GAG <sup>b</sup> <i>AGT AAC CCA TAC GCA TGC TGATGATGCCTCTTCACATT</i> GCTCC <sup>b</sup>

<sup>a</sup>CT-R as degenerated primer with M = A or C; Y = T or C

<sup>b</sup>5'-Fused CT-F and one of the CT-R primers

- 8.4, 1.6 mM dNTPs, iTaq DNA polymerase (50 units/mL), 6 mM MgCl<sub>2</sub>, and stabilizers) (see Note 3).
10. PCR Purification Kit (e.g., Nucleo Spin of Macherey & Nagel, Dueren, Germany).
11. Conventional PCR cycler and corresponding PCR tubes (0.2 mL).
12. pGem-T cloning vector system I (Promega, Madison, WI, USA).
13. Transformation-competent *E. coli* DH5 $\alpha$ .
14. SOC-medium (2 % (w/v) Tryptone, 0.5 % (w/v) Yeast Extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl<sub>2</sub>, 20 mM Glucose, pH 7.0) (see Note 4).
15. LB-plates with 0.1 g/L Ampicillin, 0.01 % (w/v) X-Gal (bromo-chloro-indolyl-galactopyranoside), and 0.5 mM IPTG (Isopropyl- $\beta$ -D-1-thiogalactopyranoside) (see Note 5).
16. 42 °C water bath.
17. Ice.
18. Shaker.
19. Loosely capped glass or plastic tubes (5–15 mL).
20. UV-photometer (e.g., nanoDrop).
21. Toothpicks.
22. LB-medium with 0.1 g/L Ampicillin.
23. Plasmid DNA-Preparation Kit (e.g., Roche).
24. Fridge (2–8 °C).
25. Freezer (–20 °C).
26. Incubator (37 °C).

### **2.3. Pre-PCR Processing of Clinical Specimens**

1. Pipettes (0.5–10  $\mu$ L; 10–100  $\mu$ L; 100–1,000  $\mu$ L).
2. Sterile, nuclease-free filter-pipette tips.
3. Sterile, nuclease-free 1.5 mL microtubes with screw cap or 0.2 mL PCR tube.
4. Clinical specimens: e.g., swabs of ocular, urethral, cervical, and rectal specimens in M4-transport medium (Remel, Lexana, KS) which contains three to four glass beads or respective Nylon-flocked e-swabs in Amies transport medium (Copan Italia, Brescia, Italy), urine, or tissue (see Note 6).
5. Phosphate-buffered saline (PBS): 10 mM phosphate buffer, 2.7 mM potassium chloride, and 137 mM sodium chloride, pH 7.4.
6. 10 mM Tris-HCl, pH 7.5.
7. Proteinase K-solution (0.1 mg/mL Proteinase K, 0.1 mM EDTA, 1 mM Tris-HCl, pH 8.0) (see Note 7).
8. Microfuge (e.g., MiniSpin of Eppendorf).

9. Vortexer.
10. Water bath (56 °C and 95 °C) or conventional PCR cyclor (see Subheading 2.2).
11. Freezer (−20 °C).

#### **2.4. qPCR**

1. Pipettes (0.5–10 µL; 10–100 µL; 100–1,000 µL).
2. Sterile, nuclease-free filter-pipette tips.
3. Sterile, nuclease-free 1.5 mL microtubes.
4. qPCR Master Mix No ROX: 2× reaction buffer with dNTPs (including dUTP), HotGoldStar DNA Polymerase, 10 mM MgCl<sub>2</sub>, Uracil-N-glycosylase, and stabilizers (Eurogentec, Seraing, Belgium) (see Note 8).
5. Primers and probes (see Table 1).
6. Sterile, nuclease-free water.
7. 0.2 mL thin-wall 8-tube strips with appending caps, DNase-free or thin-wall plates 96×0.2 mL, DNase-free and qPCR optical 8-cap strips (flat or semidome), DNase-free (see Note 9).
8. Microfuge.
9. Vortexer.
10. PCR cabinet with UV light.
11. Real-time PCR system (e.g., IQ5-cycler of Biorad, PC and IQ5 optical system software; version 2.0).

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### **3. Methods**

#### **3.1. Handling of Primers and Probes**

1. Reconstitute primers and probes that have been commercially purchased as HPLC-purified lyophilisates to 100 µM stock solutions.
2. If Master Mixes are generated for each single run, stocks are diluted in 10 mM Tris–HCl, pH 7.5 as indicated in Table 2 and stored at −20 °C (see Note 10). With few exceptions working concentration of primers is 3 µM and probes 2 µM.
3. Probes should be stored in the dark to prevent bleaching.

#### **3.2. Generation of Plasmids as Positive (Quantification) and Inhibition Controls**

The positive control plasmids for the detection of the human *gap*-gene or one of the pathogens were constructed by ligating the respective PCR product in a cloning vector such as pGemT. The internal inhibition control plasmid IKC (see Note 11) was constructed based on a description of Rosenstraus et al. in 1998 (6). The IKC-F and -R primers (Table 1) used in PCR were composed of the respective 5'-sequence of the primers CT-F and CT-R fused to downstream sequences of the ninja transposon of *Drosophila*



**(b) (50x reactions)**

<b>Ingredients</b>	<b>hGAP<sup>FAM</sup></b>	<b>IKC<sup>HEX/CT<sup>FAM</sup></sup></b>	<b>Up<sup>HEX/Uu<sup>FAM</sup></sup></b>	<b>Mg<sup>HEX/Mh<sup>FAM</sup></sup></b>	<b>LGV<sup>HEX</sup></b>	<b>L1<sup>HEX</sup></b>	<b>L2<sup>FAM</sup></b>	<b>L3<sup>FAM</sup></b>
2x qPCR Master Mix No ROX	625 µL	625 µL	625 µL	625 µL	625 µL	625 µL	625 µL	625 µL
Forward primers (100 µM)	3.75 µL gap-F	3.75 µL CT-F	1.9 µL Up-F 1.9 µL Uu-F	1.9 µL Mg-F 7.5 µL Mh-F <sup>c</sup>	3.75 µL LGV-F	3.75 µL L1-F	3.75 µL L2-F	3.75 µL L3-F
Reverse primers (100 µM)	3.75 µL gap-R	3.75 µL CT-R	1.9 µL Up-R 1.9 µL Uu-R	1.9 µL Mg-R 7.5 µL Mh-R <sup>c</sup>	3.75 µL LGV-R	3.75 µL L1-R	3.75 µL L2-R	3.75 µL L3-R
Probes (100 µM)	2.5 µL gap-P <sup>FAM</sup>	2.5 µL CT-P <sup>FAM</sup> 2.5 µL IKC-P <sup>HEX</sup>	1.25 µL Up-P <sup>HEX</sup> 1.25 µL Uu-P <sup>FAM</sup>	1.25 µL Mg-P <sup>HEX</sup> 1.25 µL Mh-P <sup>FAM</sup>	2.5 µL LGV-P <sup>HEX</sup>	2.5 µL L1-P <sup>HEX</sup>	2.5 µL L2-P <sup>FAM</sup>	2.5 µL L3-P <sup>FAM</sup>
IKC plasmid (10 <sup>2</sup> /µL)	-	50 µL	-	-	-	-	-	-
Aqua dest.	500 µL	450 µL	500 µL	500 µL	500 µL	500 µL	500 µL	500 µL
<i>Volume/reaction</i>	22.5 µL	22.5 µL	22.5 µL	22.5 µL	22.5 µL	22.5 µL	22.5 µL	22.5 µL

<sup>a</sup>Primer pair Mh-F and Mh-R in fourfold concentration (stock and master mix)

<sup>b</sup>IKC-probe in fivefold concentration (stock)

<sup>c</sup>Primer pair Mh-F and Mh-R in fourfold concentration (master mix)

*simulans* (not found in *C. trachomatis*). The plasmid pSARM [5] was used as a template which consists of a 276 bp sequence of the ninja transposon. The resulting 88 bp PCR product is composed of a 44 bp region of the transposon, containing the 29 bp region recognized by the Dros-probe, and flanked by the primer sequences CT-F and CT-R.

1. To amplify the human *gap*-gene, or the species-specific gene regions of pathogens, conduct each PCR in 100  $\mu$ L 1 $\times$  PCR mix (e.g., Biorad iQ-Supermix) with 3 mM MgCl<sub>2</sub>, 1  $\mu$ M of the respective primer pair (without using the probe), and 10–100 ng genomic DNA of the respective pathogen under the following thermal cycling conditions: 1 cycle at 95 °C for 5 min followed by 30 cycles at 95 °C for 15 s, 60 °C for 1 min.
2. For the construction of the internal inhibition control plasmid IKC, conduct the PCR in 100  $\mu$ L 1 $\times$  Biorad iQ-Supermix with 3 mM MgCl<sub>2</sub>, 1  $\mu$ M primer pair IKC-F and IKC-R, and 10 ng pSARM plasmid DNA under the following thermal cycling conditions: 1 cycle at 95 °C for 5 min followed by 40 cycles at 95 °C for 15 s, 48 °C for 30 s, and 72 °C for 30 s (see Note 12).
3. Purify the amplicons using a PCR purification kit (e.g., Nucleo Spin of Macherey and Nagel).
4. Determine the concentration of amplicon solutions using a photometer (e.g., by nanoDrop) (see Note 13).
5. Adjust for each control plasmid in a 1.5 mL microfuge tube 10–20 ng respective amplicon to 3.0  $\mu$ L with 10 mM Tris-HCl, pH 8.0.
6. Add 25 ng/0.5  $\mu$ L pGemT-easy, 1  $\mu$ L 5 $\times$  DNA dilution buffer, 5  $\mu$ L 2 $\times$  T4 DNA ligation buffer, and 0.5  $\mu$ L T4 DNA ligase of the Rapid DNA ligation kit from Roche.
7. Incubate the ligation assays for 10–30 min at room temperature (see Note 14).
8. Add 100  $\mu$ L transformation-competent *E. coli* DH5 $\alpha$  to each tube and incubate the transformation assays for 30 min on ice to enable uptake of the plasmid.
9. After 90 s at 42 °C and 1–2 min on ice transfer each sample to 800  $\mu$ L SOC medium in a loosely capped glass or plastic tube, shake for 90 min at 37 °C, plate 80  $\mu$ L and 800  $\mu$ L of a culture on LB-Amp agar plates, and incubate overnight at 37 °C.
10. Choose 3–6 white colonies per ligation reaction, number them consecutively on the LB-plate, and add a toothpick of material of each single colony as target to the respective qPCR.
11. Prepare Plasmid-DNA from overnight cultures of PCR-positive clones in 5 mL LB-Amp medium and control the insert sequence by commercial sequencing before using as control plasmids (see Note 15).

12. Based on the plasmid-DNA concentration (see Note 16) and insert length calculate the copy numbers and prepare dilutions of  $10^5$ ,  $10^3$ , and  $10^2$  copies/ $\mu\text{L}$  as quantification standards and  $10^2$  copies/ $\mu\text{L}$  of the IKC plasmid (see Note 17). Plasmid DNA stocks are stored at  $-20\text{ }^\circ\text{C}$  and the dilutions, used as standards in (q)PCR, at  $2\text{--}8\text{ }^\circ\text{C}$  (see Note 18).

### **3.3. Preparation of Sample Lysates and DNA Extractions**

The following provides a quick and simple preparation of whole cell lysates that generally gives good results. In any samples containing blood, DNA isolation is necessary to clear the sample of PCR inhibitors (see Note 19).

1. Resuspend urine or specimens (ocular, urethral, cervical, and rectal) in transport medium by vortexing ( $3 \times 30$  s) (see Note 20).
2. Transfer 1 mL of each sample suspension to a labeled, sterile 1.5 mL microtube and sediment the insoluble material, including bacterial cells, by centrifugation (10 min at room temperature for  $15,000 \times g$  (corresponding to 13,400 rpm in MicroSpin)) (see Note 21).
3. Discard the supernatant and wash the sediment once with 500  $\mu\text{L}$  PBS (via resuspension and centrifugation).
4. Resuspend the sediment in 25  $\mu\text{L}$  10 mM Tris-HCl, pH 7.5 and add 50  $\mu\text{L}$  Proteinase-K-solution. (In case of biopsies, use 10 mg tissue instead of sediment.)
5. Incubate the samples for 60 min at  $56\text{ }^\circ\text{C}$  followed by inactivation of the Proteinase K at  $95\text{ }^\circ\text{C}$  for 30 min (see Note 22).
6. After a short spin of 30 s transfer the supernatants into labeled, sterile tubes and store at  $-20\text{ }^\circ\text{C}$  until use.

### **3.4. qPCR: In the DNA-Free Master Mix-Room (See Note 23)**

1. Prepare for the following qPCR-mixtures labeled, DNase-free 1.5 mL microtubes: *hgap* (as reference gene for the detection of human DNA as control of swab quality), CT/IKC (for the detection of *C. trachomatis* and amplification of the inhibition control), Up/Uu (for the detection of *Ureaplasma parvum* and *U. urealyticum*), Mh/Mg (for *M. hominis* and *M. genitalium*), LGV (for the detection of any L-serovars), L1, L2, and L3 (for serovars L1, L2, and L3) (see Notes 24–27).
2. Multiply the values for one reaction depicted in Table 2 with  $(n+2)$  for a qualitative detection of  $(n)$  samples or  $(n+4)$  for a quantifiable detection of *C. trachomatis* in  $(n)$  samples (to account for the respective positive and negative controls.)
3. Pipet the respective volumes at room temperature in the labeled microtubes using barrier pipette tips.
4. Mix the ingredients by vortexing for 2–3 s and collect the liquid by short spin (10 s) (see Note 28).
5. Assemble a 96-well plate or respective amounts of 8-tube strips with the qPCR-mixtures in rows (22.5  $\mu\text{L}$ /well) according to

the PCR plate design shown in Table 3. Using 96-well plates that could be cut between the columns enabled the generation of plates with 1 up to 12 columns (see Note 9).

6. For transportation to the PCR-Room put the plate or strips into a new rack outside the Master Mix-Room and loosely fittingly cover open tubes with 8-cap strips (to avoid contaminations with DNA) (see Note 29).

### 3.5. qPCR: In the PCR-Room

1. Add to each of the eight qPCR-mixtures 2.5  $\mu\text{L}$  of the each sample in columns as depicted in Table 3.
2. Add 2.5  $\mu\text{L}$  quantitation plasmids to the respective qPCR-mixtures. For a quantitative detection, use three concentrations of control plasmids ( $10^5$  (K1+),  $10^3$  (K2+), and  $10^2$  (K3+) copies/ $\mu\text{L}$ ) for each reaction. For a qualitative result one positive control (K3+) per PCR is sufficient.
3. As negative control (K-) use 2.5  $\mu\text{L}$  of a DNA extraction or preparation of pure transport medium.
4. Cap each tube (8-tube strips with appending caps) or the wells of a column (96-well plate) immediately after adding the DNA sample(s).

### 3.6. qPCR: Thermocycling

The following protocol describes usage of an IQ5 Cyclor of Biorad. However, most steps are transferable to other qPCR instruments.

1. Switch on the basic and optical unit of the cyclor and the affiliated computer.
2. Start the (IQ5) Software.

In *Workshop* under *Setup*

3. Create or choose the following *protocol* TaqMan 1.2 (see Note 30): 1 cycle at 50 °C for 10 min, 1 cycle at 95 °C for 10 min followed by 45 cycles at 95 °C for 15 s, 60 °C for 1 min. Ramp rate is maximal for the first and 1.2 for all other cycles and data acquisition will be done in the annealing+extension step at 60 °C.
4. Create new *plate setup* and define for each well the kind of sample (S for positive controls with indication of copy numbers; X for the samples of unknown quantity and for the negative controls) and the fluorophores to be measured (FAM for hGAP, L2, and L3; HEX for LGV and L1; and HEX/FAM for IKC/CT, Up/Uu, and Mh/Mg).
5. Enter a sample volume of 25, vessel type of *plate* and seal type of *domed* or *flat cap* before saving.
6. Start RUN and save the DATA-file in opd-format (see Note 31).  
Cycling and fluorescent data collection are then carried out automatically.

**Table 3**  
**Plate design for urogenital *Mollicutes* and *C. trachomatis* detection and LGV differentiation**

**(a) Protocol**

PCR	1	2	3	4	5	6	7	8	9	10	11	12
GAP	(K1+) <sup>a</sup> 2.5 × 10 <sup>5</sup>	(K2+) <sup>a</sup> 2.5 × 10 <sup>3</sup>	K3+2.5 × 10 <sup>2</sup>	K-	S1	S2	S3	S4	S5	S6	S7	S8
CT/IKC	-do-	-do-	-do-	-do-	-do-	-do-	-do-	-do-	-do-	-do-	-do-	-do-
Uu/Up	-do-	-do-	-do-	-do-	-do-	-do-	-do-	-do-	-do-	-do-	-do-	-do-
Mg/Mh	-do-	-do-	-do-	-do-	-do-	-do-	-do-	-do-	-do-	-do-	-do-	-do-
LGV	-do-	-do-	-do-	-do-	-do-	-do-	-do-	-do-	-do-	-do-	-do-	-do-
L1	-do-	-do-	-do-	-do-	-do-	-do-	-do-	-do-	-do-	-do-	-do-	-do-
L2	-do-	-do-	-do-	-do-	-do-	-do-	-do-	-do-	-do-	-do-	-do-	-do-
L3	-do-	-do-	-do-	-do-	-do-	-do-	-do-	-do-	-do-	-do-	-do-	-do-

(continued)

**Table 3**  
**(Continued)**

**(b) Ct-values for positive reactions**

<b>Ct</b>	<b>1</b>	<b>2</b>	<b>3</b>	<b>4</b>	<b>5</b>	<b>6</b>	<b>7</b>	<b>8</b>	<b>9</b>	<b>10</b>	<b>11</b>	<b>12</b>		
<b>Fluorophor</b>	<b>F</b>	<b>H</b>	<b>F</b>	<b>H</b>	<b>F</b>	<b>H</b>								
GAP	21-26	28-33	31-36											A
CT/IKC	22-28	28-34	31-37	<44										B
Uu/Up	23-28	23-28	29-35	32-38	32-38									C
Mg/Mh	22-28	25-29	29-33	32-37	31-36	35-41								D
LGV		24-29	29-35	32-38										E
L1		23-28	29-35	32-38										F
L2	23-28	29-35	32-38											G
L3	23-28	29-35	32-38											H

FFAM, HHEX

<sup>a</sup>(Only necessary for quantification)

### 3.7. qPCR Analysis

1. Open the respective DATA-file by double-clicking. The IQ5 Optical System Software opens automatically at the *Workshop*-sheet (see Note 32).
2. Click on the button *Analyze* in the right-hand field: *Selected Data File*.
3. The view automatically changes from *Workshop* to *Data Analysis*. With the high-lightened *PCR-Quant* button the amplification curves and setup of PCR samples on PCR plate become visible.
4. Threshold should automatically be set at the start of the linear range of amplification curves. Otherwise the Crossing Threshold could be manually positioned by Mouse (see Note 33).
5. Clicking on *Analyze Wells* opens a field *Select Wells to Analyze* where you can select all wells of quantification standards and samples belonging to the same Mono- or Duplex-PCR.
6. Choose the field *FAM* (Gap/CT/Uu/Mh/L2/L3-detection) or *HEX* (IKC/Up/Mg/LGV/L1) below the Amplification Charts to see the respective amplification curves.
7. Choose the field *FAM* (Gap/CT/L2/L3-detection) or *HEX* (IKC/LGV/L1) below the plate image to visualize respective values of the corresponding PCR.  
For qualitative detection, click on the field *Threshold Cycle* in the middle field. Then you can see the Ct-values of the samples.  
For quantitative detection, click additionally on the field *Concentration*. With the definition of three quantification standards (K1+/K2+/K3+) you will see the concentration of gene copies/qPCR of the samples.
8. First of all, check the PCR run for technical validation. Ct-values of positive and negative controls have to correspond to the intervals as depicted in Table 3b (see Note 34).
9. PCR reactions of samples without any amplification curve will be evaluated as “inhibited” if the inhibition control plasmid has not been amplified.
10. PCR reactions of samples without an amplification curve will be evaluated as negative if the inhibition control plasmid has been amplified.
11. Samples with Ct-values > 30 in *hgap*-PCR must be rejected and judged as “specimen of insufficient quality” (see Note 35).
12. PCR reactions of samples with a positive amplification curve will be evaluated as “positive” even if the inhibition control plasmid has not been amplified (see Note 36).
13. The basic prerequisite for LGV-serotyping is a positive LGV-PCR with a Ct-value comparable to that of the CT reaction. Only specimens positive in the L1-, L2-, or L3-PCR has to

be considered as L1, L2, or L3 positive that are additionally positive in CT- and LGV-PCR (see Note 37).

14. Quantities of positive qPCR reactions that are automatically calculated as copies/PCR reaction have to be adjusted to x genome equivalents/mL body fluid or y genome equivalents/swap.

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## 4. Notes

1. Purchase the primers and probes as listed in Table 1 from any commercial supplier in desalted (primers) or HPLC quality (probes) as lyophilisate. The Mh-probe was originally designed as MGB-probe by Primer Express software, but now supplied by Biospring (Heidelberg, Germany) and contains modifications in all but the first G instead of the minor groove binder.
2. Genomic DNA sources of the respective pathogens or human DNA as target for PCR can easily be produced using human specimens (hGAP) positive for the respective pathogen. Heat lysis of the specimen resuspended in 10 mM Tris-HCl, pH 7.5, for 10 min at 95 °C is usually sufficient to release enough DNA into the supernatant to serve as PCR template.
3. You can use a number of commercial Taq-Polymerases and PCR buffers except those that proofread. Cloning PCR products into T-vectors characterized by a single-base 3'-thymidine (T) overhang is favored by complementary 3'-adenine (A) overhang of PCR products. Proofreading polymerases, DNA polymerases without any DNA transferase activity, do not generate any overhang in their PCR products and thus hamper successful cloning.
4. Add glucose solution (sterilized by passing it through a 0.2 µm filter) after the solution with the remaining ingredients has been autoclaved and cooled to <56 °C.
5. Cloning of the PCR product into the pGemT-vector leads to disruption of the lacZ gene resulting in loss of β-galactosidase expression. IPTG-induced X-gal cleavage does not take place in insert-carrying plasmid clones. The cleavage product of X-Gal is the blue-colored 5-bromo-4-chloro-3-hydroxyindole and thus insert-positive clones (white) are easily distinguished from insert-free ones (blue).
6. Criteria must be evaluated and defined for suitability of specimens in terms of their source, conditions of transport (time, temperature, and transport media), and conditions of storage prior to DNA preparation.
7. In the case of inhibitors that interfere with PCR amplification, DNA of proteinase-K-derived DNA lysates can easily be

purified using Qiagen tissue kit according to the manufacturer's instructions or by an automated DNA preparation by using the EZ1 DNA Tissue Kit with EZ1 DNA Bacteria Card on an EZ1 Biorobot instrument (Qiagen).

8. Depending on the real-time instrument you use and the type of background fluorescence measurement you may need a Master Mix including ROX as a reference dye. Check this carefully before ordering as it is one of the most expensive reagents needed in real-time PCR.
9. We encourage the use of 8-tube strips with connected caps as it enables the separate opening of a PCR tube, thus decreasing the risk of contamination.
10. Storage life of lyophilized primers and probes is guaranteed by suppliers for up to 8 (primers) and 5 (probes) years and that of stock solutions restricted to 5 years for primers and 6 months for probes. Working dilutions of primers and probes as well as completed Master Mixes, both stored at  $-20^{\circ}\text{C}$ , should not be used for more than 6 months without checking them for deterioration.

As the probes are the most expensive material we checked the stability of stock solutions that are older than 6 months and prolonged the service life for further 6 months if the relative fluorescence unit is a maximum of one log-step below the original ones but distinctly above the background.

11. It is important to check for the presence of inhibitors in the PCR reaction. Therefore an internal inhibition control plasmid was constructed, which should be included in each specimen. As an alternative procedure, each specimen can be run twice and one of the two reactions is spiked by a low number of the respective PCR product (e.g., with 50 copies of the quantification plasmid). A spiked specimen which remains negative in PCR reaction indicates the presence of an inhibitor and makes a negative result in the specimen invalid.
12. pSARM plasmid will be supplied on request.
13. A semiquantitative approach may also be used to define the concentration of purified amplicons, by comparing the densities of amplicon bands derived from one-fifth of the purified PCR product to DNA bands of similar length and known quantity on ethidium bromide-stained agarose gels.
14. For higher efficiency we prolonged the time for ligation from 5 min up to 30 min.
15. To prepare a frozen stock mix 0.5 mL of the cultured clone with 0.5 mL 100 % glycerine, incubate for 20 min at room temperature to enable the uptake of glycerine in the membrane and store the frozen stock at  $-80^{\circ}\text{C}$  if possible. This stock is stable for years. Storing at  $-20^{\circ}\text{C}$  is possible if 0.8 mL of the

cultured clone is mixed with 0.2 mL glycerine followed by the same procedure as above.

16. To measure the concentration of plasmid solutions you can either use a nanoDrop instrument that will use 1  $\mu\text{L}$  of the DNA preparation and calculate purity ( $\text{OD}_{260\text{nm}}/\text{OD}_{280\text{nm}}$  ratio = 1.8–2.1) and DNA concentration ( $\text{ng}/\mu\text{L}$ ). If using a different UV photometer the following equation may be helpful:  $1 \text{ OD}_{260\text{nm}} = 50 \mu\text{g}/\text{mL DNA}$ .

In any case dilute the DNA to get  $\text{OD}_{260}$  values  $< 1$  for exact calculation.

17. To calculate the copy numbers of plasmid in 1  $\mu\text{L}$  plasmid preparation you need the following equation:

$$\text{copies of plasmid}/\mu\text{L} = X \text{ ng}/\mu\text{L} \times (9.13 \times 10^8 / Y \text{ kbp})$$

where  $X$  = amount of ng, measured in 1  $\mu\text{L}$  of plasmid preparation

and  $Y$  = length of the plasmid [pGemT + insert] in kbp.

Example:

Plasmid	$Y$ (kbp)	$\text{OD}_{260/280}$	$\text{OD}_{260}$	$X$ (ng/ $\mu\text{L}$ )	Copies/ $\mu\text{L}$	$C = 10^9$ copies/ $\mu\text{L}$
pGemT-Mh	[3.003 + 0.061]	1.83	1.981	99.1	$2.95 \times 10^{10}$	10 $\mu\text{L}$ ( $2.95 \times 10^{10}$ ) ad 295 $\mu\text{L}^a$

<sup>a</sup>Dilute with 10 mM Tris-HCl, pH 7.5

18. Most plasmid dilutions are stable for more than 1 year. Use new dilutions of quantitation standards when their Ct-values are out of range (Table 3b).
19. Alternatively, DNA preparation can be done using a Qiagen Tissue Kit on an EZ1 biorobot machine. Therefore, steps (a)–(c) have to be replaced by the following steps (a)–(d).
- Resuspend the sediment in 190  $\mu\text{L}$  G2 buffer and add 10  $\mu\text{L}$  Proteinase-K solution of the Qiagen Tissue Kit.
  - Incubate the samples for 30 min at 56  $^\circ\text{C}$  (either in thermoblock or water bath).
  - Transfer 200  $\mu\text{L}$  sample into 2 mL sample tube and start program “Bacteria protocol: 1: Bact\_200  $\mu\text{L}$ .”
  - Store the eluted DNA (100  $\mu\text{L}$ ) at  $-20$   $^\circ\text{C}$  until use.
20. As negative control an unspiked transport medium should be processed in parallel.
21. Samples should be stored at 4  $^\circ\text{C}$  and not be frozen to prevent lysis of bacterial cells. Concentration of the sample by sedimentation of the bacterial cells is only successful with intact cells.
22. Proteinase K digestion can be done either in a thermoblock or a water bath or after transfer into 0.2 mL PCR tubes in a conventional thermocycler.

23. Try to ensure a *3-Rooms Solution* (at least a 3-separate area solution) for the performance of the real-time PCR with one room for Master Mix preparation, a second room for DNA extraction, and a third PCR-room for sample processing and cycling.
24. *C. trachomatis* detection and LGV serovar subtyping can be done simultaneously (time-saving) or consecutively, in that only *C. trachomatis*-positive samples are subjected to LGV serovar differentiation (cost-saving).
25. To prevent DNA contaminations, wear a special lab coat that *never* leaves the Master Mix-Room; wear new gloves; work, if possible, under a PCR cabinet that has been decontaminated by UV irradiation; and *never* take DNA, like control plasmids or sample DNA, into the room.
26. When using in clinical diagnostics it is more comfortable to prepare mixtures for 50 reactions and to store the rest not used at  $-20^{\circ}\text{C}$  in the dark. Freezing and thawing for up to ten times does not alter the amplification results.
27. Instead of using monoplex reactions for the detection of LGV, L1, L2, and L3 these PCRs can be conducted as duplex PCR, [LGV/L2] and [L1/L3] with concentrations as described for duplex-PCRs in Table 2.
28. The IKC plasmid is not genomic DNA as it is an artificial DNA construct amplified by the CT forward and reverse primer CT-R and only detected by the Dros-probe. Thus handling of this plasmid is the only exception of DNA entering the Master Mix-Room.
29. For transportation plates could also be covered by paper sheets or adhesive films. But, be careful when removing adhesive film in the PCR room to avoid creating droplets.
30. Reduction of Ramp Rate from max to 1.2 which corresponds to a slowed activation of temperatures could be omitted working on an ABI qPCR instrument as that instrument has a naturally slowed gate drive temperature.
31. If not using an external well factor plate for fluorophore standardization, ensure that the formerly measured well factors were done with the same volume, vessel, and seal type—otherwise thermocycling will not start.
32. Data analysis can already be performed when the camera is switched off. The respective error message can be ignored and clicked off.
33. The threshold cycle (Ct) is the cycle number at which a detectable amount of amplicon (measured by an increase of fluorescence) has been generated during the early exponential phase of the reaction. Thereby the fluorescence signal crosses the fluorescence threshold line, which is automatically set as barrier to exclude background fluorescence from real exponential amplifications.

34. You can use copies of the form in Table 3 to document each PCR run. Tick the controls which are in range, and write the Ct-values of positive reactions in the respective field of the lower 96-well plate scheme with respect to the fluorophore of the distinct PCR (F=FAM/H=HEX) and the concentration (in case of a qPCR) of the pathogenic DNA below the specimen's name (S1–S8) in the upper scheme.
35. In taking swaps of human tissue surfaces and body fluids a lot of human cells were collected, too. We empirically estimated that the amount of human DNA, measured by hGAP-PCR led to Ct-values ranging from Ct=20–25. In cases of Ct-values higher than 30 the amount of swapped material seems to be decreased and the specimen has to be guessed as of “unsufficient quality.”
36. In case of *C. trachomatis* detection the primers CT-F and CT-R will be used in the amplification of DNA of the pathogen *C. trachomatis*, as well as of the cryptic inhibition plasmid. Thus both reactions may compete with each other leading to a loss of amplification of the less concentrated one. If the bacterial load of *C. trachomatis* is 100-fold higher ( $10^4$  genome equivalents/PCR) than that of the inhibition plasmid (100 copies/PCR) an inhibition of the plasmid amplification occurs. On the other hand, an assured *C. trachomatis* detection is restricted to 25 genome equivalents/PCR with respect to the simultaneous amplification of 100 copies of inhibition plasmid.
37. In contrast to the *pmpH*-gene-based LGV-qPCR which is specific for all lymphogranuloma venereum-associated L-serovars because of a characteristic gap in the targeted *pmpH*-region, the *omp1*-gene targeting L1-, L2-, and L3-PCR reactions show some cross-reaction with other *C. trachomatis* serovars. Thus, positive CT- and LGV-PCRs are a prerequisite to consider specimen with positive L1, L2, or L3 reaction as respective L-serovar positive.

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## Acknowledgements

We would like to especially thank Servas Morr e and Eberhard Straube for kindly providing the *Chlamydia* strains.

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# Chapter 16

## Protocol for the Use of Self-Reporting Duplex Mutation Primers to Detect PCR Products in the Diagnosis of HBV

Qian-Feng Xia

### Abstract

Quantitative measurements of serum hepatitis B virus (HBV) DNA are useful for tailoring of treatment schedules and the monitoring of HBV replication during therapy. We developed a novel fluorescence-based quantitative real-time PCR for quantitating HBV DNA based on the duplex mutation primers principle, in which signal is generated by melting a duplex mutation primer during renaturation. The duplex mutation primers are much more specific than double-stranded DNA dyes like SYBR Green I and, unlike other probes, do not require the double-labeled synthesis of fluorophore and quencher on the same molecule.

**Key words:** Hepatitis B virus, Quantitation, Diagnosis, Duplex mutation primer, Real-time PCR

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### 1. Introduction

Since 1993 when Higuchi et al. pioneered the analysis of PCR kinetics by constructing a system that detects PCR products as they accumulate (1), the fluorescence-based quantitative real-time PCR (qPCR) technology has successfully evolved to a reliable and easy-to-use standard method for detection and/or quantification of nucleic acid sequences. PCR product accumulation is measured by a variety of fluorescent detection chemistries, such as sequence-specific probes like hydrolysis probe (2), molecular beacon (3), sunrise primer (4), scorpion (5), adjacent hybridization probe (6) and invader (7), or generic dsDNA binding dyes like SYBR Green I (8) and ethidium bromide (9). The principal drawback to intercalator-based detection of PCR product accumulation is that both specific and nonspecific products generate signals. An alternative method, the dual hybridization probes, provides a real-time method for detecting specific amplification products only. The synthesis and purification of high-quality multilabeled probes are often

neither trivial nor inexpensive compared to fluorescent dye and quencher pair being attached to the same oligonucleotide. This prompted scientists to devise a probe with a fluorescent dye and quencher pair on different oligonucleotides such as hybridization probes (9), displacing probes (10), and Q-PNA primers (11). Both hybridization probes and displacing probes are 5'-terminal-labeled with fluorophore and blocked from extension with a 3'-phosphate group; thus they are not really single-labeled probes and therefore costly. The synthesis of peptide nucleic acid of Q-PNA primers is equally expensive.

Therefore, we have devised a new method based on a primer, with a fluorophore attached to its 5'-end, which is annealed to a complementary single-base mismatched oligonucleotide labeled with a quencher at its 3'-end. In PCR, the fluorophore-labeled primer binds preferably to the target over the quencher oligonucleotide and the primer-guided extension. Incorporation of the primer into a double-stranded amplicon causes displacement of the duplex mutation primers such that the fluorescence of the sample is a direct indication of the amplicon concentration (Fig. 1). The fluorophore

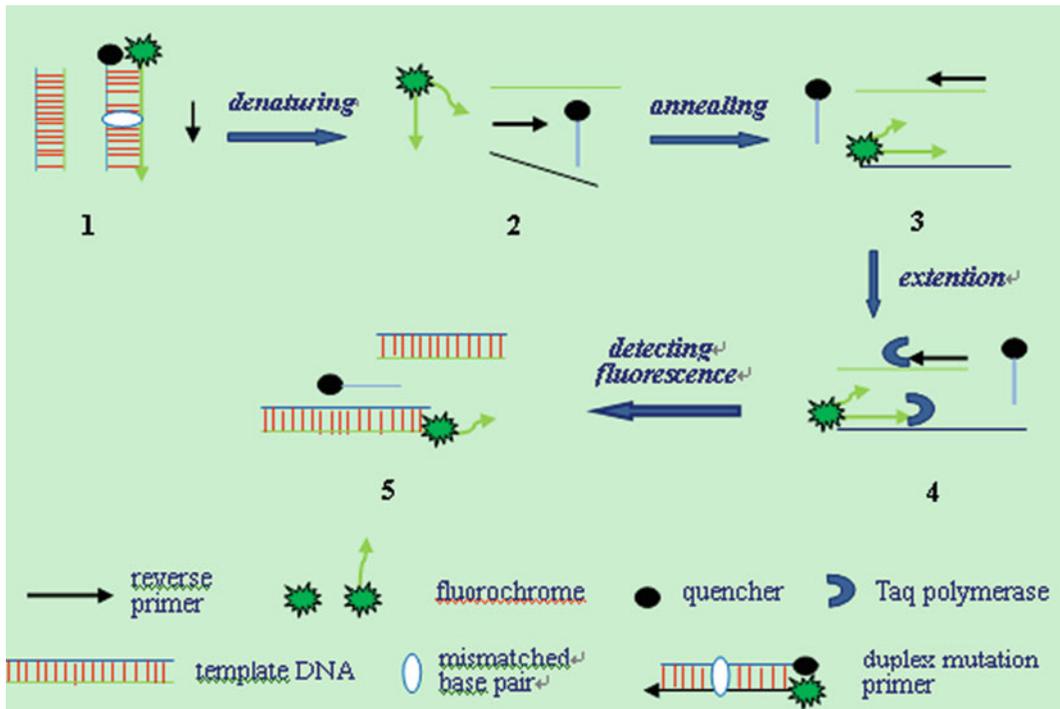


Fig. 1. Schematic of the duplex mutation primers principle. (1) The duplex mutation primers is a duplex of a single mismatch between the fluorophore primer and the quencher oligonucleotide. 3'-Quencher-labeled oligonucleotide hybridizes to 5'-fluorophore-labeled forward primer, and fluorescence resonance energy transfer (FRET) takes place. (2) All the double-strand structures are denatured, and FRET neutralized (3, 4). The fluorophore/quencher duplex is less thermodynamically favored than the fluorophore/target, the fluorophore-labeled primer anneals preferentially to the complementary template and the primer guides the extension. (5) The end product of the reaction is a double-stranded amplicon, of which one strand is fluorescently labeled, and the high fluorescence is detected because of the failure of FRET.

primer can anneal to the complementary template and guide the extension as a normal primer, whereas extension from the quencher oligonucleotides is blocked by the 3'-quencher. The duplex mutation primers are more specific than double-stranded DNA dyes like SYBR Green I and, unlike other internal probes (5–8), do not require the synthesis of a quencher on the same molecule. We report here a qPCR assay for the detection of hepatitis B virus (HBV) DNA standardized for clinical use in the sera of patients with HBV disease.

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## 2. Materials

Prepare all solutions using double-distilled water (DDW) and analytical grade reagents. Prepare all reagents or samples at room temperature (unless indicated otherwise). Diligently follow all waste disposal regulations when disposing waste materials.

### 2.1. DNA Preparation

1. QIAamp DNA Blood Mini kit (Qiagen Inc., Germany).
2. Serum samples, obtained from patients and from blood donors diagnosed with HBV infection (see Note 1).
3. 1.5 mL microcentrifuge tubes.
4. Micropipettes and tips.
5. Heater or water bath.
6. Ethanol (96–100 %).
7. Vortexer.
8. Microcentrifuge.
9. Freezer (–20 °C).

### 2.2 qPCR

1. Fluorophore primer: 5'-FAM-CCAGGGAATTAGTAGTCAGC-3', Reverse primer: 5'-AAAGTAAGGCAGGAAATGTG-3', Quencher oligonucleotide: 5'-GCTGACTAATAATTCCTGG-Dabcyl-3'. Each oligonucleotide is supplied as 2 μM solution in water, pH 7.5. Mix each 1:10 and pool (see Note 2). Store at –20 °C.
2. 100 mM dNTP set. Store at –20 °C.
3. Taq DNA-polymerase 250 U (Invitrogen) with supplied 10× PCR reaction buffer. Store at –20 °C.
4. 25 mM MgCl<sub>2</sub> (Takara). Store at –20 °C.
5. Distilled water.
6. Negative and positive control DNA.
7. Commercially available panels of HBV standards at concentrations of 10<sup>7</sup>, 10<sup>6</sup>, 10<sup>5</sup>, 10<sup>4</sup>, 10<sup>3</sup>, 10<sup>2</sup>, and 0 IU/mL (Da'an Ltd.,

Guangzhou, China) were used in this test (see Note 3). Aliquots of the HBV standards were stored at  $-70^{\circ}\text{C}$ .

8. PCR tubes (100  $\mu\text{L}$ ).
9. Micropipettes and tips.
10. Real-time PCR machine (e.g., Rotor Gene 3000).
11. Freezer ( $-70^{\circ}\text{C}$ ).

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### 3. Methods

Carry out all procedures at room temperature unless otherwise specified.

#### **3.1. Isolation of DNA from Serum**

1. Pipet 10  $\mu\text{L}$  QIAGEN Protease (or proteinase K) into the bottom of a 1.5 mL microcentrifuge tube.
2. Add 100  $\mu\text{L}$  serum to the microcentrifuge tube.
3. Add 100  $\mu\text{L}$  Buffer AL to the sample. Mix by pulse-vortexing for 15 s.
4. Incubate at  $56^{\circ}\text{C}$  for 10 min.
5. Briefly centrifuge the 1.5 mL microcentrifuge tube to remove drops from the inside of the lid.
6. Add 100  $\mu\text{L}$  ethanol (96–100 %) to the sample, and mix again by pulse-vortexing for 15 s. After mixing, briefly centrifuge the 1.5 mL microcentrifuge tube to remove drops from the inside of the lid.
7. Carefully apply the mixture from step 6 to the QIAamp Mini spin column (in a 2 mL collection tube) without wetting the rim. Close the cap, and centrifuge at  $6,000\times g$  (8,000 rpm) for 1 min. Place the QIAamp Mini spin column in a clean 2 mL collection tube (provided), and discard the tube containing the filtrate.
8. Carefully open the QIAamp Mini spin column and add 250  $\mu\text{L}$  Buffer AW1 without wetting the rim. Close the cap and centrifuge at  $6,000\times g$  for 1 min. Place the QIAamp Mini spin column in a clean 2 mL collection tube (provided), and discard the collection tube containing the filtrate.
9. Carefully open the QIAamp Mini spin column and add 250  $\mu\text{L}$  Buffer AW2 without wetting the rim. Close the cap and centrifuge at full speed ( $20,000\times g$ ) for 3 min.
10. Recommended: Place the QIAamp Mini spin column in a new 2 mL collection tube and discard the old collection tube with the filtrate. Centrifuge at full speed for 1 min.

11. Place the QIAamp Mini spin column in a clean 1.5 mL microcentrifuge tube, and discard the collection tube containing the filtrate. Carefully open the QIAamp Mini spin column and add 100  $\mu$ L Buffer AE or distilled water. Incubate at room temperature (15–25  $^{\circ}$ C) for 1 min, and then centrifuge at  $6,000 \times g$  for 1 min.
12. Total DNA was extracted from 100  $\mu$ L of serum with a QiaAmp DNA mini eluted in 100  $\mu$ L of Qiagen elution buffer, and stored at  $-20^{\circ}$ C (see Note 4).

### 3.2. qPCR

1. PCR reaction mix (15  $\mu$ L) containing 4  $\mu$ L duplex mutation primers (2.0  $\mu$ M of the fluorophore primer, 2.0  $\mu$ M of the quencher strand), 2.0  $\mu$ M of the reverse primer, 1.6 mM of each dNTP, 1U of Taq polymerase, and 2.5 mM  $MgCl_2$ , adding distilled water to 20  $\mu$ L (see Note 5).
2. Add 5  $\mu$ L of extracted DNA for each testing sample and mix by pipetting. Briefly centrifuge. For each set of samples, a negative control and a positive control (serum with known HBV DNA concentration) should also be included (see Note 6).
3. Reactions will take place at the following thermal cycle conditions: 94  $^{\circ}$ C for 4 min, 40 cycles of 93  $^{\circ}$ C for 15 s, and 42  $^{\circ}$ C for 15 s. The signal detection was performed at 42  $^{\circ}$ C during each cycle.
4. Using the tenfold serial diluted HBV standards from  $10^2$  to  $10^7$  IU/mL as the reference standards, the standard curve was established by software and the linear range was judged by the standard curve that the R (relativity) value must be larger than 0.99 (Fig. 2).
5. Results of clinical samples can be calculated automatically according to the standard curve by software.

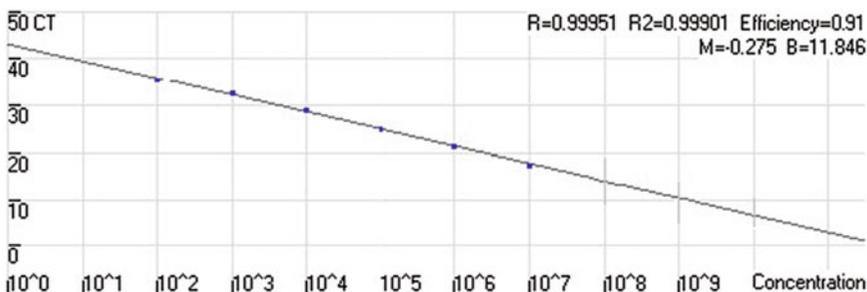


Fig. 2. The standard curve. Typical standard curve for the template concentrations from  $10^7$  to  $10^2$  IU/mL.

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## 4. Notes

1. Blood samples should be centrifuged within 4 h to obtain the serum fractions, which were then divided into aliquots and kept at  $-70^{\circ}\text{C}$  before testing.
2. Aliquots of 100  $\mu\text{L}$  master mix, including 10 mM pH 8.0 Tris-HCL, and 10 nmol of fluorophore and quencher strand, can be denatured in a thermal cycler at  $94^{\circ}\text{C}$  for 2 min, annealed at  $40^{\circ}\text{C}$  for 5 min and allowed to cool to room temperature. The solution should be prepared in advance and stored at  $-20^{\circ}\text{C}$ .
3. The World Health Organization (WHO) Collaborative Study Group and the WHO Expert Committee on Biological Standardization have recommended and made available the first international standard, 97/746, for HBV DNA for NAT assays (nucleic acid testing, NAT). It is hoped that the WHO international standard will be adopted universally as a reference and that consistent reporting of results in IU per milliliter will emerge.
4. Disposable cotton-plugged pipette tips and autoclaved microtubes should be used.
5. Pipetting scheme with the volumes of each reagent: 4  $\mu\text{L}$  duplex mutation primers, 4  $\mu\text{L}$  reverse primer, 0.8  $\mu\text{L}$  dNTP, 0.2  $\mu\text{L}$  Taq polymerase, 1  $\mu\text{L}$   $\text{MgCl}_2$ , and 5  $\mu\text{L}$  target DNA. The enzymes required for PCR should be kept on ice during handling.
6. The extraction and qPCR procedure for clinical serum samples and HBV standards in each test should be simultaneous operations.

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# Chapter 17

## Protocol for the Use of a Rapid Real-Time PCR Method for the Detection of HIV-1 Proviral DNA Using Double-Stranded Primer

Chou-Pong Pau, Susan K. Wells, and Timothy C. Granade

### Abstract

This chapter describes a real-time PCR method for the detection of HIV-1 proviral DNA in whole blood samples using a novel double-stranded primer system. The assay utilizes a simple commercially available DNA extraction method and a rapid and easy-to-perform real-time PCR protocol to consistently detect a minimum of four copies of HIV-1 group M proviral DNA in as little as 90 min after sample (whole blood) collection. Co-amplification of the human RNase P gene serves as an internal control to monitor the efficiency of both the DNA extraction and amplification. Once the assay is validated properly, it may be suitable as an alternative confirmation test for HIV-1 infections in a variety of HIV testing venues including the mother-to-child transmission testing sites, clinics, and diagnostic testing centers.

**Key words:** HIV-1 proviral DNA, Real-time PCR, Double-stranded primer, Rapid PCR, Nucleic acid testing

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### 1. Introduction

Nucleic acid amplification technology (NAAT) is the method of choice for the detection of early HIV-1 infection, especially in high-risk populations (1) and in infants born to infected mothers (2). Recent studies have demonstrated that NAAT increased the HIV-1 detection by 23–25 % over antibody testing in sites for men who have sex with men (MSM) and in substance abuse treatment centers (3). These results suggest that the implementation of NAAT in areas with high HIV-1 incidence rates could enhance early detection of HIV-1-infected individuals and improve intervention strategies. Globally, the populations with the highest HIV-1 incidence rates are located in resource-poor regions, and the implementation of NAAT in those areas is challenging due to the high cost of

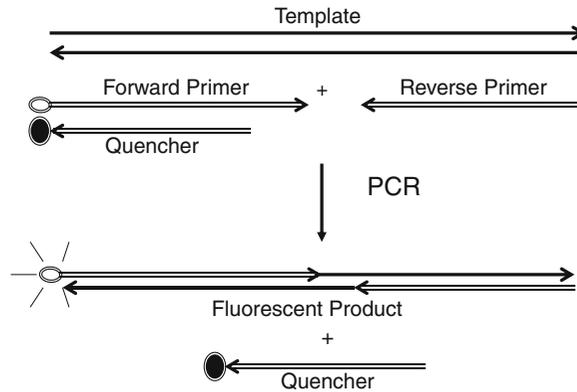


Fig. 1. Schematic of PCR amplification using double-stranded primer. The double-stranded primer consists of the forward primer, which is labeled with a fluorophore at the 5' end, and a quencher, which is labeled with a nonfluorescent acceptor at the 3' end. The quencher sequence is reverse complementary to the 5' end portion of the forward primer. The forward primer and the quencher form a double-stranded structure which is not fluorescent due to the close proximity of the fluorophore and the acceptor. After PCR amplification, the quencher is displaced by the product generated from the reverse primer, and the resulting amplicon becomes fluorescent and can be monitored fluorometrically.

laboratory infrastructure, the need for highly trained personnel, and the complexity of existing commercial assays (4, 5).

Currently, most NAAT tests are performed in central laboratories which may be hundreds of miles away from treatment centers or sample collection sites. In these settings, the collection, storage, and transport of specimens further complicate the effective use of NAAT for diagnostic purposes and for therapeutic monitoring. Results may take days or even weeks before being returned to the physician, thus delaying treatment and care for infected individuals and hindering local prevention efforts.

In-house assays that use relatively inexpensive instrumentation and simplified testing protocols could alleviate some of these concerns and permit the expansion of HIV-1 NAAT in resource-poor countries. As these technologies improve and are properly validated, implementation of rapid NAAT testing close to the treatment and care centers or sample collection sites will become feasible, and the testing can be decentralized and be more accessible to the affected populations in a cost-effective manner.

In this chapter, a rapid real-time PCR protocol to detect HIV-1 proviral DNA in whole blood is described. DNA is extracted easily using a commercially available extraction kit and the extracted DNA from the HIV-1 long terminal repeat (LTR) region is amplified using a novel double-stranded primer (DSP) system (Fig. 1) (6). In this system, the forward primer is modified with a fluorophore (ROX for HIV-1, FAM for RNase P) at the 5' end, while the quencher, which is reverse complementary to the 5' portion of the forward primer, is modified with a nonfluorescent

acceptor (BHQ2) at the 3' end. Before PCR amplification, the forward primer and the quencher form a double-stranded structure which is nonfluorescent due to the close proximity of the fluorophore and the acceptor. During PCR amplification, the quencher is displaced by the increasing concentration of the product generated from the reverse primer, and the resulting double-stranded amplicon becomes fluorescent and can be monitored fluorometrically after each PCR cycle using a real-time thermocycler. A real advantage of PCR using DSP is that it does not require a conserved probe-binding region in the target sequence, thus eliminating potential sequence mismatches in the probe region. This is useful especially for HIV-1 detection since it is well known that HIV-1 sequences are extremely divergent among strains from different geographic regions. In addition, a shorter target sequence is amplified, which may lead to faster (~50 min) and more efficient amplification (~100 %) (6).

Since the HIV-1 LTR is highly conserved among HIV-1 group M isolates, this method detects all HIV-1 group M subtypes. Using a DNA standard panel from the US National Institutes of Health, the assay could detect a single copy of HIV-1 DNA 64 % of the time (7/11) and four copies in all attempts ( $n=11$ ) within 90 min after sample (whole blood) collection. Co-amplification of the human RNase P gene served as an internal control to monitor the efficiency of both the nucleic acid extraction and template amplification.

---

## 2. Materials

### 2.1. DNA Extraction

1. QIAamp® DNA Blood Mini Kit (Qiagen). Store at room temperature except for the reconstituted protease which is stored at 4°C for up to 2 months.
2. Additional 2-mL sample collection tubes from Qiagen (see Note 1).
3. Sterile microcentrifuge tubes (1.5- and 2.0-mL).
4. Sterile pipette tips with aerosol barriers.
5. Benchtop centrifuge with rotor for 2-mL tubes.
6. Heated water bath at 56°C.
7. Vortexer.
8. Ethanol (100 %).
9. Timer/clock.
10. Solid polypropylene 96-well microcentrifuge tube rack (see Note 2).
11. Known HIV-1-infected and noninfected whole blood as the positive and negative control, respectively (see Note 3).

**2.2. Real-Time PCR**

1. QuantiFast® Multiplex PCR+R Kit (Qiagen). Store working master mix at 4°C for up to 1 month (see Note 4).
2. Primer and quencher oligonucleotides (see Note 5):  
 HIV-1 forward primer: 5'-ROX-TIAAGCCTCAATAAAGCT TGCCTTG-3'  
 HIV-1 quencher: 3'-BHQ2-ACTTCGGAGTTATTTTCGAAC-5'  
 HIV-1 reverse primer 1: 5'-CACAAACAGACGGGCACACACT ACT-3'  
 HIV-1 reverse primer 2: 5'-AACACAGACGGGCACACA CCACT-3' (see Note 6)  
 RNase P forward primer: 5'-FAM-GTGTTCGAGATTTGG ACCTGCG-3'  
 RNase P quencher: 3'-BHQ2-CACAAACGTCTAAACCTG-5'  
 RNase P reverse primer: 5'-GTGAGCGGCTGTCTCCAC-3'  
 Store stock oligonucleotide solutions at -20°C.
3. Nuclease-free water.
4. Working primer mix. Prepare HIV-1 and RNase P primer mix according to Table 1. Aliquot primer mixes (0.1 mL) into sterile 1.5-mL microcentrifuge tubes and store at -20°C. Store working solutions at 4°C for 2 weeks.
5. Sterile pipette tips with aerosol barriers.
6. Sterile microcentrifuge tubes (1.5 mL).
7. 0.2-mL PCR tubes and optical cap (8-well strip).
8. FlipStrip™ microtube rack (holds 1.5-mL and 0.2-mL strips, Molecular BioProducts) for storage of working primer mix and 2× master mix. Store at 4°C (see Note 8).
9. Mini centrifuge for 0.2-mL PCR tubes.

**Table 1**  
**Composition of HIV-1 and RNase P working primer mix**

	Oligonucleotide	Concentration
HIV-1 primer mix	HIV-1 forward primer	5 μM
	HIV-1 reverse primer 1	5 μM
	HIV-1 reverse primer 2	5 μM
	HIV-1 quencher	12.5 μM (see Note 7)
RNase P primer mix	RNase P forward primer	5 μM
	RNase P reverse primer	5 μM
	RNase P quencher	12.5 μM (see Note 7)

Nuclease-free water is used to dilute the oligonucleotides

10. Real-time thermocycler Model MX3000P (Stratagene) with computer system and MXPro V.4.01 software or equivalent (see Note 9).
11. Two PCR cabinets equipped with a UV light (see Note 10).

---

### 3. Methods

#### 3.1. DNA Extraction

DNA extraction is performed at room temperature (15–25°C) under BSL-2 safety requirements as described in the Spin Protocol of the QIAamp DNA Blood Mini Kit instruction manual. Each extraction run should include at least one positive and one negative whole blood control (see Note 3).

1. Pipette 20  $\mu\text{L}$  of the protease solution into the bottom of a 1.5-mL microcentrifuge tube.
2. Add 200  $\mu\text{L}$  of the whole blood sample.
3. Add 200  $\mu\text{L}$  of Buffer AL and vortex for 10 s.
4. Incubate at 56°C for 10 min in a heated water bath.
5. Add 200  $\mu\text{L}$  of ethanol and vortex for 15 s.
6. Centrifuge for 5 s and transfer the sample mixture to a Spin Column (supplied with kit) with a 2-mL collection tube.
7. Close the cap and centrifuge the Spin Column at  $6,000 \times g$  for 1 min.
8. Transfer the Spin Column to a clean collection tube and discard the collection tube with filtrate.
9. Add 500  $\mu\text{L}$  of Buffer AW1, close the cap, and centrifuge at  $6,000 \times g$  for 1 min.
10. Transfer the Spin Column to a clean collection tube and discard the collection tube with filtrate.
11. Add 500  $\mu\text{L}$  of Buffer AW2, close the cap, and centrifuge at  $20,000 \times g$  for 3 min.
12. Transfer the Spin Column to a clean collection tube and discard the collection tube with filtrate.
13. Centrifuge at  $20,000 \times g$  for 1.5 min.
14. Discard the collection tube and place the Spin Column on a clean 2.0-mL microcentrifuge tube.
15. Add 200  $\mu\text{L}$  of Buffer AE and incubate for 1 min at RT.
16. Centrifuge at  $6,000 \times g$  for 1 min.
17. Transfer the filtrate to a clean 1.5-mL microcentrifuge tube.
18. Store the eluted DNA at 4°C for same day testing (see Note 11) or at -20°C for future use.

**3.2. Real-Time PCR**

1. Turn on the MX3000P real-time thermocycler and computer.
2. Start the MxPro program and establish the PCR Thermal Profile as follows:
  - Segment 1: 1 cycle, 95°C for 1.5 min (enzyme activation).
  - Segment 2: 42 cycles of 94°C for 1 s, then 62°C for 20 s (PCR).

Choose ROX (HIV-1) and FAM (RNase P) channels for data acquisition at the end of each cycle of Segment 2.
3. Remove the 2× master mix, HIV-1, and RNase P primer mixes from the refrigerator and place them in a cold (4°C) FlipStrip™ microtube rack.
4. Prepare the PCR mix in a 1.5-mL microcentrifuge tube in the cold FlipStrip™ microtube rack. Determine the number of samples, including the positive and negative controls, to be tested and add reagents for two extra samples to the total to ensure that an adequate amount of reagents is available. Prepare the master mix as described in Table 2 in a PCR cabinet dedicated to reagent preparation (see Note 10).
5. Place the PCR tubes in the cold FlipStrip™ microtube rack and aliquot 14.0 µL of the PCR master mix into each tube.
6. Place the optical cap strip over the tubes without closing tightly, and move the rack to a separate PCR cabinet located in a different room (see Note 10).
7. Remove the DNA extracts from the refrigerator, add 11 µL of each sample to the PCR tube, and mix the content by pipetting up and down three times.
8. Close the cap tightly and spin on a mini centrifuge for 5 s.
9. Place the tubes in the MX3000P real-time thermocycler and click “Start” to initiate the PCR reaction immediately.

**Table 2**  
**Pipetting scheme for HIV-1/RNase P real-time PCR setup**

Reagents	Volume (µL) required for number of reactions					
	1	5	10	15	20	25
2× Kit master mix	12.5	62.5	125	187.5	250	312.5
HIV-1 primer mix <sup>a</sup>	1	5	10	15	20	25
RNase P primer mix <sup>a</sup>	0.5	2.5	5	7.5	10	12.5

<sup>a</sup>See Table 1

All values are in microliters

10. At the end of PCR amplification, perform data analysis and record the HIV-1 and RNase P  $C_t$  values for each sample and control (see Note 12).
11. Compare the  $C_t$  values of the controls with those of historical data (see Note 3).
12. Inspect the HIV-1  $C_t$  and RNase P  $C_t$  for each sample. If (a) the RNase P  $C_t$  is over that of the negative control by more than three cycles or RNase P is not amplifiable, and (b) HIV-1 test is negative, the DNA extraction may be insufficient. Repeating the test with a fresh DNA extract is recommended.

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## 4. Notes

1. The QIAamp DNA Blood Mini Kit provides three sets of 2-mL collection tubes, and a fourth set is needed for this procedure.
2. A solid polypropylene tube rack is used for sample storage and transportation. Once prechilled to 4°C, the rack will keep the samples cool without using an ice bucket.
3. Since universal positive and negative controls are not available, each laboratory may need to identify and characterize its own controls. A pool of whole blood from several HIV-1 sero-positive individuals can serve as a positive control. The pool should be aliquoted (220  $\mu$ L) into microcentrifuge tubes and stored at -70°C for future use.

The positive and negative controls should be characterized and tested repeatedly prior to actual sample testing. Ideally, a positive control should contain an HIV-1 copy number just over the assay's lower limit of detection. However, proviral copy number measurement in whole blood is not a simple task; therefore, we recommend to characterize the positive control by  $C_t$  measurement. From our previous study, the lower limit of detection was approximately four copies per reaction with a  $C_t$  of 37.8. If the control  $C_t$  is significantly lower than 37.8, the specimen should be diluted with a noninfected whole blood to give a  $C_t$  value slightly below 37.8.

After the appropriate controls are identified, they should be extracted and tested at least ten times in separate runs. The HIV-1  $C_t$  and RNase P  $C_t$  values for each extract should be measured using the standard PCR protocol described in Subheading 3. Average  $C_t$  values and standard deviations for the positive and negative controls can then be established. The control  $C_t$  values in the subsequent tests are then compared to the average  $C_t$  values determined previously to evaluate the performance of each individual run. Control  $C_t$  values differing

by more than three cycles are considered significant, and the specimen should be retested using a fresh extract.

4. The 2× master mix from the QuantiFast Multiplex kit contains all reagents, except the primers and DNA templates, that are needed for the reaction. In addition, no optimization of the magnesium ion concentration is needed (as stated in the QuantiFast Multiplex Kit Handbook), resulting in less pipetting steps during assay preparation. The working 2× master mix should be stored at 4°C to avoid freezing and thawing which can denature the polymerase enzyme that is already incorporated into the kit master mix.
5. The HIV-1 forward and reverse primers bind to sites located in a conserved LTR region of the genome. The quencher sequences are shown in reverse complementary configurations to the corresponding forward primers. Oligo Primer Analysis Software for Windows Version 6 (Molecular Biology Insights) was used to design the primers to ensure that there is no primer dimer formation between the primers.
6. Minor sequence variations in the HIV-1 reverse primer region, especially in the Clade AE strains, were noted using the QuickAlign algorithm from the Los Alamos National Laboratory (<http://www.hiv.lanl.gov>). Therefore, a second primer (HIV-1 reverse primer 2) is included to ensure amplification of divergent strains.
7. A slight excess of quencher compared to the forward primer is needed to ensure quenching of the fluorescent primer. However, a large excess of quencher may compete with the template for the primer and thus inhibit the PCR reaction. A two- to threefold excess of quencher was found to be optimal for this assay.
8. The prechilled rack keeps the reagents cool while setting up the assay. Return all reagents to 4°C immediately after use.
9. This assay has been optimized for use with an MX3000P thermocycler. If a different real-time thermocycler is used, re-optimization may be needed. In addition, the HIV-1 primer was labeled with ROX; other real-time thermocyclers that require ROX as the reference dye are not suitable for this assay.
10. The first cabinet is used for reagent preparation only. The second cabinet located in a separate room is for addition of the DNA extracts to the PCR reaction mix in order to avoid contamination of the reagents.
11. For same day analysis, tubes containing the DNA extracts may be stored in a cold, solid polypropylene rack at 4°C until use. The rack will keep the samples cool during sample addition to the reaction mix without using an ice bucket.

12. Although this assay is not classified as probe-based real-time PCR since a target-specific fluorescent probe is not required, the amplification plot is similar to that of a probe-based assay such as the TaqMan probe assay. Therefore, routine procedures for data analysis may be used.

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## Protocol for the Use of Light Upon Extension Real-Time PCR for the Determination of Viral Load in HBV Infection

Guimin Li, Wangfeng Li, and Lixia Liu

### Abstract

Real-time PCR has engendered wide acceptance for quantitation of hepatitis B virus (HBV) DNA in the blood due to its improved rapidity, sensitivity, reproducibility, and reduced contamination. Here we describe a cost-effective and highly sensitive HBV real-time quantitative assay based on the light upon extension real-time PCR platform and a simple and reliable HBV DNA preparation method using silica-coated magnetic beads.

**Key words:** LUX primers, HBV DNA, Real-time PCR, Magnetic beads, Viral DNA preparation

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### 1. Introduction

Hepatitis B virus (HBV) is an important cause of viral hepatitis in humans worldwide, especially in Asia, Africa, southern Europe, and Latin America. It is estimated that over two billion people have evidence of previous HBV infection and more than 400 million people have become chronic carriers of the virus (1). Approximately one-third of HBV carriers will progress to cirrhosis and 25 % will develop hepatocellular carcinoma (HCC) (2). There is increasing evidence that measuring the level of HBV DNA in serum has become an important tool to identify individuals with high viral replication, to monitor disease progression and the efficacy of anti-viral therapy, to detect the occurrence of drug-resistant mutants, and to detect relapse after discontinuing antiviral therapy (3–5).

In recent years, real-time PCR has been accepted widely as a gold standard for quantitation of viral nucleic acids due to its improved rapidity, sensitivity, reproducibility, and reduced contamination (6, 7). Several real-time PCR methods for detecting HBV DNA with high levels of sensitivity and specificity have been

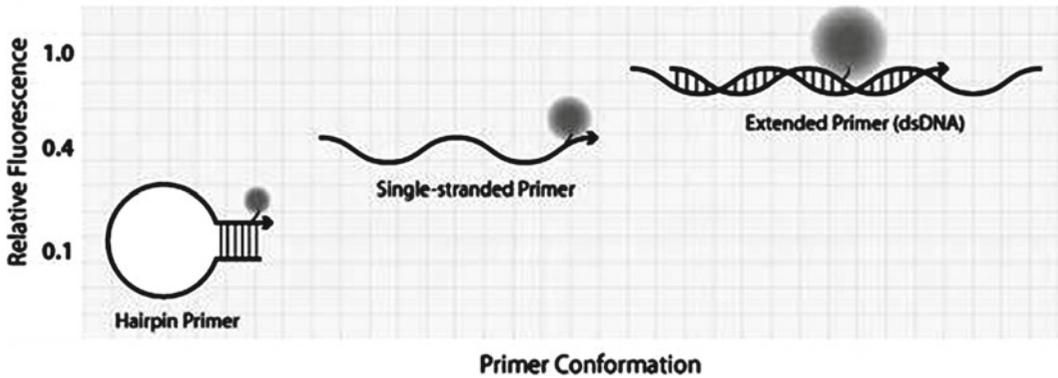


Fig. 1. The LUX™ (Light Upon eXtension) effect.

developed (8–11). Most of the reports used either SYBR green intercalating dye or probes with a fluorophore and a quencher moiety. However, the former has limited specificity while the latter may face increasing cost of the assays.

A novel real-time PCR technique using light upon extension (LUX) primer was recently developed as a cost-effective alternative to other fluorescence-based PCR techniques (12, 13). LUX detection technique provides sequence-specific detection without the need for probes. Each LUX primer set includes one primer labeled with a single fluorophore (either the forward or the reverse primer) and a corresponding unlabeled primer, both synthesized to the target of interest. On its own, the fluorogenic primer is designed to be “self-quenched” and does not emit notable fluorescence. When this primer is incorporated into a double-stranded PCR product, a significant increase in fluorescent signal will be observed (Fig. 1) (<http://www.invitrogen.com/lux>).

Here, we describe a cost-effective and highly sensitive HBV real-time quantitative assay based on the LUX platform and a new viral DNA preparation method using silica-coated magnetic beads. The sensitivity of LUX-PCR defined as the lowest concentration of HBV DNA quantified at a frequency of 100 % was found to be 5 copies/reaction. The new silica bead method is simple and efficient and is comparable to the spin-column-based QIAamp Blood Mini Kit. Thus, the assay possessed high sensitivity and the detection limit of this system was as few as 25 copies/mL of serum.

## 2. Materials

Prepare all solutions using ultrapure water (18 M $\Omega$  cm at 25 °C) and analytical grade reagents. Prepare all reagents at room temperature.

**2.1. Viral DNA  
Preparation  
Components**

1. HBsAg-positive and -negative serum or plasma samples. All samples were stored at  $-70\text{ }^{\circ}\text{C}$  until use.
2. Proteinase K solution: 10 mg/mL.
3. Stock solution of silica coated magnetic beads (Allrun, Shanghai, China): 50 mg/mL.
4. Magnetic rack (Allrun, Shanghai, China).
5. Tris-HCl: 1 M, pH 8.0.  
Dissolve 121.1 g Tris base in 800 mL of purified water in a beaker. Adjust pH to 8.0 with concentrated HCl. Transfer solution to a 1 L graduated cylinder and adjust the volume to 1 L with purified water. Cover cylinder with Parafilm and mix thoroughly. Filter with 0.5  $\mu\text{m}$  filter and autoclave. Store at  $4\text{ }^{\circ}\text{C}$  (see Note 1).
6. Tris-HCl: 1 M, pH 6.6.  
Prepare a 1 L solution as in previous step adjusting the pH to 6.6 with concentrated HCl (see Note 1).
7. Tris-HCl: 1 M, pH 7.5.  
Prepare a 1 L solution as in previous step adjusting the pH to 7.5 with concentrated HCl (see Note 1).
8. EDTA: 0.5 M, pH 8.0.  
Add 186.1 g EDTA to 800 mL of ddH<sub>2</sub>O. Add about 20 g of NaOH pellets while stirring to bring the pH to 8.0. Add the last few grams slowly to avoid overshooting the pH. Note that the EDTA will not completely dissolve until the pH is around 8. Adjust the volume to 1 L with purified water. Filter with 0.5  $\mu\text{m}$  filter and autoclave. Store at  $4\text{ }^{\circ}\text{C}$ .
9. NaCl: 2.5 M.  
Weigh 141.1 g NaCl and transfer to the cylinder. Add water to a volume of 900 mL. Mix well and adjust the volume to 1 L with purified water. Filter with 0.5 micron filter and autoclave. Store at  $4\text{ }^{\circ}\text{C}$ .
10. 10 $\times$  lysis buffer: 100 mM Tris-HCl (pH 8.0), 10 mM EDTA (pH 8.0), 5 % SDS, 5 % Triton X-100.  
Dissolve 50 g SDS in 800 mL of purified water, then add 100 mL of 1 M Tris-HCl (pH 8.0), 20 mL of 0.5 M EDTA (pH 8.0), and 5 mL of Triton X-100 to it. Mix thoroughly and filter with 0.5 micron filter. Store at room temperature (see Note 2).
11. Binding buffer: 5 M GuSCN, 20 mM Tris-HCl (pH 6.6), 10 % ethanol.  
Heat in a  $60\text{ }^{\circ}\text{C}$  water bath to dissolve 590 g GuSCN in 300 mL of purified water, then add 20 mL of 1 M Tris-HCl (pH 6.6) and 100 mL of pure ethanol. Adjust the volume to 1 L with purified water and filter with 0.5 micron filter. Store at room temperature.

12. Washing buffer: 20 mM NaCl, 2 mM Tris-HCl (pH 7.5), 80 % ethanol.  
Transfer 2 mL of 1 M Tris-HCl (pH 7.5), 8 mL of 2.5 M NaCl, and 800 mL of pure ethanol to 190 mL pure water. Mix thoroughly and store at room temperature.
13. Elution buffer: 10 mM Tris-HCl (pH 8.0).  
Transfer 10 mL of 1 M Tris-HCl (pH 8.0) to 990 mL pure water. Mix thoroughly and store at room temperature.
14. Heater block (BIOER, Hangzhou, China).
15. Vortex (IKA, Germany).
16. 1.5 mL Eppendorf tubes.
17. Micropipettes and tips.  
Filter-plugged pipette tips of 10, 100, and 1,000  $\mu$ L.

## **2.2. HBV LUX Real-Time Assay Components**

1. H<sub>2</sub>O  
Ultrapure water (18 M $\Omega$  cm at 25 °C) was sterilized at 121 °C for 20 min. Store at -20 °C.
2. The plasmid containing Chinese HBV complete genome.  
A plasmid containing Chinese HBV genome (pHBV-adr) was kindly donated by Chinese Centers for Disease Control and Prevention (CDC).
3. MiniBEST Plasmid Purification Kit (TaKaRa, Dalian, China).
4. *Eco*R I restriction enzyme (TaKaRa).
5. Agarose gel DNA purification Kit (TaKaRa).
6. Primers.  
Search for the highly conservative part in HBV polymerase gene sequence using the NCBI Blast (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Design LUX™ Primer Sets using LUX™ Designer online (<http://www.invitrogen.com/lux>). Enter the specific sequence of the target region, and the software will generate one primer set. The sequence of the primers used in this protocol: forward primer (labeled primer) 5'-cggaaCCCC TATCTTATCAACACTTCC(FAM)G-3', reverse primer (unlabeled primer) 5'-CGAGGCGAGGGAGTTCTTCT-3' (see Note 3).
7. Bovine serum albumin (BSA): 10 mg/mL.  
Dissolve 100 mg BSA in 9.5 mL water. Adjust the volume to 10 mL with water. Store in aliquots at -20 °C.
8. dNTP solution: 200  $\mu$ M of each dGTP, dATP, dCTP and 400  $\mu$ M dUTP.  
Transfer 20  $\mu$ L each of dGTP, dATP, dCTP, 40  $\mu$ L dUTP solution (100 mM), and 900  $\mu$ L water. Mix thoroughly and store at -20 °C.
9. 10 $\times$  PCR buffer (TaKaRa, Dalian, China):  
100 mM Tris-HCl (pH 8.3), 500 mM KCl, 15 mM MgCl<sub>2</sub>.

10.  $\text{MgCl}_2$ : 50 mM.  
Weigh 10.165 g  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$  and transfer to the cylinder. Add water to a volume of 900 mL. Mix it up and adjust the volume to 1 L with purified water. Filter with 0.5 micron filter and autoclave. Store at  $-20^\circ\text{C}$ .
11. Hot start Taq DNA polymerase (TaKaRa, Dalian, China).
12. Uracil DNA glycosylase (UDG) (MBI Fermentas, Burlington, ON, Canada).
13. iCycler iQ5 and software, version 2.0 (Bio-Rad, Hercules, CA, USA).
14. 96-well PCR plates.
15. Micropipettes and tips.

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### 3. Methods

To obtain reliable quantitation results all pipettes have to be checked regularly for precision and, if necessary, have to be calibrated. In order to avoid possible cross-contamination, this assay needs to be run according to Good Laboratory Practice (see Notes 4–11).

#### **3.1. HBV DNA Extraction with a New Magnetic Bead Method (See Note 12)**

1. Mix 200  $\mu\text{L}$  of serum (or plasma), 20  $\mu\text{L}$  of 10 mg/mL of Proteinase K solution, and 20  $\mu\text{L}$  of lysis buffer in 1.5-mL Eppendorf tubes. Place the tubes into holes in heater block. Incubate at  $65^\circ\text{C}$  for 10 min (see Notes 13 and 14).
2. Add 400  $\mu\text{L}$  of binding buffer and 25  $\mu\text{L}$  stock solution of silica-coated magnetic beads to each tube and vortex briefly. Incubate at room temperature for 3 min (see Notes 15–17).
3. Place the tubes on a magnetic rack for about 30 s. Discard the supernatant with a micropipette.
4. Add 750  $\mu\text{L}$  of washing buffer to suspend the magnetic beads pellet. Place the tubes on a magnetic rack for about 30 s. Discard the supernatant with micropipette.
5. Repeat step 4.
6. Add 50  $\mu\text{L}$  elution buffer and resuspend the magnetic beads. Incubate at  $65^\circ\text{C}$  for 2 min.
7. Place the tubes on a magnetic rack for about 30 s. Transfer the supernatant as eluate to a clean tube. Perform real-time PCR assay directly or store the extracted DNA at  $-70^\circ\text{C}$  for later analysis.

#### **3.2. HBV LUX Real-Time Assay**

1. Preparation of standard HBV DNA:  
Isolate the plasmid containing Chinese HBV complete genome with TaKaRa MiniBEST Plasmid Purification Kit. Digest the plasmid with *EcoR* I at  $37^\circ\text{C}$  for 2 h. Verify it by agarose gel

electrophoresis. Cut the target band out of gel and recover HBV DNA with Agarose gel DNA purification Kit. Measure the HBV DNA concentration at 260 nm with spectrophotometer. Calculate the copy number using the following formula:

Formula: Copy number = (ng of dsDNA/660 × 3,200) × 6.02 × 10<sup>14</sup> (see Note 18).

Make a serial tenfold dilution from 2 × 10<sup>7</sup> copies/mL to 2 × 10<sup>2</sup> copies/mL and use as standards.

- Set up the qPCR reaction at room temperature (see Notes 19–23).

Real-time PCR reaction	μL/reaction
H <sub>2</sub> O	6.2
10× PCR buffer	5
BSA (10 mg/mL)	2.5
MgCl <sub>2</sub> (50 mM)	3.5
dNTP solution 200 μM(G, A, C)/400 μM(U)	5
Unlabeled primer (10 pmol/μL)	1
FAM-labeled D-LUX primer (10 pmol/μL)	1
UDG (1 U/μL)	0.4
Hot start Taq DNA polymerase (5 U/μL)	0.4
DNA template	25
Total	50 μL

Set up negative control, positive control, standard samples, and test samples in duplicate in a 96-well plate.

- Mix well and centrifuge briefly. Place the plate into the Bio-Rad iQ5 instrument (see Note 24).
- Programme the real-time equipment as follows:  
Select FAM as the reporter dye. Enter the sample names. Specify thermal cycling conditions.

Cycling program

Stage I (UNG step): 1 cycle

50 °C 2 min

Stage II (hot start activation): 1 cycle

95 °C 5 min

Stage III (PCR): 45 cycles

95 °C 10 s

60 °C 30 s

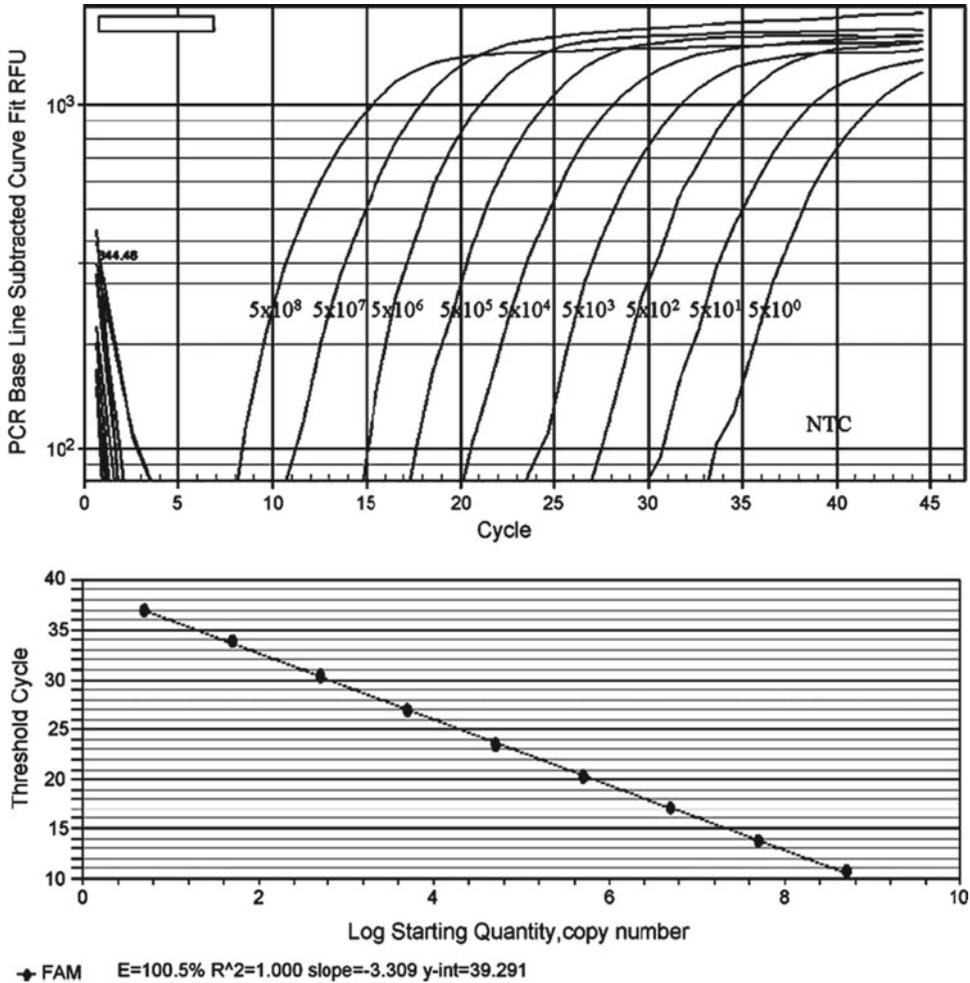


Fig. 2. Amplification plots and standard curves of genomic HBV DNA of plasmid standard ranging from  $5 \times 10^0$  to  $5 \times 10^8$  copies/reaction. *NTC* no template control.

72 °C 30 s

The data were collected at the extension step (72 °C) of every cycle.

Stage IV (*melting curve*)

95 °C 1 min

55 °C 1 min

80 cycles of 0.5 °C increments (10 s each) beginning at 55 °C

- After run is complete go to analysis of the results. Plot the standard curve (Fig. 2) and get the samples' values. Transfer to an excel file and plot graphs. Evaluate the specificity of the LUX assay according to melt curve analysis (see Note 25).

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## 4. Notes

1. Concentrated HCl (12 N) can be used at first to narrow the gap from the starting pH to the required pH. From then on it would be better to use a series of HCl dilutions (e.g., 6 and 1 N) with lower ionic strengths to avoid a sudden drop in pH below the required pH value.
2. Wear a mask and gloves when working with SDS powder or use commercial stock solutions.
3. Invitrogen can label only three fluoresceins (FAM, JOE, or Alexa 546) for LUX primer.
4. Maintain separate working areas for solution preparation, DNA preparation, reaction setup, and amplification.
5. The procedure requires experience of working under sterile conditions.
6. Clean lab benches and equipment periodically with 10 % sodium hypochlorite solution (bleach). Bleach will very effectively destroy contaminating DNA.
7. UV is irradiated periodically in working areas.
8. Wear separate coats and gloves in each area.
9. Use only powder-free gloves and change them frequently.
10. Use filter-plugged pipette tips.
11. Be careful to avoid any aerosol formation.
12. Clinical samples should be regarded as potentially infectious materials and should be prepared in a laminar flow hood. All samples and associated wastes must be autoclaved before disposal.
13. Serum and plasma specimens must be equilibrated for 15–30 min at room temperature before use.
14. If the sample volume is larger than 200  $\mu\text{L}$ , the amount of lysis buffer and Proteinase K should be increased proportionally. For example, a 400  $\mu\text{L}$  sample will require 40  $\mu\text{L}$  lysis buffer and Proteinase K.
15. Magnetic bead will sediment in stock solution. Resuspend it completely to create a homogenous dispersion of the beads before use.
16. GuSCN will crystallize if binding buffer was stored at 15 °C and below. Hold in 60 °C water bath to dissolve it.
17. The binding buffer contains GuSCN which is an irritant. Wear gloves and follow usual safety precautions when handling.
18. Average MW of a DNA base pair = 660 Da; number of full-length HBV-DNA base pairs = 3,200 bp; 1 nmol =  $6.02 \times 10^{14}$  copies.

19. All amplification and detection reagents must be at room temperature before use. Once the reagents have been thawed, vortex and centrifuge briefly the tubes.
20. Avoid repeated thawing and freezing of the reagents; this may reduce the sensitivity of the test.
21. For each run, a standard curve was created in a 6-log-unit range (from 5 to  $5 \times 10^5$  copies/tube) by 1:10 serial dilutions of the HBV plasmid DNA standard. If real-time PCR data of the samples are  $>5 \times 10^5$ , dilute the samples with negative serum and repeat the experiment.
22. All samples were run in duplicates.
23. The UDG (or UNG) and dUTP were used to minimize carryover contamination from previous PCR amplifications.
24. The PCR assay has been optimized for use in a Bio-Rad iQ5 detection system. Other systems may be used, but thermal cycling conditions must be verified.
25. Melt curve analysis can reveal the presence of nonspecific products such as primer dimers. The LUX primer assay supports melting curve analysis. It is essential in designing an efficient and specific quantitative PCR assay and plays an important role in real-time PCR optimization. Only one peak at 82 °C in melting curve analysis demonstrates specific amplification of HBV DNA. In contrast, the presence of other peaks indicates the presence of primer-dimers and/or nonspecific PCR products.

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## Protocol for the Use of a Silica Nanoparticle-Enhanced Microcantilever Sensor-Based Method to Detect HBV at Femtomolar Concentrations

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and Tae Song Kim

### Abstract

DNA sensors that are capable of detecting specific DNA sequences in a bio-sample have recently been highlighted as a powerful and sensitive approach to detect infectious diseases caused by pathogens such as viruses and bacteria. Generally, DNA samples extracted from biological fluids are amplified by PCR prior to analysis by DNA sensors or directly analyzed by DNA sensors equipped with a signal amplification process. Nanoparticles have recently been used to amplify the sensor signal and have been shown to play an important role in improving the sensitivity of mechanical resonating sensors. This is because the weight of the nanoparticle can increase the change in the resonance response of the mechanical sensor since this signal change is closely related to mass. Here, we introduce an experimental method to detect HBV at femtomolar concentrations using a silica nanoparticle-enhanced microcantilever resonating sensor. This method includes the preparation of detection probe-conjugated silica nanoparticles, immobilization of capture probe on the microcantilever sensor and sandwich type detection of HBV DNA.

**Key words:** Microcantilever sensor, Silica nanoparticles, HBV DNA, Sensitivity enhancement, Sandwich assay

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### 1. Introduction

Hepatitis B virus (HBV) infection is one of the most severe viral infectious diseases worldwide, with an estimated 400 million people chronically infected. Approximately, 70 % of hepatocellular carcinoma cases develop from the chronic hepatitis type B. HBV also has the capacity to escape immune surveillance by mutating structural genes encoding epitopes recognized by the immune system, resulting in a quasi-species population (1, 2). Because of the clinical reasons described above, it is highly important to diagnose HBV at the

early stage before chronicity and fatal complications occur (3, 4). The diagnosis of viral infectious diseases has been mainly performed by quantitative DNA analysis methods including the use of polymerase chain reaction (PCR) amplification, transcription-mediated amplification (TMA) and branched DNA (bDNA) amplification (5–8). These techniques are based on detecting the multiplied DNA molecules or applying specific amplification probes to DNA captured on probe DNA. Here, we developed an experimental method to detect HBV at femtomolar concentrations using a silica nanoparticle-enhanced microcantilever sensor (9, 10). A 243 bp sequence of HBV DNA precore/core region was used as the target DNA. In this assay, the capture probe on the microcantilever surface and the detection probe conjugated to silica nanoparticles were specifically designed for the target DNA. The applied nanoparticle concentrations and the resonant frequency shifts of the microcantilever were strongly correlated and the quantitative relationship between the mass and resonant frequency shift was validated.

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## 2. Materials

Prepare all solutions using ultrapure water (prepared by purifying deionized water to attain a resistance of 18 M $\Omega$  cm at 25 °C), analytical grade solvents, and reagents with purity of >99 %. Prepare reagents at room temperature and store all products in the dark at 4 °C (unless indicated otherwise).

### 2.1. Silica Nanoparticle Components

1. Rhodamine B isothiocyanate (RITC), 3-aminopropyltriethoxysilane (APTS), *N,N'*-dimethylsulfoxide (DMSO), tetraethoxyorthosilicate (TEOS), succinic anhydride, *N,N*-diisopropylethylamine (DIEA), ammonium hydroxide (NH<sub>4</sub>OH, 25 %), ethanol (EtOH, 99.0 %), and water can be purchased from Sigma-Aldrich.
2. 1.5 mL conical tubes (e.g., Eppendorf Safe-Lock Microcentrifuge Tubes).
3. Magnetic stirrer.
4. Triangular and egg-shaped magnetic bars can be purchased from Thermo Fisher Scientific.
5. 50 mL conical tubes (e.g., BD Falcon™ 50 mL conical tubes).
6. Microcentrifuge (e.g., Eppendorf Refrigerated Microcentrifuge Model 5417 R).
7. 2-[Methoxy(polyethyleneoxy)propyl]trimethoxysilane (MPPTS): 9–12 C<sub>2</sub>H<sub>4</sub>O groups (e.g., Gelest, Morrisville, PA, USA).

### 2.2. Components for Detection Probe Conjugation

1. MES buffer (pH 6.0, 50 mM): 50 mM 2-(*N*-morpholino) ethanesulfonic acid. Dissolve 195.2 g of MES in 0.9 L water and adjust pH with 1 N NaOH. Adjust volume to 1 L with additional water.
2. Detection probe (100  $\mu$ M): Dissolve 0.05  $\mu$ mol of the detection probe (5'-ATCTGGCCACCTGGGTGGGAAGTAAT<sub>10</sub>-(NH<sub>2</sub>)-3') in 500  $\mu$ L of water and keep it in a freezer (-20 °C). Thaw immediately before use.
3. 1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS) can be purchased from Sigma-Aldrich.
4. Phosphate buffered saline (PBS): 150 mM PBS, pH 7.4. Dissolve 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na<sub>2</sub>HPO<sub>4</sub>, and 0.24 g of KH<sub>2</sub>PO<sub>4</sub> in 0.9 L H<sub>2</sub>O and adjust pH with 1 N NaOH and 1 N HCl. Adjust volume to 1 L with additional water.

### 2.3. Components for Capture Probe Immobilization

1. Lead zirconate titanate (PZT)-embedded microcantilever: Use fabricated piezoelectric (PZT) thin layer-embedded microcantilevers (see Note 1). Sequentially deposit the chrome and gold layer with thickness of 10 and 50 nm using an e-beam evaporator (SME-200E, ULVAC Corporation, Japan) (see Note 2).
2. 48-Well plate (e.g., BD Falcon™ 48-well Multiwell Plate).
3. Capture probe (1  $\mu$ M): Dissolve 0.05 mol of capture probe (5'-(SH)-(CH<sub>3</sub>)<sub>18</sub>-T<sub>10</sub>TGGAGCTTCCGTGGAGTTACTCTCT-3') in 500  $\mu$ L of water and keep it in a freezer (-20 °C). Before use, thaw the solution (1 mM) immediately and mix 1  $\mu$ L with 990  $\mu$ L of TE buffer (see Note 3).
4. TE buffer (pH 8.0, 10 mM): Dissolve 1.58 g of Tris-HCl and 0.292 g of *N,N,N',N'*-ethylenediaminetetraacetate (EDTA) in 0.9 L water and adjust pH with 0.1 N NaOH. Adjust volume to 1 L with additional water.
5. Orbital shaker: Compact open air shakers (Eppendorf Inc., Hamburg, Germany).
6. Backfiller (5 mM): Dissolve 16.8 mg of (11-mercaptopundecyl) tri(ethylene glycol) (e.g., Sigma-Aldrich) in 10 mL of EtOH freshly before use.
7. Ethanol 95 %.
8. Water.
9. Nitrogen gas: 99.9 %.
10. Impedance analyzer (e.g., Agilent Technologies Model 4294A).

### 2.4. HBV Target DNA for Standard Curve

1. HBV PCR product (243 bp) of 2.31 nM (see Note 4).
2. TE buffer (pH 8.0, 10 mM): Dissolve 1.58 g of Tris-HCl and 0.292 g of *N,N,N',N'*-EDTA in 0.9 L water and adjust pH with 0.1 N NaOH. Adjust volume to 1 L with additional water.

3. Orbital shaker: Compact open air shakers (Eppendorf Inc., Hamburg, Germany).
4. Impedance analyzer (e.g., Agilent Technologies Model 4294A).

### 2.5. HBV DNA Samples

1. HBV PCR products of clinical sera with suspected HBV infection (see Note 4).

---

## 3. Methods

All procedures are performed under room temperature and room humidity if not specified.

### 3.1. Preparation of Dye-Incorporated Silica Nanoparticle

1. Dissolve 2.2 mg (4  $\mu\text{mol}$ ) of RITC and 1.88  $\mu\text{L}$  (8  $\mu\text{mol}$ ) of APTS (RITC:APTS = 1:2) with 0.5 mL DMSO in a 1.5 mL conical tube and stir the solution with egg-shaped magnetic bar on the magnetic stirrer for 12 h at room temperature.
2. Transfer this solution to a round-bottomed glass flask containing 5 mL of TEOS, 1 mL of  $\text{NH}_4\text{OH}$ , 10 mL water, and 78 mL of EtOH. Stir the solution with egg-shaped magnetic bar on the magnetic stirrer for 12 h at room temperature (see Note 5).
3. Transfer the final solution into the 50 mL conical tube, centrifuge the solution at  $24,000\times g$  for 10 min, and discard the supernatant. Pour 50 mL of EtOH into the tube and re-disperse the pink-colored pellet of RITC-incorporated silica nanoparticles (RITC-SiNPs) (see Note 6). Repeat the centrifugation and dispersion steps five times ( $\sim 100$  nm silica nanoparticles).
4. Transfer 55 mg of RITC-SiNPs dispersed in 1 mL EtOH to a 1.5 mL conical tube (see Note 6) and add 55 mg of MPPTS and 4.4 mg of APTS. Stir the solution using a triangular magnetic bar on the magnetic stirrer for 12 h. Centrifuge the solution at  $24,000\times g$  for 10 min and discard the supernatant. Pour 1 mL of EtOH into the tube and re-disperse the pellet of nanoparticles (RITC-SiNP-PEG600/ $\text{NH}_2$ ) (see Note 6). Repeat the centrifugation and dispersion steps five times.
5. Transfer 18  $\mu\text{L}$  of the RITC-SiNP-PEG600 solution into a new 1.5 mL conical tube and add 482  $\mu\text{L}$  EtOH. Add 2.5 mg of succinic anhydride and 4.1  $\mu\text{L}$  of DIEA into the solution. Stir the solution with triangular magnetic bar on the magnetic stirrer for 12 h at room temperature. Centrifuge the solution at  $24,000\times g$  for 10 min and discard the supernatant. Pour 1 mL of EtOH into the tube and re-disperse the pellet of silica nanoparticles (RITC-SiNP-PEG600/ $\text{COOH}$ ). Repeat the centrifugation and dispersion steps five times.

### 3.2. Conjugation of the Detection Probe to Silica Nanoparticles (See Fig. 1a)

1. Disperse 1 mg of RITC-SiNP-PEG600/COOH in 1 mL of MES buffer.
2. Add 50  $\mu\text{L}$  of 50 mg/mL EDC and 50  $\mu\text{L}$  of 50 mg/mL NHS into the solution to activate carboxylic acid group on of RITC-SiNP-PEG600/COOH.
3. Stir the solution using triangular magnetic bar on the magnetic stirrer for 1 h at room temperature.
4. Add 50  $\mu\text{L}$  of 100  $\mu\text{M}$  detection probe and stir for 2 h at room temperature (see Table 1, detection probe).
5. Centrifuge the solution at  $24,000 \times g$  for 10 min and discard the supernatant. Pipette 1 mL of 150 mM PBS into the tube and re-disperse the cake of silica nanoparticles. Repeat the centrifugation and dispersion steps five times (see Note 7).

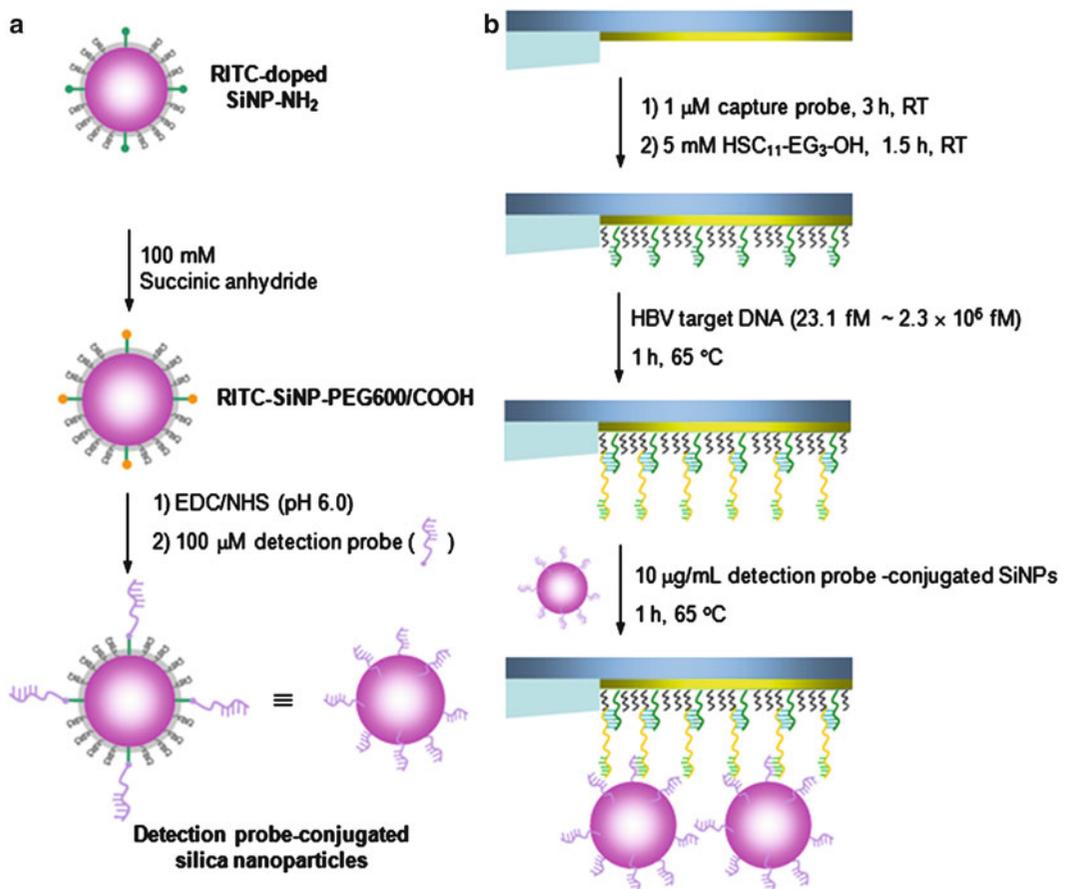


Fig. 1. (a) Preparation of detection probe-conjugated silica nanoparticles and (b) detection protocol of HBV target DNA using silica nanoparticle-enhanced microcantilevers. Reproduced from (10) with permission from Elsevier.

**Table 1**  
**Sequences of probe DNAs and target DNA**

Probes	Sequences	Length (mer)
Capture probe	5'-(SH)-(CH <sub>3</sub> ) <sub>18</sub> -T <sub>10</sub> TGGAGCTTCCGTGGAGTTACTCTCT-3'	35
HBV target DNA	TCCCTGGATGCTGGGTCTTCCAAATTACTTCCCACCCAGG TGGCCAGATTCATCAACTCACCCCAACACAGAATAGCTT GCCTGAGTGCTGTATGGTGAGGTGAACAATGTTCCGGA GACTCTAAGGCCTCCCGATACAAAGCAGAGGCGGTGTC GAGGAGATCTCGAATAGAAGGAAAGAAGTCAGAAGGC AAAAAAGAGAGTAACTCCACGGAAGCTCCAAATTCTTT ATACGGGTCAATG	243
Detection probe	5'-ATCTGGCCACCTGGGTGGGAAGTAA T <sub>10</sub> -NH <sub>2</sub> -3'	35

All DNA probes are HPLC grade and over 1 OD

**3.3. Immobilization  
of the Capture Probe  
on Microcantilever  
(See Fig. 1b)**

1. Place the microcantilever device with fresh gold layer in a well of a 48-well plate.
2. Spot 100  $\mu$ L of a 1  $\mu$ M capture probe solution on the device and incubate it for 3 h at room temperature (see Note 8).
3. (Washing) Transfer the device to another well containing 500  $\mu$ L of fresh TE buffer and shake the plate at 600 rpm for 5 min using the orbital shaker (1 $\times$ ). Transfer the device to another well containing 500  $\mu$ L of fresh water and shake the plate at 600 rpm for 5 min using the orbital shaker (2 $\times$ ).
4. (Backfilling) Transfer the washed device to another well containing 100  $\mu$ L of 5 mM backfiller solution and incubate for 1.5 h at room temperature.
5. (Washing) Transfer the device to another well containing 500  $\mu$ L of 95 % ethanol and shake the plate at 600 rpm for 5 min using the orbital shaker (2 $\times$ ). Transfer the device to another well containing 500  $\mu$ L of water and shake the plate at 600 rpm for 5 min using the orbital shaker (2 $\times$ ).
6. Dry the washed device under flow of nitrogen (see Note 9).
7. To measure the resonant frequency, connect between two electrodes the microcantilever (30  $\mu$ m $\times$ 90  $\mu$ m) and impedance analyzer. Enter the input signal condition (0.5 V<sub>pp</sub>) of impedance analyzer. Enter the frequency range with condition of  $\pm$ 1.5 kHz span from center frequency. Press the button with auto-scaling function. Find the frequency at the peak value of impedance phase signal.

**3.4. Plot of Standard  
Curves**

1. Prepare 100  $\mu$ L HBV Target DNA (in TE buffer) of the following concentrations: 23.1, 231 fM, 2.31, 23.1, 231 pM, and 2.31 nM for each microcantilever (see Table 1, HBV Target DNA and Note 4).

- Place six devices with the capture probe-immobilized microcantilever in well of a 48-well plate.
- Carefully spot 100  $\mu\text{L}$  HBV DNA solution on each microcantilever to immerse the microcantilever completely.
- Incubate the device at 65  $^{\circ}\text{C}$  for 1 h (see Note 8).
- (Washing) Transfer the device to another well containing 500  $\mu\text{L}$  of fresh TE buffer and shake the plate at 600 rpm for 5 min using the orbital shaker (2 $\times$ ). Transfer the device to another well containing 500  $\mu\text{L}$  of fresh water and shake the plate at 600 rpm for 5 min using the orbital shaker (2 $\times$ ).
- Dry the devices under flow of nitrogen.
- Measure the resonant frequency of microcantilevers (30  $\mu\text{m} \times 90 \mu\text{m}$ ) using impedance analyzer (see step 8 of Subheading 3.3).
- (Signal processing) Plot the differences between the resonant frequencies from these measurements and those from step 8 of Subheading 3.3 at each concentration (see Fig. 2, filled square).
- Place the device treated with HBV DNA solution to another well and carefully spot 100  $\mu\text{L}$  of 10  $\mu\text{g}/\text{mL}$  detection probe-conjugated silica nanoparticles in TE buffer to immerse microcantilever completely.
- Incubate 65  $^{\circ}\text{C}$  for 1 h (see Note 8).
- (Washing) Transfer the device to another well containing 500  $\mu\text{L}$  of fresh TE buffer and shake the plate at 600 rpm for 5 min using the orbital shaker (2 $\times$ ). Transfer the device to

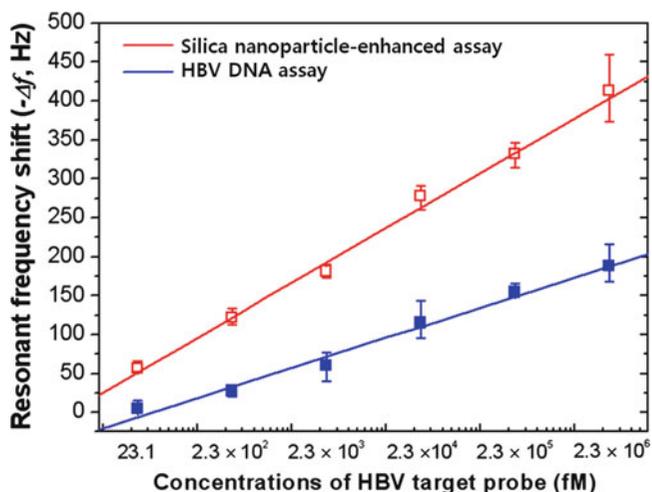


Fig. 2. Plots of the resonant frequency shifts acquired from the HBV DNA assay (*filled square*) and the silica nanoparticle-enhanced HBV DNA assay (*empty square*) in the concentration range from 23.1 fM to 2.3 nM. Reproduced from (10) with permission from Elsevier.

another well containing 500  $\mu\text{L}$  of fresh water and shake the plate at 600 rpm for 5 min using the orbital shaker ( $2\times$ ).

12. Dry the devices under flow of nitrogen (see Note 9).
13. Measure the resonant frequency of microcantilevers ( $30\ \mu\text{m}\times 90\ \mu\text{m}$ ) using impedance analyzer (see step 8 of Subheading 3.3).
14. (Signal processing) Plot the differences between the resonant frequencies from these measurements and those from step 8 of Subheading 3.3 for each concentration (see Fig. 2, empty square).

### **3.5. HBV DNA Assay on Microcantilever**

1. Perform HBV DNA assay using HBV PCR product of clinical serum by following method of steps 3–14 of Subheading 3.4. Obtain the difference of the resonant frequencies between the capture probe-immobilized microcantilever (before assay) and silica nanoparticles-enhanced microcantilever (after assay) (see Note 10). Match the difference with the concentration of HBV DNA using the graph of Fig. 2.

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## **4. Notes**

1. The procedure used to fabricate the piezoelectric layer-embedded microcantilever is as follows (see Fig. 3a): the piezoelectric (PZT) thin layer-embedded microcantilever structures with silicon nitride ( $\text{SiN}_x$ ) supporting layer are fabricated by a surface and bulk micromachining process. The substrates are 100 mm-diameter p-doped Si (100) wafers ( $525\pm 20\ \mu\text{m}$  thickness) covered with a 1  $\mu\text{m}$ -thick low stress  $\text{SiN}_x$  layer deposited by low pressure chemical vapor deposition (LPCVD). Then, the platinum layer with thickness of 150 nm, which functions as the bottom electrode is prepared by sputtering on a thin tantalum (Ta) adhesion layer of 30 nm. The PZT films are deposited with a thickness of 1  $\mu\text{m}$  using the diol-based sol-gel method. The PZT films are deposited by spin coating of a mixed PZT solution at  $1,536\times g$  for 30 s. The films are then heated at 400  $^\circ\text{C}$  for 5 min and annealed at 650  $^\circ\text{C}$  for 10 min. To assemble the metal-ferroelectric-metal (MFM) capacitor structure, a platinum layer is deposited as the top electrode by sputtering on the PZT layer. Using a multilayer deposited substrate, the microcantilevers are fabricated through photolithography and etching by repeating the patterning using masks for each layer. The platinum layer for the top electrode and PZT layers are etched using an advanced oxide etcher (AOE). After the top electrode and PZT layer are etched, a silicon dioxide ( $\text{SiO}_2$ ) thin film with thickness of 200 nm is deposited by plasma enhanced chemical vapor

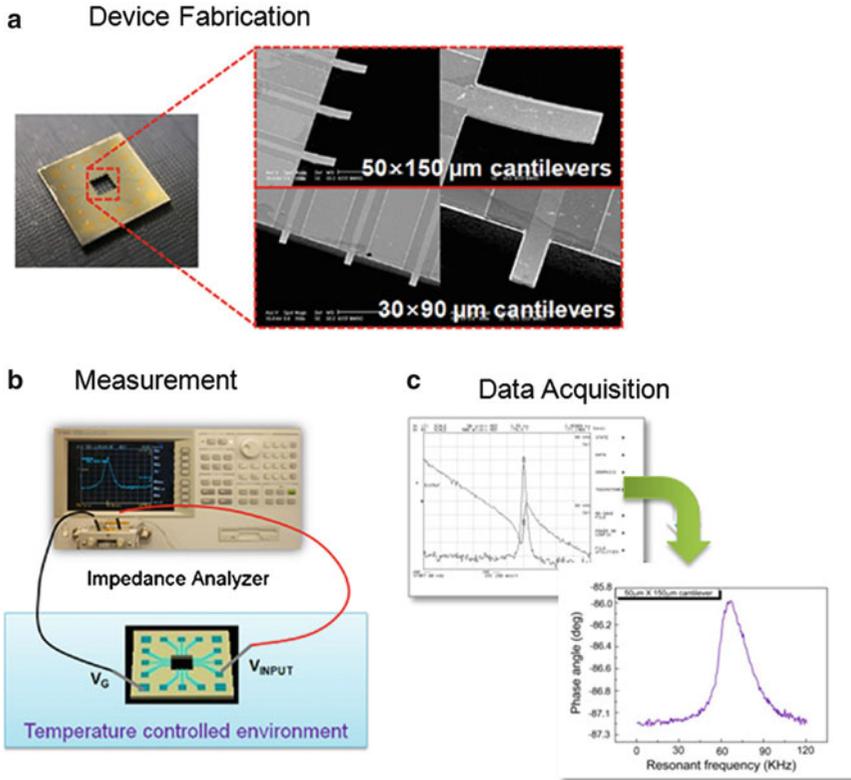


Fig. 3. (a) Photograph of the single device with 12 microcantilevers and SEM images of two types of microcantilevers (six microcantilevers of  $30\ \mu\text{m} \times 90\ \mu\text{m}$  and six microcantilevers of  $50\ \mu\text{m} \times 150\ \mu\text{m}$ ), (b) schematic diagram of the equipment (the impedance analyzer) used to measure the resonant frequency of the microcantilevers and (c) peak of resonant frequency obtained from the impedance signal. Reproduced from (10) with permission from Elsevier.

deposition (PECVD) to provide electrical insulation in the buffer solutions. The contact holes on the top and bottom electrode are then etched and connected through a gold lift-off process. Patterning of the bottom electrode is carried out with AOE. The back  $\text{SiN}_x$  window is patterned by reactive ion etching (RIE) and the bulk silicon is wet-etched using a KOH silicon etchant at a concentration of 30 %. Finally, the upper side of the  $\text{SiN}_x$  is etched by RIE to release the cantilever. A single device contains six cantilevers of  $30\ \mu\text{m} \times 90\ \mu\text{m}$  and six cantilevers of  $50\ \mu\text{m} \times 150\ \mu\text{m}$ .

2. Deposit a gold film freshly before capture probe immobilization. Otherwise, use the piranha solution: mix 15 mL of concentrated  $\text{H}_2\text{SO}_4$  and 5 mL of 30 %  $\text{H}_2\text{O}_2$  in a 50 mL Pyrex beaker. Dip the gold-coated microcantilever devices into the solution for 5 min. Transfer the device to a Petri dish containing 2 mL water and shake it for 1 min on the orbital shaker (3 $\times$ ). Dry the device under a flow of nitrogen gas and transfer it to the capture probe solution as soon as possible.

3. The concentration of silica nanoparticles can be measured by weighing the solid pellet which is dried from 1 mL of the nanoparticles solution. The sample should be weighed at least five times and the average value should be regarded as the weight of the silica nanoparticles in 1 mL EtOH.
4. DNA of an HBV positive serum was extracted from 140  $\mu\text{L}$  serum using the QIAamp DNA Mini-Kit, and 4.5  $\mu\text{L}$  DNA sample was amplified in a 20  $\mu\text{L}$  reaction containing 0.5 U Taq polymerase and 1 $\times$  PCR buffer with 1.5 mM  $\text{MgCl}_2$ , 0.2 mM dNTP and 10 pmol of each primer (anti-sense: 5'-TCC CTG GAT GCT GG(G/A) TCT TCC AAA-3' and sense: 5'-CAT TGA CCC (C/T)AT AAA GAAT T-3'). PCR conditions were 5 min at 94  $^\circ\text{C}$  followed by 35 cycles of (45 s at 94  $^\circ\text{C}$ , 45 s at 56  $^\circ\text{C}$  and 45 s at 72  $^\circ\text{C}$ ) with a final extension step of 72  $^\circ\text{C}$  for 5 min. PCR product was purified using the QIAquick PCR purification kit and DNA concentration measured photometrically (1 OD = 50  $\mu\text{g}/\text{mL}$  DNA). Preparation of genomic DNA of clinical sera and subsequent HBV PCR were carried out accordingly.
5. The reaction should be performed in the dark place or the glass flask should be wrapped with aluminum foil.
6. Use a bath-type ultrasonicator to disperse the pellet well.
7. Keep the detection probe-conjugated silica nanoparticles in the dark at 4  $^\circ\text{C}$ .
8. Confirm the devices are fully dipped in the solution and the lid of the 48-well plate must be firmly sealed before incubation at 65  $^\circ\text{C}$ .
9. Store the devices in a drying desiccator before measuring the resonant frequency.
10. General measurement of resonant frequency: Measure the resonant frequency of the microcantilevers (30  $\mu\text{m} \times 90 \mu\text{m}$ ) using an impedance analyzer (4294A, Agilent Technologies, CA, USA), which monitors the phase angle change of impedance in the frequency domain. Induce an ac signal of 0.5  $V_{\text{pp}}$  (peak to peak) on the top and bottom electrode of the microcantilever. The scan range should be 3 kHz and the regime should be  $\pm 1.5$  kHz from the resonant frequency (see Fig. 3b, c).

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## Protocol for the Detection of *Treponema pallidum* in Paraffin-Embedded Specimens

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### Abstract

Formalin-fixed paraffin-embedded (FFPE) tissue blocks are routinely used for histopathological examination and are also useful for specific pathogen detection by polymerase chain reaction (PCR). FFPE tissue is stable at ambient temperature for an extended period of time and relatively easy to transport compared to fresh tissue, which has to be processed or frozen immediately. In addition, archival material is an invaluable source for retrospective molecular and clinical investigation. This chapter describes detailed procedures for nucleic acid extraction and PCR detection of *Treponema pallidum* using FFPE tissue.

**Key words:** Polymerase chain reaction, PCR, Real-time PCR, *Treponema pallidum*, FFPE, Paraffin-embedded specimen

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### 1. Introduction

Syphilis, caused by the spirochete *Treponema pallidum*, is a multi-stage sexually transmitted disease that continues to be a public health concern due to its increasing incidence worldwide. Serology is routinely used for syphilis testing with a sensitivity ranging from 70 to 100 % (1); however, an accurate diagnosis is dependent upon a combination of clinical presentation, patient history, serologic testing, and/or direct detection methods. Darkfield (DF) microscopy or PCR are most useful for the detection of *T. pallidum* in lesion exudates from patients with primary, secondary, and early congenital syphilis. DF and PCR are probably more sensitive than serology in very early syphilis infection before seroconversion occurs (2). DF is a simple yet effective technique for detection of *T. pallidum* in fresh lesion material. It can be useful in busy clinics and in resource poor settings but its sensitivity and specificity is usually low compared to PCR, especially if the microscopist is relatively inexperienced (2, 3). PCR is more versatile and can be

used to detect treponemal DNA extracted from FFPE tissue, CSF, blood, and other specimens (4–14). Thin-sections of FFPE tissue from secondary, tertiary, or neuro-syphilis are often used to visualize spirochetes by histologic or immunohistochemical methods (11, 15–18), which include silver staining (Warthin-Starry or Steiner method), direct fluorescent antibody tissue test for *T. pallidum* (DFAT-TP), and immunohistochemistry (IHC). IHC using a monoclonal antibody specific for *T. pallidum* has been reported to be more sensitive and specific than silver staining for detection of spirochetes in FFPE tissue (17). PCR is becoming increasingly common for the laboratory diagnosis of syphilis because of its high sensitivity and specificity. While it is regarded as the new gold standard for the detection of *T. pallidum*, there is no commercial PCR test for syphilis, but a number of *in-house* conventional PCR and real-time PCR tests have been applied to various types of specimen (3–14, 16). This chapter describes the methodology for a diagnostic real-time PCR for *T. pallidum* using FFPE specimens. Because some laboratories may not have real-time PCR capability, a conventional method is also described.

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## 2. Materials

The ChargeSwitch gDNA Tissue Kit (Invitrogen) or the QIAamp DNA Mini Kit (Qiagen) is used for DNA extraction from FFPE specimens. These kits can also be used for unfixed tissue specimens. All reagents in the kits are stored at room temperature except proteinase K from the Invitrogen Kit, which is stored at 4 °C. PCR reagents, especially *Taq* polymerase must be stored at –20 °C in a non-frost free freezer. Hazardous waste should be disposed in accordance with local waste disposal regulations. Additional information on specific chemicals can be obtained from the material safety data sheets provided by the product supplier.

### 2.1. DNA Extraction by QIAamp DNA Mini Kit (See Note 1)

1. QIAamp DNA Mini Kit.
2. Water bath or dry bath at 55 °C/56 °C, and 70 °C.
3. 10–25 mg tissue.
4. Sterile 1.5- to 2.0-mL microcentrifuge tubes.
5. Sterile, disposable Petri dishes.
6. Sterile, disposable scalpels.
7. Vortex or shaking water bath.
8. Microcentrifuge.
9. Ethanol (96–100 %).
10. Phosphate-buffered saline (PBS).

**2.2. DNA Extraction by ChargeSwitch gDNA Mini Tissue Kit (See Note 1)**

1. ChargeSwitch gDNA Mini Tissue Kit.
2. MagnaRack or other magnetic separation rack.
3. Water bath or dry bath at 55 °C/56 °C, and 70 °C.
4. 10–25 mg tissue.
5. Sterile 1.5- to 2.0-mL microcentrifuge tubes.
6. Sterile, disposable Petri dishes.
7. Sterile, disposable scalpels.
8. Vortex.
9. Microcentrifuge.

**2.3. Real-Time TaqMan PCR (See Note 2)**

All PCR reagents must be kept on ice during master mix preparation.

1. DNA samples.
2. Positive control (DNA from a laboratory strain of *T. pallidum*).
3. *T. pallidum* DNA target: DNA polymerase I gene (*polA*, *tp0105*).
  - (a) Forward primer: 5'CAGGATCCGGCATATGTCC3'.
  - (b) Reverse primer: 5'AAGTGTGAGCGTCTCATCATTCC3'.
  - (c) Probe: 5'FAM-CTGTCATGCACCAGCTTCGACGTCTT-BHQ3'.
4. Internal DNA control (human ribonuclease P gene, *RNase P*).
  - (a) Forward primer: 5'CCAAGTGTGAGGGCTGAAAAG3'.
  - (b) Reverse primer: 5'TGTTGTGGCTGATGAACATAAAAGG3'.
  - (c) Probe: 5'CY5-CCCCAGTCTCTGTCAGCACTCCCTTC-BHQ3'.
5. DNA polymerase (5 U/μL, AmpliTaq Gold, Applied Biosystems).
6. Deoxyribonucleoside 5'-triphosphate mix with dUTP (2.5 mM of dATP, dCTP, dGTP, and 5.0 mM dUTP (Applied Biosystems)).
7. AmpErase Uracil-*N*-glycosylase (1 U/μL, UNG).
8. 10× PCR buffer (supplied with *Taq* polymerase).
9. Magnesium chloride (25 mM, supplied with *Taq* polymerase).
10. DNase and RNase-free water.
11. 0.2 mL microcentrifuge tubes (flat top, thin walled).
12. Real-time PCR instrument (see Note 3).

**2.4. Conventional PCR**

1. DNA samples.
2. Positive control (DNA from a laboratory strain of *T. pallidum*).
3. DNA target: DNA polymerase I gene (*polA*, *tp0105*).

4. Forward (5'TGCGCGTGTGCGAATGGTGTGGTC3') and reverse (5'CACAGTGCTCAAAAACGCCTGCACG3') primers.
5. DNA polymerase (3.5 U/ $\mu$ L, Expand high-fidelity *Taq* polymerase, Roche Diagnostics).
6. PCR Nucleotide Mix (10 mM each of dATP, dCTP, dGTP, and 30 mM dUTP).
7. Uracil-*N*-glycosylase (1 U/ $\mu$ L, UNG).
8. PCR reaction buffer with 15 mM MgCl<sub>2</sub> (supplied with DNA polymerase).
9. 0.2 mL microcentrifuge tubes.
10. Thermocycler (see Note 4).

### **2.5. Agarose Gel Electrophoresis**

1. Agarose.
2. SYBR<sup>®</sup> Gold Nucleic Acid Gel Stain (10,000 $\times$  in DMSO, Invitrogen Corp.) (see Note 5).
3. 50-bp DNA size marker (1  $\mu$ g/ $\mu$ L).
4. 10 $\times$  Loading buffer (0.3 % (w/v) bromophenol blue).
5. TBE buffer (89 mM Tris-base, 89 mM boric acid, 1 mM ethylenediaminetetraacetic acid [EDTA], pH 8.0).
6. TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5–8.0).
7. Power source.
8. UV transilluminator.

### **2.6. Agilent 2100 Bioanalyzer**

1. Agilent DNA 1000 kit.
2. Sterile deionized water.
3. Vortex with DNA chip adapter (see Note 6).
4. Agilent 2100 Bioanalyzer instrument.

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## **3. Methods**

For isolation of genomic DNA from *paraffin-embedded tissue* using the QIAamp DNA Mini Kit, the use of xylene extraction protocol is not necessary. For isolation of genomic DNA from *formalin-fixed tissues*, wash tissue samples twice in PBS at room temperature to remove fixative first and process samples for DNA extraction according to the steps in Subheading 3.1. Extracted DNA should be used immediately or placed at 4 °C if PCR is performed on the same day—otherwise, samples should be stored at –20 °C or –70 °C. Avoid repeated freezing and thawing as this will lead to degradation of the DNA.

### 3.1. DNA Extraction by QIAamp DNA Kit

1. Weigh an empty sterile 1.5 mL microcentrifuge tube using an analytical balance with a precision of  $\pm 1$  mg.
2. Excise a small piece of tissue and place it in the microcentrifuge tube. Determine the weight of tissue by subtracting the weight of microcentrifuge tube from the total weight. Use up to 25 mg of tissue for extraction.
3. Transfer the tissue into a sterile Petri dish and use a sterile disposable scalpel to cut up the tissue into tiny pieces. Transfer the tissue pieces back into the tube, and add 180  $\mu$ L of Buffer ATL.
4. Add 20  $\mu$ L proteinase K (20 mg/mL, not protease), mix briefly by vortexing, and incubate at 56 °C until the tissue is completely lysed. Vortex occasionally during incubation to disperse the sample, or place in a shaking water bath or on a rocking platform. Lysis usually takes 1–3 h but overnight incubation may be necessary in some cases.
5. Briefly centrifuge the 1.5 mL microcentrifuge tube to remove drops from inside of the lid.
6. Add 200  $\mu$ L Buffer AL to the sample, mix by pulse-vortexing for 15 s, and incubate at 70 °C for 10 min. Briefly centrifuge the 1.5 mL microcentrifuge tube to remove drops from inside the lid. The white precipitate does not interfere with subsequent steps.
7. Add 200  $\mu$ L ethanol (96–100 %) to the sample and mix by pulse-vortexing for 15 s.
8. After mixing, briefly centrifuge the 1.5 mL microcentrifuge tube to remove drops from inside the lid.
9. Carefully apply the mixture from step 6 (including the precipitate) to the QIAamp Mini spin column (in a 2 mL collection tube) without wetting the rim. Close the cap, and centrifuge at  $6,000 \times g$  (8,000 rpm) for 1 min. Place the QIAamp Mini spin column in a clean 2 mL collection tube (provided) and discard the tube containing the filtrate.
10. Carefully open the QIAamp Mini spin column and add 500  $\mu$ L Buffer AW1 without wetting the rim. Close the cap and centrifuge at  $6,000 \times g$  (8,000 rpm) for 1 min.
11. Place the QIAamp Mini spin column in a clean 2 mL collection tube and discard the collection tube containing the filtrate.
12. Carefully open the QIAamp Mini spin column and add 500  $\mu$ L Buffer AW2 without wetting the rim. Close the cap and centrifuge at full speed ( $20,000 \times g$ ; 14,000 rpm) for 3 min.
13. Place the QIAamp Mini spin column in a new 2 mL collection tube and discard the old collection tube with the filtrate. Centrifuge at full speed for 1 min to ensure that there is no carryover of Buffer AW2.

14. Place the QIAamp Mini spin column in a clean 1.5 mL microcentrifuge tube and discard the collection tube containing the filtrate. Carefully open the QIAamp Mini spin column and add 200  $\mu$ L Buffer AE. Incubate at room temperature for 5 min and then centrifuge at  $6,000\times g$  (8,000 rpm) for 1 min. The eluted DNA can be used immediately for PCR or stored at  $-20\text{ }^{\circ}\text{C}$  until needed (see Note 7).

**3.2. DNA Extraction  
by ChargeSwitch  
gDNA Mini Tissue Kit**

1. Weigh and cut up tissue as described in Subheading 3.1 (steps 1–3).
2. Add 1 mL of lysis buffer (L13) to the microcentrifuge tube containing tissue pieces.
3. Add 20  $\mu$ L proteinase K (20 mg/mL), mix by vortexing or inverting for 10–15 s, and incubate at  $55\text{ }^{\circ}\text{C}$  until the tissue is completely lysed. Perform the lysis overnight if necessary.
4. Vortex the tube containing the ChargeSwitch magnetic beads to fully resuspend and submerge them in the storage buffer.
5. Add 120  $\mu$ L of beads to the digested tissue sample and pipette up and down gently five times to mix. Use a 1 mL pipette tip set to 900  $\mu$ L to mix the sample. Avoid creating bubbles as this may result in shearing of the DNA.
6. Add 100  $\mu$ L of purification buffer (N5) and pipette up and down gently ten times to mix.
7. Place the sample in the MagnaRack for 2 min or until the beads have formed a tight pellet and the supernatant is clear.
8. Without removing the tube from the MagnaRack, carefully remove the supernatant and discard. Take care not to disturb the pellet.
9. Remove the tube containing the pellet from the MagnaRack and add 1 mL of wash buffer (W12). Pipette up and down gently twice to resuspend the beads.
10. Place the sample in the MagnaRack for 1 min or until the beads have formed a tight pellet.
11. Repeat steps 8–10.
12. Without removing the tube from the MagnaRack, carefully remove the supernatant and discard. Take care not to disturb the pellet.
13. Remove the tube containing the pellet from the MagnaRack and add 60–80  $\mu$ L of elution buffer (E5) or TE buffer, pH 8.5 to the tube. Pipette up and down gently twice to resuspend the beads (see Note 8).
14. Incubate at  $55\text{ }^{\circ}\text{C}$  for 5 min and place the sample in the MagnaRack for 2 min or until the beads have formed a tight pellet.
15. Leave the tubes in the MagnaRack but carefully remove the supernatant containing the DNA and transfer to a sterile

microfuge tube. Take care not to disturb the pellet. Discard the used beads.

### 3.3. Real-Time PCR

1. Set up a 25  $\mu\text{L}$  reaction volume containing 2  $\mu\text{L}$  of dNTPs, 5  $\mu\text{L}$  of  $\text{MgCl}_2$ , 0.5 U UNG, 5 U of AmpliTaq Gold polymerase, 2.5  $\mu\text{L}$  of 10 $\times$  PCR buffer, a final concentration of 300 nM of each primer and 200 nM of probe for the *T. pallidum* target, 80 nM of each primer and probe for *RNase P*, and 10  $\mu\text{L}$  of template DNA.
2. Include a positive and negative (no template) control in each run.
3. Initial hold cycle: 50  $^\circ\text{C}$  for 2 min. This allows UNG to digest any carryover product from previous PCR amplifications (see Note 9).
4. Initial denaturation: 95  $^\circ\text{C}$  for 10 min (see Note 10).
5. The following steps are repeated for 45 cycles:

Denaturation at 95  $^\circ\text{C}$  for 20 s.

Primer annealing and extension at 60  $^\circ\text{C}$  for 1 min for TaqMan probe displacement and cleavage to release the fluorescent signal.

6. Interpretation of results:

*T. pallidum*-positive sample: A positive fluorescent signal indicated by a threshold cycle (Ct) value above the manually set cut-off in both the FAM and CY5 channels (see Note 11).

*T. pallidum*-negative sample: No fluorescent signal or Ct value in the FAM channel but having a positive fluorescent signal in the CY5 channel.

Invalid result: No fluorescent signal in the CY5 channel indicates the possibility of PCR inhibition and/or inadequate sample. Repeat DNA extraction and real-time PCR testing.

### 3.4. Conventional PCR (Follow This Protocol if a Real-Time PCR Instrument and Reagents Are Unavailable)

1. Set up a 50  $\mu\text{L}$  reaction containing 5  $\mu\text{L}$  of 10 $\times$  PCR buffer, 1  $\mu\text{L}$  (50 nM final concentration) of forward and reverse primer, 1  $\mu\text{L}$  of PCR Nucleotide Mix<sup>PLUS</sup>, 1 U of UNG, 10  $\mu\text{L}$  of template DNA, and 1.75 U of Expand *Taq* polymerase in a sterile 0.2 mL PCR tube.
2. Transfer the tubes to a thermocycler and run the PCR using the following conditions:
  - (a) Initial hold cycle: 50  $^\circ\text{C}$  for 5 min.
  - (b) Initial denaturation: 94  $^\circ\text{C}$  for 5 min.
  - (c) 45 Cycles of:
    - Denaturation at 94  $^\circ\text{C}$  for 1 min.
    - Primer annealing at 65  $^\circ\text{C}$  for 1 min.
    - Primer extension at 72  $^\circ\text{C}$  for 1 min.

- (d) Final extension at 72 °C for 7 min.
- (e) The reaction should be held at 4 °C until removed from the cycler.

### **3.5. Agarose Gel Electrophoresis of PCR Products**

1. Prepare a 1.5 % (w/v) agarose gel by adding 1.5 g of agarose to 100 mL of TBE (see Note 12).
2. Heat in a microwave until boiling.
3. Leave to cool for 2–3 min or place in a 50 °C water bath for 5 min.
4. Swirl the mixture gently without creating air bubbles.
5. Insert a gel comb into a gel tray and pour the agarose.
6. Wait approximately 30–40 min for the agarose to solidify.
7. Fill the electrophoresis tank with TBE buffer.
8. Insert the gel tray into the tank and gently remove the comb.
9. Mix 5  $\mu\text{L}$  of PCR product with 1.5  $\mu\text{L}$  of loading buffer and 1  $\mu\text{L}$  SYBR Gold stain (Make a 1:5,000 or 1:10,000 dilution of the 10,000 $\times$  concentrate in TE or TBE buffer).
10. Load samples onto the gel.
11. Add 0.5  $\mu\text{L}$  of DNA size marker (1  $\mu\text{g}/\mu\text{L}$ ) and 1  $\mu\text{L}$  of SYBR Gold stain (diluted as indicated above) to 1.5  $\mu\text{L}$  of loading buffer.
12. Load the mixture in one of the outer wells of the gel.
13. Connect the electrophoresis tank to the power source. Set the voltage to 100 V and let it run for 45–60 min (see Note 13).
14. Remove the gel and place on a UV transilluminator. Identify the 377-bp PCR product by comparing to the DNA size marker.

### **3.6. Analysis of PCR Products on an Agilent 2100 Bioanalyzer (See Note 14)**

1. Remove reagents from the fridge and allow to equilibrate to room temperature for 30 min (see Note 15).
2. To prepare the gel-dye mix, add 25  $\mu\text{L}$  of the dye concentrate to a DNA gel matrix vial (red top tube) (see Note 16).
3. Vortex the solution well, centrifuge briefly and transfer to a spin filter (supplied with Agilent 1000 kit).
4. Centrifuge the spin filter at  $2,240 \times g \pm 20\%$  for 15 min. Protect the solution from light.
5. Put a new DNA chip on the chip priming station.
6. Pipette 9  $\mu\text{L}$  of gel-dye mix in the well marked with a G in a black circle  (see Note 17).
7. Make sure that the plunger is positioned at the 1 mL mark then close the chip priming station.

8. Press the plunger smoothly and firmly until it is held by the clip (see Note 18).
9. Wait for exactly 60 s then release clip.
10. Wait for 5 s. Slowly pull back the plunger to the 1 mL position.
11. Open the chip priming station and pipette 9  $\mu$ L of gel-dye mix in the wells marked "G." 
12. Pipette 5  $\mu$ L of marker (green top tube) in all 12 sample wells.
13. Pipette 1  $\mu$ L of DNA ladder (yellow top tube) in the well marked with a ladder symbol. 
14. Pipette 1  $\mu$ L of sample in each of the 12 wells. Add 1  $\mu$ L of deionized water to unused wells.
15. Put the chip horizontally in the adapter and vortex (provided with Agilent 2100 Bioanalyzer) for 1 min at the indicated setting (2,400 rpm).
16. Run the chip in the Agilent 2100 Bioanalyzer within 5 min.
17. Identify the 377-bp PCR product by comparing the size of the PCR amplicon to the DNA ladder.

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#### 4. Notes

1. We have not compared the performance of the QIAamp DNA mini kit (silica membrane-based) and the ChargeSwitch gDNA Tissue Kit (magnetic bead-based) for DNA extraction; however, the DNA quality using both methods should be comparable. The QIAamp DNA FFPE Tissue Kit (Qiagen) is also specifically designed for extraction of DNA from FFPE specimens; however, we have not evaluated it for *T. pallidum* testing. The kit uses special lysis conditions to release DNA from tissue sections and to overcome inhibitory effects caused by formalin cross-linking of nucleic acids.
2. The limit of detection of this PCR assay is 1–10 *T. pallidum* genomic copies per reaction. Both the real-time and conventional PCR assays described here cannot distinguish *T. pallidum* subsp. *pallidum* (venereal syphilis) from the causes of non-venereal treponematoses (*T. pallidum* subsp. *pertenue*, and subsp. *endemicum*).
3. The real-time TaqMan assay is run on a Rotor-Gene 6000 (Qiagen). If a different instrument or fluorescent reporter molecule is used, additional validation or optimization may be required. We highly recommend the use of an internal control (e.g., human *RNase P* gene) to monitor PCR inhibition.

4. Conventional PCR is run on an Applied Biosystems 9700 thermocycler. Use of a different instrument may require minor optimization.
5. No data are available addressing the mutagenicity or toxicity of SYBR<sup>®</sup> Gold nucleic acid gel stain; however, because this reagent binds to nucleic acids, it should be treated as a potential mutagen and handled appropriately.
6. Make sure that the sample chip fits snugly into the adapter; otherwise, it may come loose while vortexing.
7. Use of a 200  $\mu$ L volume of Buffer AE for elution will increase the efficiency. Elution with a smaller volume (50–150  $\mu$ L) will increase the DNA concentration but might decrease the overall yield.
8. Use the recommended volume of E5 (a minimum volume of 60  $\mu$ L, Mini Kit) to ensure that the magnetic beads are in suspension during elution. Use of water for elution is not recommended because the elution efficiency will be low due to its poor buffering capacity.
9. We highly recommend the use of UNG to prevent carryover contamination from previous PCR amplification products. In the event that UNG is not used in the reaction, the initial PCR hold cycle of 50 °C for 2 or 5 min can be omitted. The master mix should preferably be prepared in a designated PCR clean room.
10. This step is to activate AmpliTaq Gold<sup>®</sup> DNA polymerase and simultaneously inactivate the UNG.
11. The Ct value is where the threshold line crosses the amplification curve. By setting a threshold line, the Ct value for each sample is established. Depending on the instrument, the threshold can be set manually and/or automatically. In addition, the Ct value can also be affected by software features of different instruments for data normalization and analysis. Regardless of fluorescent signal strength, the presence of a Ct value indicates a positive PCR.
12. Add the weighed agarose to the required volume of TBE buffer rather than the other way around. This will prevent clumping and make microwaving easier.
13. The 377-bp product can be monitored by observing the bromophenol blue (loading dye) which migrates at the same distance as a 400-bp DNA fragment in a 1.5 % agarose gel.
14. PCR products can be analyzed on an Agilent 2100 Bioanalyzer as an alternative to agarose gel electrophoresis.
15. The DNA dye concentrate and dye mixture (gel-dye mix) should be kept in the dark since the dye decomposes when exposed to light.

16. The prepared gel-dye mix can be used for 1 month but must be stored at 4 °C away from light.
17. Always insert the pipette tip to the bottom of the well when dispensing gel-dye mix or reagents. Also, the gel-dye mix is very viscous and should be dispensed slowly to avoid creating air bubbles.
18. Make sure that the syringe clip in the chip priming station is at the lowest setting. Avoid sudden release of the plunger, once pressed, as this may cause the gel-dye mix to get onto the o-ring or into the chip priming station, which may affect future runs.

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# Chapter 21

## Guidelines for the Use of Molecular Biological Methods to Detect Sexually Transmitted Pathogens in Cases of Suspected Sexual Abuse in Children

Margaret R. Hammerschlag and Charlotte A. Gaydos

### Abstract

Testing for sexually transmitted infections (STIs) in children presents a number of problems for the practitioner that are not usually faced when testing adults for the same infections. The identification of an STI in a child, in addition to medical implications, can have serious legal implications. The presence of an STI is often used to support the presence or allegations of sexual abuse and conversely, the identification of an STI in a child will prompt an investigation of possible abuse. The significance of the identification of a sexually transmitted agent in such children as evidence of possible child sexual abuse varies by pathogen.

While culture has historically been used for the detection of STIs in cases of suspected abuse in children, the increasing use of nucleic acid amplification tests (NAATs) in adults and the increasing proliferation of second-generation tests with better sensitivity and specificity has made inroads into the use of such tests in children, especially for diagnostic and treatment purposes. Acceptance by the medicolegal system for sexual abuse cases is still controversial and more test cases will be necessary before definitive use becomes standard practice. In addition, if these assays ever become legally admissible in court, there will be recommendations that more than one NAAT assay be used in order to assure confirmation of the diagnostic result.

**Key words:** *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, Nucleic acid amplification tests, Polymerase chain reaction, Sexual abuse, Strand displacement amplification, Transcription-mediated amplification, *Trichomonas vaginalis*

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### 1. Introduction

The introduction of NAATs for detection of *Neisseria gonorrhoeae* and *Chlamydia trachomatis* has been a major advance in the diagnosis of STIs. Use of NAATs has supplanted standard culture methods for *N. gonorrhoeae* and *C. trachomatis* in many laboratories. Currently there are four commercially available NAATs for *N. gonorrhoeae* and *C. trachomatis*: polymerase chain reaction (PCR) (Amplicor, Roche Molecular Diagnostics, Indianapolis, IN), strand displacement

amplification (SDA) (ProbeTec, Becton-Dickson, Inc., Sparks, MD), transcription-mediated amplification (TMA) (Aptima C2, GenProbe Inc., San Diego, CA), and real-time m2000 PCR (Abbott Molecular Diagnostics, Des Plaines, IL) (1–4). NAATs are currently recommended by Centers for Disease Control and Prevention (CDC) as the diagnostic assays of choice for screening purposes (5). Use of such assays for sexual abuse is discussed in the new laboratory guidelines as useful in adults and potentially useful in children for diagnostic testing (5). Point-of-care tests (POC), which can be used by minimally trained healthcare workers, are not yet of sufficient sensitivity to be recommended for any screening or testing, but newer improved assays are under development. PCR, realtime PCR, and SDA are DNA amplification assays. TMA is an RNA amplification assay. All four assays have FDA approval for use in genital sites (cervix, vagina, self-collected vaginal swabs, and male urethra) and urine from adolescents and adults. None are currently approved for extragenital sites (pharynx or rectum) or have approval for any site in children. In the USA, since no company has sought FDA clearance for such sites, individual laboratories can perform independent verification for using amplification assays for testing such specimens for diagnostic purposes in order to remain Clinical Laboratories Improvement Act (CLIA) compliance (6). These diagnostic and controversial legal issues are discussed in this chapter (7).

### **1.1. NAATs for *Gonococcus***

Although NAATs offer several advantages over culture-based methods including higher sensitivity and enabling the use of non-invasive specimens (urine, vaginal swabs), these assays have some limitations, especially for detection of *N. gonorrhoeae*. The gonococcus (GC) has the capacity for genetic variation and recombination that can affect the genetic sequences that are targets for amplification, leading to potentially false-negative results. In addition *Neisseria* species also have the relatively rare characteristic of being fully competent for exogenous DNA uptake throughout their life cycle. This enables frequent horizontal interspecies exchange of genetic material between *Neisseria* species leading to false positive amplification results for gonorrhea, when nonpathogenic, commensal *Neisseria* species acquire gonococcal sequences and vice versa (8–10). All NAATs do not have the same performance parameters (11). PCR and SDA have both been demonstrated to have cross-reactivity with other *Neisseria* species including *N. cinerea*, *N. flavescens*, *N. lactamica*, *N. sicca*, and *N. subflava* (12). This has important diagnostic implications especially when testing extragenital sites including pharynx and rectum.

Several recent studies have examined the performance of NAATs for detection of *N. gonorrhoeae* from the pharynx and rectum, primarily in men who have sex with men (MSM). McNally et al. (13) reported that SDA had a low positive predictive value

(PPV) for oral (30.4 %) and rectal specimens (73.7 %) in a population of MSM in Australia. Similar findings were reported by Schachter et al. (14) comparing PCR, SDA, and TMA in oral and pharyngeal specimens from MSM in California. Although the sensitivities of the NAATs were better than culture for detection of *N. gonorrhoeae* from both sites, PCR had a specificity of 78.9 % with oropharyngeal swabs. Specificities of SDA and TMA were  $\geq 99.4$  % for both anatomic sites. The overall prevalence of pharyngeal and rectal gonococcal infection in this population was 8.3 and 8.2 %, respectively. Bachmann et al. (15) examined the performance of PCR, SDA, and TMA compared to culture in pharyngeal specimens from males and females  $\geq 15$  years of age who acknowledged performing fellatio or cunnilingus. PCR was found to have a specificity of 73.0 % compared to 96.3 % for SDA and 98.6 % for TMA. The prevalence of oral infection in this population was 7.0 % in men and 9.1 % in women. Ota et al. also compared the detection of *N. gonorrhoeae* and *C. trachomatis* in pharyngeal and rectal specimens using the BD Probetec ET system, the Gen-Probe Aptima combo2 assay and culture, demonstrating the superiority of NAATs over culture (16). These prevalences in MSM and attendees at STD clinics are significantly higher than one would observe in children being evaluated for suspected sexual abuse. Therefore, the PPV of a positive result is highly relevant and significant in such a sexual abuse population, since low prevalence in a population directly lowers the probability (PPV) of a positive result being correct.

To date, there have been three published studies that compared NAATs to *N. gonorrhoeae* culture in children being evaluated for suspected sexual abuse (17–19). All included urine specimens as well as vaginal swabs. Although the results of two of these studies suggested that the sensitivity of NAATs for *N. gonorrhoeae* was similar to culture, they both had several serious limitations (18, 19). The populations studied included adolescents up to 18 and 20 years of age. In a study by Girardet et al. (20), only 48 of 203 (23.7 %) of the children enrolled were prepubertal and only 13 were male. Kellogg et al. (19) only evaluated girls; 58.5 % were  $\geq 13$  years of age, a large proportion of whom reported consensual sexual activity. Both studies utilized ligase chain reaction (LCR, LCx, Abbott Diagnostics), which was taken off the market in 2002 due to specificity concerns in the detection of *N. gonorrhoeae* (21). Kellogg et al. also evaluated PCR in addition to LCR (19). Other study limitations included the failure to use an independent reference standard in estimating test performance, failure to separately analyze test performance by age and gender (when applicable). The prevalence of *N. gonorrhoeae* infection in both studies was low (1.9 and 3.2 %) reducing the precision of sensitivity estimates. The number of extragenital specimens was also too low to assess test performance at those sites. Black et al. (17) recently evaluated the use of SDA and TMA using urine and genital swabs vs. culture for

the diagnosis of *N. gonorrhoeae* and *C. trachomatis* in children, 0–13 years of age, evaluated for sexual abuse in four US cities. All children were tested at multiple sites for *N. gonorrhoeae* and *C. trachomatis* by culture and vaginal, urethral swabs and urine were also tested with SDA and TMA. Cultures of *N. gonorrhoeae* were performed at all sites using Thayer-Martin agar medium and positive results were confirmed at all sites by Gram stain, oxidase test, enzyme detection, and/or biochemical tests according to the site's standard protocol. Positive NAATs for *N. gonorrhoeae* were confirmed by an in-house PCR using an alternative target, the *HinfI* fragment of the 4.2-kb cryptic plasmid (22). Of the 536 participants with complete data, none of the male children ( $n=51$ ) were positive for *N. gonorrhoeae* by any test at any site. Of the 485 female participants with complete data, 16 (3.3 %) had a positive result for *N. gonorrhoeae* by any test: 12 (2.5 %) by culture, 14 (2.9 %) by vaginal NAAT, and 14 (2.9 %) by urine NAAT. All participants who had a positive vaginal culture for *N. gonorrhoeae* had positive urine NAATs. There were discrepant results in two cases (both SDA-positive and TMA-negative). One of these girls was positive in urine and negative by vaginal swab, the other was positive both by urine and swab. All SDA-positive results for *N. gonorrhoeae* were confirmed to be true positives by a species-specific *N. gonorrhoeae* PCR. Three girls had discrepant results by site: two were vaginal swab positive and urine negative; one was vaginal swab negative and urine positive.

The 2010 CDC STD Treatment Guidelines now recommend that NAATs can be used for detection of *N. gonorrhoeae* in vaginal swabs and urine from girls being evaluated for suspected sexual abuse. However, NAATs were not recommended for use in boys or extragenital specimens, as there are no supporting data. As some NAATs cross-react with other *Neisseria* species, it was also recommended that clinicians consult with an expert before selecting an assay for use in this population. Although confirmatory testing was not specifically mentioned, it was suggested that specimens be retained for further testing if necessary (23).

## **1.2. NAATs for *C. trachomatis***

As described previously in the section on gonorrhea, there are several FDA approved NAATs for the simultaneous detection of *N. gonorrhoeae* and *C. trachomatis*: PCR, SDA, and TMA. NAATs are currently approved by the FDA for detection of *C. trachomatis* from genital sites (cervix, vagina, urethra) and urine from adolescents and adults. None are approved for extragenital sites (pharynx or rectum) or have approval for any site in children. These methods have been found to have excellent sensitivity for detection of *C. trachomatis*, usually well above 90 %, in genital specimens and urine from adult men and women, while maintaining high specificity (14). A new genetic variant of *C. trachomatis* was discovered in Sweden in 2006, which was found to have a mutation in the

sequence of the cryptic plasmid at the target site for Roche PCR rendering the organism undetectable by this assay (24). Recent data from Sweden reports that this variant is now responsible for 20–65 % of all detected chlamydial infection in counties where PCR was used. So far, this variant appears to be limited primarily to Sweden with a few isolates being identified in Norway and Denmark. Spread of the variant in Sweden was associated with use of PCR as the NAAT for diagnosis of *C. trachomatis* infection. It has not yet been detected in the United States (25).

The recent multicenter study by Black et al. (17) mentioned above, also evaluated the use of SDA and TMA using urine and genital swabs (vagina and urethra) compared to culture for diagnosis of *C. trachomatis* in children, 0–13 years of age. Cultures for *C. trachomatis* were performed at the clinical or hospital laboratories of each center, according to their own standard protocols. All sites transported swab specimens at 4 °C for *C. trachomatis* culture in either commercial Chlamydial or viral transport medium. Culture protocols at all sites included the isolation of *C. trachomatis* in cycloheximide-pretreated McCoy cells, either in shell vials, 24-well, or 96-well tissue culture plates. The inoculated cell monolayers were incubated at 35–37 °C for 48–72 h followed by fixation with ethanol, methanol, or acetone. The fixed monolayers were stained to detect chlamydial inclusions with fluorescein-conjugated Chlamydia genus-specific or *C. trachomatis* species-specific monoclonal antibodies. One laboratory also performed a single passage of the inoculated cell monolayers onto a fresh monolayer after 48 h of incubation. The commercial NAAT tests were performed at the CDC (SDA and TMA). All samples were processed and tested according to manufacturer's protocols except for the TMA tests which were performed on previously frozen urine or swabs collected in the BD ProbeTec sample collection medium. Test results that were positive by SDA for *C. trachomatis* were confirmed using an in-house PCR targeting the *ompA* gene, performed at the CDC (26). Fifteen (3.1 %) of 485 female participants had a positive result for *C. trachomatis* by any test (7 [1.4 %] by culture; 11 [2.3 %] by vaginal NAAT; 13 [2.7 %] by urine NAAT). None of the male participants had any positive cultures or NAATs for *C. trachomatis*. All participants who had a positive vaginal culture for *C. trachomatis* also had positive urine NAAT. Two prepubertal female children had positive *C. trachomatis* cultures from rectal swab specimens, but negative vaginal swab specimens by both culture and NAATs, and negative urine NAATs. No other participants had positive rectal cultures. There were no discrepant results in any of the participants tested by two commercial NAATs for *C. trachomatis* (ProbeTec and Aptima Combo 2). All *C. trachomatis*-positive results were confirmed to be true positives by DNA sequence genotyping. When NAAT results were compared by the type of specimen, only one girl had a discrepant result for *C. trachomatis*

(vaginal swab negative, urine positive). The sensitivity of vaginal culture for *C. trachomatis* was 39 % in all girls studied ( $n=485$ ). In contrast, the sensitivities of urine and vaginal swab NAATs were 100 and 85 % in all female children, respectively, for detection of *C. trachomatis*.

The results of Black et al. (17) suggest that NAATs, specifically SDA and TMA, can be used for detection of *C. trachomatis* in girls being evaluated for suspected sexual abuse. However, the same limitations apply as for use of these assays for detection of *N. gonorrhoeae*: (1) as the prevalence of *C. trachomatis* in this population is low, confirmatory testing is necessary. (2) One cannot extrapolate from these results to other NAATs, specifically PCR and use in specimens other than vagina and urine in girls. (3) One cannot make any recommendations on the use of these assays in prepubertal boys. Performing a confirmatory NAAT may be problematic as most hospital laboratories only use one assay. Some of the more recently available commercial NAATs, such as TMA (Aptima Combo 2), offer an alternate target confirmation method that can be used on the same testing platform; however, there are no data on the use of this confirmatory test in this setting. Additional options include sending blinded specimens to an independent or reference laboratory for confirmation testing, confirming a NAAT-positive result by culture test (requires a separate, invasive specimen), or use of a second, alternate technology commercial NAAT (probably the preferred option). Confirmatory testing originally recommended by CDC is no longer recommended by CDC for routine genital samples from adults and sexually active adolescents being testing for routine screening and diagnostic testing (27). However, for cases of suspected sexual abuse, confirmatory testing by a second NAAT should be performed and the laboratory should always use a newer “second generation” NAAT with the highest sensitivity possible, preferably with a different target (11). The 2010 CDC STD Treatment Guidelines recommended that NAATs can be used to detect *C. trachomatis* in vaginal swabs and urine from girls being evaluated for suspected sexual abuse (23). However, NAATs were not recommended for use in boys or extragenital specimens, as there are no data. Specimens collected from children for forensic applications should be retained in the laboratory for purposes of additional testing, in accordance with local policies and procedures (see Notes 1–5).

### **1.3. NAATs for *T. vaginalis***

*Background.* Trichomonas infections, caused by the parasite *Trichomonas vaginalis*, are highly prevalent sexually transmitted infections (STIs) worldwide, with estimates of 7–8 million infections annually in the United States (28). As such, they represent the most common curable STI in young, sexually active women (29, 30). Trichomonas infections have been associated with poor reproductive outcomes such as low birth weight (LBW)

and premature birth (31, 32). However data on trichomonas infections in the setting of child sexual abuse are limited. Most published studies of STIs in sexually abused children have testing for *T. vaginalis* has been limited to girls presenting with vaginal discharge (20, 33–36).

Rarely *T. vaginalis* can be transmitted vertically from mother to infant (vaginal, urine) during parturition (37, 38). These infections may persist for several months after birth. Care should be taken in interpretation when trichomonads are reported present in urine specimens from children collected for another purpose. As the morphology of *Pentatrichomonas (Trichomonas) hominis*, a nonpathogenic intestinal flagellate, is very similar to that of *T. vaginalis*, care must be taken to make sure that specimens are not contaminated with fecal material which can occur with bagged urine specimens.

Trichomoniasis has primarily been screened for at gynaecologic visits in the antenatal or family planning setting by visualization of the trichomonads on wet mount microscopy, but this methods in relatively insensitive; culture is more sensitive, but not as sensitive as NAAT assays, which are not yet FDA cleared.

*POC assays.* There are several FDA-cleared POC tests available including the Affirm VP III Microbial Identification System (Becton Dickinson) test, which is a direct nucleic acid probe hybridization test for detection of *T. vaginalis*, *Gardnerella vaginalis*, and *Candida* spp. (39). It has been reported to have sensitivities of 80–90 % in adult women with vaginitis compared to culture as the reference standard. Culture, however, has now been demonstrated to be of lower sensitivity than NAAT assays (40, 41). The Affirm VP III has not been validated or approved for use in genital specimens from prepubertal girls or urethral specimens from men.

A relatively new POC test (Genzyme, Inc.), test shows promise with better sensitivity than wet preparation and culture, but it has not been studied in prepubertal girls (42).

*NAAT assays.* As previously mentioned, at present there is no FDA-cleared commercial NAAT assay for trichomonas. Several research PCR assays have been reported and appear to perform with sensitivities >90 %; greater sensitivity than wet preparation or culture (42–49). There is one commercially available analyte-specific reagent (ASR) based on TMA (GenProbe, Inc., San Diego, CA) that is FDA cleared for purchase of reagents, but not in a “kit” format (49). Clinical trials for FDA clearance of this NAAT assays have been performed and have been submitted to the FDA. More research is needed to ascertain how this test performs with samples from prepubertal girls and in men.

*Sensitivity comparisons using NAATs.* When comparisons have been performed with NAATs, the sensitivity and specificity of

wet-preparation microscopy for trichomonas have been estimated to be between 50–60 % and >90 %, respectively, whereas sensitivity and specificity of PCR for trichomonas have been shown to be both >90%, respectively (46, 47, 50). A study of multiple etiologies of cervicitis in STD clinics, using both a TMA-based research NAAT (49) and another research PCR-based NAAT (45) for trichomonas, demonstrated an overall prevalence of 15.3 % for trichomonas compared with 11.9 % using wet-preparation microscopy (51). Because of the lower sensitivity of the wet-preparation method for diagnosis of TV, a significant percentage of infections may be routinely missed, which is of concern in cases of suspected sexual abuse. The increased sensitivity of NAAT assays in women over traditional methods of diagnosis such as wet preparation is evidence for the need for more future research determining use of NAAT assays for the diagnosis of trichomonas.

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## 2. Materials

The materials for commercially available and FDA-cleared diagnostic tests are provided in the kits provided by the manufacturer. For NAAT assays, these include positive and negative controls, primers, polymerase enzyme, buffers, and reagents. For culture, additional supplies such as Thayer Martin plates for gonorrhea culture, as well as generic biochemical tests for gonorrhea and Diamonds media for trichomonas culture are required. Additional materials needed are consistent with standard laboratory supplies and include items such as the equipment platform for the particular commercial assay, in addition to standard laboratory supplies such as pipettes, tips, plastic ware and tubes, gloves, biohazard bags, and miscellaneous equipment such as plate washers, incubators, refrigerators, freezers, etc.

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## 3. Methods

Methods differ for each diagnostic test being processed (gonorrhea, chlamydia, or trichomonas) and are rigorously standardized for commercially available and FDA-cleared assays. Manufacturer's package inserts must be followed exactly according to the provided protocol. If manufacturer's directions are altered as in the case of using a chlamydia or gonorrhea for rectal samples for example, each laboratory must perform their own verification study and document it carefully in order to remain CLIA compliant for laboratory inspectors (see Notes 1–3) (6).

## 4. Notes

1. Troubleshooting may be necessary when assays are not performing correctly as when positive and negative controls for a particular kit are performing incorrectly. In cases such as these, companies have technical representatives, who will work with laboratory personnel to correct the problem.
2. One method to ascertain laboratory and assay expertise is to participate in proficiency surveys. Several of these exist and most laboratories are required by their institutions to participate. The surveys provided by the Clinical Association of Pathologists (CAP) are the most commonly used.
3. Another method to maintain laboratory and test excellence for amplified tests is to routinely perform “swipe” tests by collecting moistened swab samples from the environmental surfaces in the laboratory and equipment on a monthly basis and to run them in an amplified test that is in use to monitor for amplicon contamination in the environment. If any such samples test positive, rigorous cleaning is required and a repeat monitoring “swipe” test should be performed.
4. If cultures are being performed for any of the aforementioned organisms, proficiency can also be measured by participating in CAP surveys for the particular organism.
5. As always, for diagnostic laboratories, quality assurance measures should be in continual use for each diagnostic assay, continued laboratory staff proficiency should be maintained, and adequate training of new laboratory professionals is required.

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## Protocol for the Molecular Detection of Antibiotic Resistance Mechanisms in *Neisseria gonorrhoeae*

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### Abstract

Gonorrhoea is no longer an easily treatable ailment but rather is now a challenging disease in terms of antimicrobial resistance (AMR) with treatment options rapidly diminishing. The causative agent of gonorrhoea, *Neisseria gonorrhoeae*, has managed to develop resistance to almost every single drug used against it with the sole exception of extended spectrum cephalosporins. The situation is further exacerbated by the fact that not only are the rates of gonococcal infections on a steady rise globally, but tracking AMR is being undermined by the growing popularity of molecular methods at the expense of traditional bacterial culture in diagnostic laboratories. Recently, concerns have been raised over the emergence of a multi-resistant gonococci and the potential for untreatable gonorrhoea. Maintaining optimal epidemiological surveillance of gonococcal AMR remains an important aspect of gonorrhoea control. The development of molecular tools for tracking AMR in *N. gonorrhoeae* has the potential to further enhance such surveillance. In this chapter, we discuss nucleic acid amplification-based detection of AMR in gonorrhoea with a particular emphasis on chromosomal-mediated resistance to beta-lactam antibiotics.

**Key words:** Antibiotic, Resistance, Gonorrhoea, Beta-lactam, Culture independent resistance determination, DNA, NAAT, SYBR-green real time PCR, HRM (high resolution melting curve), Allele-specific PCR

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### 1. Introduction

*Neisseria gonorrhoeae* is an obligate human pathogen and the causative agent of gonorrhoea, the second most common sexually transmitted infection in the world. The most common manifestation of gonorrhoea is the acute urethritis involving the mucous membranes of the urethra and is generally present with mucopurulent discharge in males. In addition, the gonococcus can also cause pharyngitis, epididymitis, proctitis, arthritis, and disseminated gonococcal infection (DGI) among other afflictions. In females, untreated cervicitis may proceed to pelvic inflammatory disease (PID) (1).

According to World Health Organization (WHO) recommendations, only drugs with a cure rate of 95 % and above should

be used in treating gonorrhoea. Unfortunately, very few anti-gonorrhoeal drugs now fit that description. The first beta-lactam antibiotic used against gonorrhoea was penicillin, but was discontinued after a couple of decades due to widespread resistance and resultant treatment failures. Similar problems have since been encountered with quinolones and tetracyclines. Third-generation cephalosporins, including cefixime and ceftriaxone, are now the mainstay of anti-gonococcal therapy. However, as seen with other antibacterials, we are now beginning to witness what appears to be the emergence of resistance to third-generation cephalosporins. Marked increases in the minimum inhibitory concentration (MIC) of cefixime and ceftriaxone have been observed in gonococci from around the world. Albeit at low numbers, speculation is that these strains are the precursors to fully resistant gonococci (2–4).

### **1.1. Resistance to Beta-Lactam Antibiotics**

Penicillin resistance in *N. gonorrhoeae* is relatively well characterized and can either be (a) plasmid-mediated via the production of beta-lactamase enzymes or may be (b) chromosomally mediated resistance (CMR) via altered penicillin-binding proteins (PBP), over expression of the MtrCDE efflux pump through decreased activity of the *mtrR* repressor protein and reduced permeability of the *porB* outer membrane protein. Specific gonococcal markers of penicillin-CMR include L421P in PBP-1, 345A insertion in PBP-2, G45D in *mtrR*, an adenine deletion in the *mtrR* promoter affecting efflux pump activity and substitutions at positions 120 and 121, particularly G120K and A121D, of the *porB1b* protein. These mechanisms of CMR have limited influence on penicillin susceptibility on their own, but when present together can act synergistically to produce high level resistance (4, 5).

Although full-fledged resistance to a third-generation cephalosporin has not yet been reported in the gonococcus, current evidence indicates that raised MICs to cefixime and ceftriaxone are driven by similar mechanisms to that of chromosomally mediated penicillin resistance. Alterations in PBP-2 are currently a prime focus, particularly the mosaic PBP-2 which is thought to have arisen from recombination of PBP-2 proteins from several *Neisseria* species. Of further interest are substitutions in PBP-2, namely A501V, as well the adenine deletion in the *mtrR* promoter and substitutions at positions 120 and 121 of the *porB1b* protein (6–9). Of concern is a recent study demonstrating that a combination of A501V with mosaic PBP-2 can experimentally confer resistance to ceftriaxone (7).

### **1.2. Molecular Detection of Gonococcal Resistance Mechanisms**

Molecular characterization of AMR mechanisms of the gonococcus is a relatively new field which is garnering increasing interest due to increase in the popularity of NAAT tests and with decreasing availability of isolates. Rather than replacing phenotypic AMR surveillance, molecular AMR strategies should, in our opinion, be aimed at enhancing current surveillance methods. Such a system would

effectively complement the movement of diagnostic laboratories towards molecular methods while also addressing the current limitations of AMR surveillance, particularly in remote or otherwise difficult-to-reach populations.

In this chapter, we present a simple, rapid, and inexpensive method of detecting resistance markers in *N. gonorrhoeae* with a focus on CMR resistance to beta-lactam antibiotics. Key elements of this method include use of a simple heat-denaturation step for isolate preparation combined with an SYBR green-based real-time PCR approach mutation detection (10) (see Note 1).

### 1.3. Assay Designs

Two different SYBR green-based approaches were used for these protocols, comprising high resolution melting curve (HRM) analysis and allele-specific PCR (ASP).

HRM detects differences in melting curve characteristics of sequences based on their nucleotide composition (11). In these protocols, we use HRM to distinguish mutations in small PCR products (generally <50 bp) whereby forward and reverse primers are designed to directly flank the mutations of interest (10). For example, for the assay targeting the PBP2-345A insertion (PBP2-345HRM assay), the forward and reverse primers directly flanked the insertion site of the “CGA” codon. Thus, wild-type strains have a PCR product of 38 bp compared to 41 bp for strains with the insertion, enabling ready discrimination by HRM (Fig. 1).

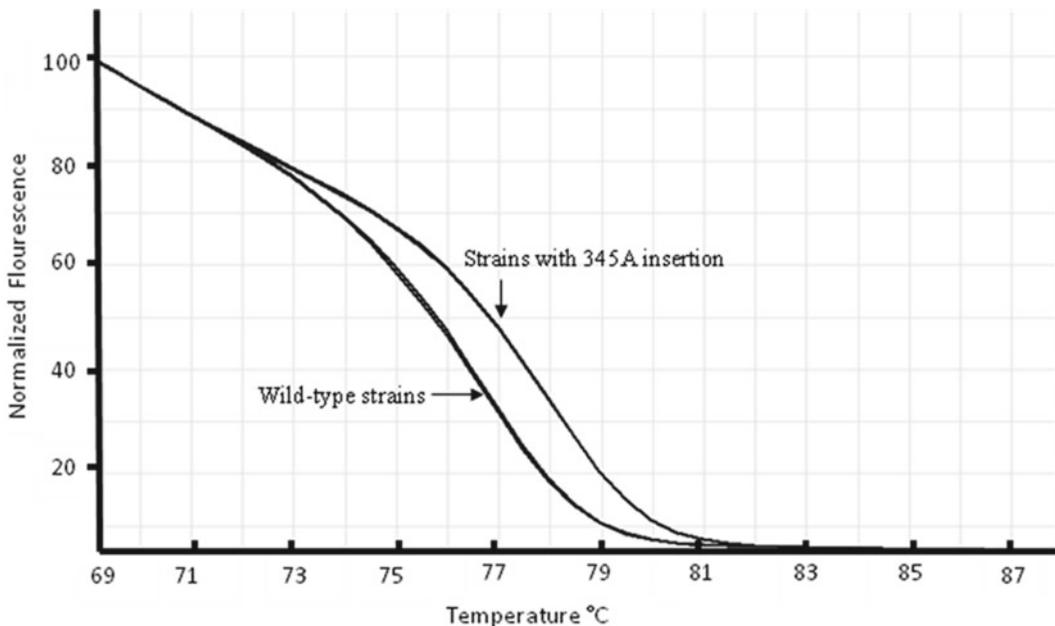


Fig. 1. An example of HRM analysis. PBP2-345HRM assay: wild-type strains can be readily distinguished from strains with the PBP2 345A codon insertion.

For the remaining HRM assays, the PCR product sizes were the same for both wild-type and mutant, but differences in GC content enabled HRM discrimination.

An ASP method was used to detect the adenine deletion in the *mtrR* promoter (10). This method used two real-time PCR reactions for each isolate. The first reaction (control reaction mix) used *mtrR*-Adel-ASP forward-1 primer and *mtrR*-Adel-ASP reverse primer (Table 1). The second reaction (test reaction mix) was performed as per the first mix except that a different forward primer (*mtrR*-Adel-ASP forward-2 primer; Table 1) was used. The primers from the “control reaction mix” were designed for a 100 % match to the *mtrR* promoter sequence of both wild-type and adenine-deletion gonococcal strains, whereas forward-2 primer from the “test reaction” possessed a two-base mismatch with strains harboring the adenine deletion (Fig. 2). These mismatches result in a delay in amplification (generally around 10 cycles) in the test reaction compared to the control reaction, whereas wild-type strains provide similar amplification characteristics in both mixes.

**Table 1**  
**Primers and control strains used for these assays**

Assay	Primers	Control strains (mutation)	References
<i>mtrR</i> -45HRM	Forward CCGGCGTAACGCGC Reverse TTTTCAAATGCCAATAGAGCGC	Wild-type (GGC) G45D (GAC)	(10)
PBP1-421-HRM	Forward GTGGTTCAAGAGCCGTTG Reverse GAAACCAAAGCCCCCTG	Wild-type (CTG) L421P (CCG)	(10)
PBP2-345HRM	Forward CGTCTCCCGTGCGCA Reverse CAAAGAGGGTAAACATGGGTA	Wild-type (no insertion) 345A insertion (codon CGA)	(10)
PBP2-501HRM	Forward CGGCGCTAAAACCGGTACG Reverse GCCATTGACCAGTTTGCGC	Wild-type (GCG) A501V (GTG)	(12)
<i>mtrR</i> -Adel-ASP	Forward-1 CATACACGATTGCACGGATAAAA Forward-2 TACACGATTGCACGGATAAAAAG Reverse CGTTTCGGGTCGGTTTGA	Wild-type (no deletion) Adenine deletion in <i>mtrR</i> promoter	(10)
porB-120/1HRM	Forward GTGGTTCAAGAGCCGTTG Reverse GAAACCAAAGCCCCCTG	GS (GGCAGC) DA (GACGCC) KG (AAGGGC) KD (AAGGAC) KN (AAGAAC) NA (AACGCC) GA1 (GGTGCC) GA2 (GGCGCC)	(10)

```

Forward-2 primer          TACACGATTGCACGGATAAAAAG
                          |||
Wild-type strain          TCATTATACATACACGATTGCACGGATAAAAAGTCTTTT
                          |||
                          |||
Forward-2 primer          TACACGATTGCACGGATAAAAAG
                          |||
Adenine-deletion strain  TCATTATACATACACGATTGCACGGATAAAAAGTCTTTT
                          |||
                          |||

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Fig. 2. The forward-2 primer used in the *mtrR*-Adel-ASP test reaction had 100 % match with wild-type strains but two mismatches with strains harboring the adenine deletion.

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## 2. Materials

### 2.1. Preparation of Bacterial Lysates

1. Heating block.
2. 1.5 mL tubes.
3. Saline; 0.9 % sodium chloride solution (Baxter, Australia).
4. Chocolate agar plates (bioMérieux, Australia).
5. Reference controls: Appropriate heat-denatured isolate controls of known sequence type as determined by DNA sequencing were included for all test runs. These are summarized in Table 1 and were prepared and stored as per the isolate preparation protocol. For all assays, excluding the porB-120/1HRM assay, only two isolates were used; representing one of each sequence type. Eight controls were used for the porB-120/1HRM assay (see Note 2).
6. Negative controls: Negative controls should be used in all test runs. These are prepared as per the isolate preparation protocol except that no colonies are added to the saline. We recommend that all test runs comprise at least 5 % negative controls. For example, a run of 72 reactions on a RotorGene instrument should comprise at least four negative controls (see Note 3).

### 2.2. Reaction Mix

1. SYBR green qPCR SuperMix-UDG (Invitrogen, Australia).
2. Qiagen Quantitect SYBR kit (Qiagen, Australia).
3. Forward and reverse primers (see Table 1).
4. 0.1 mL RotorGene tubes (Qiagen, Australia).
5. PCR-grade water.

### 2.3. Real-Time PCR

RotorGene Q instrument and software (Qiagen, Australia) (see Note 4).

### 3. Methods

#### 3.1. Preparation of Bacterial Lysates

By eliminating the need for a commercial nucleic acid extraction step from the protocol, we were able to avoid a time consuming and costly step from the method. Here, we employed a simple method of heat denaturing isolates to release DNA.

1. For each isolate, 3–6 colonies from a pure 24 h culture on chocolate agar were suspended in 1.0 mL of saline in a 1.5 mL tube.
2. This suspension was heated at 100 °C for 8 min.
3. The suspensions were then ready for testing (see Note 5).

#### 3.2. Reaction Mix

A standard reaction mix protocol using the SYBR green qPCR SuperMix-UDG (Invitrogen, Australia) was used for all assays except the PBP2-501HRM assay (see Note 6). Also, a single PCR reaction was used for all assays except the *mtrR*-Adel-ASP method (see Note 7). Reactions were prepared in batches of 80 tests for each test run.

1. Each batch contained 800 µL of 2× SYBR green qPCR SuperMix-UDG (Invitrogen), 80 µL of forward and reverse primers (at 1.0 µM) and made up to a total volume of 1.8 mL with water (see Note 8). Primers for each assay are described in Table 1.
2. Each test run comprised 72× 0.1 mL RotorGene tubes (Qiagen, Australia).
3. A total of 18.0 µL of reaction mix and 2.0 µL of heat-denatured lysate of isolate or control was added to each 0.1 mL tube.

#### 3.3. Real-Time PCR

##### 3.3.1. Real-Time PCR Cycling

Cycling was performed on a RotorGene instrument (Qiagen, Australia).

1. PCR was performed using the following conditions for all assays except the PBP2-501HRM assay (see Note 9): an initial enzyme activation step at 95 °C for 2 min, followed by 40 cycles of denaturation at 95 °C for 10 s, and primer annealing and elongation at 60 °C for 30 s, with fluorescence signal read on the green channel at the 60 °C step.
2. HRM analysis was performed on all reactions except the ASP reactions using the following conditions: post-PCR cycling, reaction mixes were continuously analyzed for one cycle from 60 to 95 °C with temperature increments of 0.5 °C/s.

##### 3.3.2. Data Analysis

HRM results were determined using the RotorGene HRM software (Qiagen, Australia).

1. For each assay, the respective controls from Table 1 were used as reference strains by specifying them as “genotypes” in the “HRM Analysis” tool.
2. “Normalisation regions” were set at 69 and 71 °C for the leading range and 86 and 88 °C for the trailing range.
3. “Confidence percentage” threshold was set at 90 %.
4. Results are then provided by the HRM results tool.
5. Samples for which genotype results are determined by the software (i.e., genotype at or more than 90 % confidence) are considered valid.
6. Samples providing a calling of “variation” (i.e., genotype <90 % confidence) are considered invalid and need to be resuspended and repeated.

ASP results were determined on the basis of cycle threshold (Ct) values as determined using the “Quantitation” tool.

1. For each isolate, the Ct value obtained in the “test reaction mix” was subtracted from the Ct value obtained in the “control reaction mix” to provide a delta-Ct value.
2. If the delta-Ct value was >5 cycles, then the isolate was considered to harbor an adenine deletion in the *mtrR* promoter, where isolates with a delta-Ct <2.5 cycles were considered wild-type.
3. Isolates falling outside of this range were repeated as above.

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## 4. Notes

1. Additional comments. We have described a simple, rapid, and cheap method of detecting resistance markers linked with decreased susceptibility to extended spectrum cephalosporins and penicillin in *N. gonorrhoeae*. The assays described here can be readily used in a diagnostic microbiology laboratory for both screening of routine isolates for the presence markers or general epidemiological surveillance in the local population. The results from our studies show that this technology provides a high degree of success in characterizing antibacterial resistance arising from chromosomal mutations. We are of the opinion that the methodology can be applied for a wide range of other resistance makers and organisms and may open further avenues for antibiotic susceptibility data generation by molecular means. Currently, commercial assays offering antimicrobial sensitivity data from specimens collected for nucleic acid analysis are not widely available. Moreover, these assays can be readily adapted to include other mutations as newer mechanisms

behind antibacterial resistances are being discovered, especially in case of the gonococcus. These assays do, however, have some limitations, and other techniques such as DNA sequencing may be more appropriate in certain circumstances.

- (a) Mutations with the same GC content can be assigned an incorrect genotype using the HRM methods. For example, we previously observed that an isolate with a rare AGC (G45S) codon was incorrectly assigned a G45D genotype by the *mtrR*-45HRM assay (10). Similarly, isolates with an A501T alteration may be incorrectly assigned an A501V genotype using the PBP2-501HRM assay. The prevalence of genotypes with similar GC content and their relevance therefore needs to be considered when developing HRM methods. ASP could be used as an alternative to HRM in these circumstances. This also highlights that previously undescribed genotypes may not be discriminated if they are of similar GC content to reference controls. It should be noted that HRM can certainly be used to distinguish sequence types of the same GC content where the DNA is carefully purified (i.e., using a commercial extraction protocol rather than isolate heat-denaturation) and quantified. However, we consider this to be an acceptable limitation of our protocol given its low-cost and simplicity.
- (b) Our HRM protocol may also be of limited utility where there are multiple genotypes such as in the *porB*-120/1HRM method. We have found that certain *porB* genotypes GS (GGCAGC) and DA (GACGCC) could not be distinguished, also because of overlapping GC contents. However, again this was not considered a limitation of this assay given the key “KD” alteration (AAGGAC) could readily be differentiated owing to its differing GC content (10).
- (c) Nonspecific amplification products may also hinder HRM analysis. It is for this reason that we used the Qiagen mix rather than the Invitrogen mix for the PBP2-501HRM assay. Using the Invitrogen mix for the PBP2-501HRM assay, two PCR products of differing melting temperature were generated for each strain. Agarose gel electrophoresis analysis indicated the presence of nonspecific product approximately twice the expected size. This nonspecific product was not observed using the Qiagen kit for the PBP2-501HRM assay. The basis of this anomaly was not investigated; however, it could be attributed to differences in PCR stringency of each mix.
- (d) The impact of sequence variation needs to be considered for all PCR assays targeting infectious agents. Here, the presence of SNPs in HRM assays primer targets may affect PCR amplification or adversely affect melting curve characteristics. Likewise, additional SNPs in ASP primer

targets may delay Ct values and hence lead to erroneous results. For these reasons, we recommend analysis of Ct values of all assays as part of quality control procedures. The amount of bacterial lysate added to these mixes should provide Ct values of <20 cycles in all assays (except the *mtrR*-Adel-ASP mix “test reaction mix”). Therefore, Ct >20 cycles may represent a problem with sample preparation or mismatches in assay primer target sequences.

Overall, our results have shown that these methods can be readily applied for detecting chromosomal markers associated with penicillin resistance and increased tolerance to extended spectrum cephalosporins. With diminishing treatment options for gonorrhoea, the need to closely monitor the emergence and spread of resistance to antimicrobials currently in therapeutic use is widely acknowledged. Combining the superior sensitivity and specificity of NAATs with an effective nucleic acid-based antimicrobial resistance (AMR) screening approach will inevitably enhance AMR surveillance systems and disease control for gonorrhoea.

2. It is important to note that the controls must be prepared using the same protocol as per the test isolates. Using a different preparation step, such as a commercial extraction method, may change the melting characteristics of the reference controls compared to the test isolates and adversely affect test performance.
3. Heat-denatured isolate suspensions contain high concentrations of gonococcal DNA and so may facilitate cross-contamination if care is not taken when pipetting and emphasize the need for appropriate numbers of negative controls.
4. Other real-time PCR instruments may be suitable but this has not been assessed.
5. If suspensions are not used immediately, then they are stored at 4 °C overnight or –20 °C for a maximum of 3 months. Longer storage may be possible however has not been assessed.
6. The PBP2-501HRM assay was performed as described at Subheading 3.2 except that the Qiagen Quantitect SYBR kit (Qiagen, Australia) was used as the basis of the reaction mix (12). This was done because our initial attempts using the Invitrogen mix produced a secondary larger product that interfered with HRM result analysis.
7. The *mtrR*-Adel-ASP mix was prepared as above except that two separate reactions were used for each isolate; one “control reaction mix” containing the *mtrR*-Adel-ASP forward-1 and reverse primers and a “test reaction mix” containing the *mtrR*-Adel-ASP forward-2 and reverse primers. Each isolate was tested in both *mtrR*-Adel-ASP reaction mixes.

8. If reaction mix from Subheading 3.2 is not used immediately, then it may be stored in dark at 4 °C overnight or –20 °C for a maximum of 1 week. Keep the reaction mix away from light during storage as this may affect fluorescent signal. For example, avoid fridges or freezers with glass doors that enable light access.
9. The PBP2-501HRM assay was performed as per Subheading 3.3 except that an initial enzyme activation step at 95 °C for 15 min (rather than 2 min) was used, as per the Qiagen Quantitect SYBR kit user manual.

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## Protocol for a Facile Multiplex PCR for Multi-antimicrobial Resistance and Gonococcus Detection

Ratana Lawung, Rungrot Cherdtrakulkiat, and Virapong Prachayasittikul

### Abstract

Gonorrhoea is a continuing problem worldwide particularly in terms of the spread of multiple drug resistance. We have successfully developed an efficient PCR method for the simultaneous identification of gonococci and detection of the antimicrobial-resistant profile. By this method, penicillinase-producing *Neisseria gonorrhoeae* (PPNG), high-level tetracycline-resistant *N. gonorrhoeae* (TRNG), and ciprofloxacin-resistant *N. gonorrhoeae* (CRNG) can be clearly identified. Moreover, the plasmid-types of penicillin and tetracycline resistance are also characterized. The method has 100 % sensitivity and specificity. It is also time- and labor-saving compared to the conventional method. Thus, the procedure is suitable for epidemiological surveillance.

**Key words:** *Neisseria gonorrhoeae*, Multiplex PCR, Multiple antimicrobial resistances, PPNG (penicillinase-producing *N. gonorrhoeae*), TRNG (tetracycline-resistant *N. gonorrhoeae*), CRNG (ciprofloxacin-resistant *N. gonorrhoeae*)

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### 1. Introduction

Gonorrhoea remains one of the most common bacterial sexually transmitted diseases (STDs), especially in Africa and South East Asia. The World Health Organization (WHO) estimated that gonorrhoea attributed approximately 62 million to the total of 340 million new cases of bacterial STDs (1). Persons who have multiple sex partners and engage in unprotected sexual intercourse are at highest risk. Furthermore, gonococcal infections in women are mostly asymptomatic which contributes to the spread of disease. STD prevention programs, such as HIV prevention activities may help to decrease the rates of gonorrhoea infection; however, the prevalence of gonorrhoea remains high and antimicrobial-resistant *Neisseria gonorrhoeae* isolates are frequent. Therefore, the correct

use of condoms and rapid and widespread use of diagnostic methods of detection and identification of antimicrobial resistance are essential for gonorrhoea control.

In accordance to the importance of gonorrhoea, all microbiological laboratories have the responsibility to identify the gonococcal isolates and their resistance profiles. Several methods have been applied to detect this pathogen. The simplest method is direct microscopy which is rapid and highly specific for the detection of gonococci in urethral discharges from male patients. However, this method is insensitive and nonspecific for rectal and pharyngeal specimens and specimens from asymptomatic patients. At present, cultivation is still regarded as the gold standard. This method has a high specificity for detection of the viable pathogen and has the advantage that one can perform susceptibility testing. Nevertheless, the method has a low sensitivity, especially in clinical specimens which have a long transport time. As a result, the noncultivation has become the method of choice for these fastidious bacteria. Various procedures have been developed to detect gonococcal disease. Nucleic acid-based techniques such as nucleic acid-based hybridization testing or nucleic acid-based amplification assay have been described (2). Several commercial kits based on nucleic acid detection have been promoted such as Cobas Amplicor test, LightCycler 16S rRNA test (3, 4). However, most commercial kits have a high false positive rate due to commensal *Neisseria* species and have a high cost. Another important disadvantage of these kits is that they do not address the need for surveillance and control of the increasing spread of antimicrobial resistance of gonococci (5).

Antimicrobial resistance in *N. gonorrhoeae* remains an important challenge to control gonorrhoea worldwide. Resistance to penicillin, tetracyclines, spectinomycin, and fluoroquinolones has been reported (6). Resistant genes carried on plasmids have been described for penicillin and tetracyclines while resistance to tetracyclines, spectinomycin, fluoroquinolones as well as penicillin are chromosomally mediated. Most *N. gonorrhoeae* isolates commonly carry the penicillin and tetracycline resistance plasmids. Seven  $\beta$ -lactamase-producing plasmid types (Asia, Africa, Toronto, Rio, Nimes Johannesburg, and New Zealand) have been reported. The Asia, Africa, and Toronto plasmid types are linked to the spread of penicillinase-producing *N. gonorrhoeae* (PPNG) whereas the Rio, Nimes Johannesburg, and the New Zealand plasmid types in PPNG are sporadic (7). High-level tetracycline-resistant *N. gonorrhoeae* (TRNG) isolates possess a 25.2 megadalton (MDa) conjugative plasmid carrying the *tetM* gene. The tetracycline resistance plasmids are categorized into two types, American and Dutch (8). Fluoroquinolone resistance is mainly caused by *gyrA* and *parC* mutations. The fluoroquinolone resistance in *N. gonorrhoeae* (QRNG) frequently occurs at Ser-91 of GyrA (9, 10). The Ser-91 of *gyrA* is naturally contained in the *HinfI* restriction enzyme site which assists the monitoring of the gene mutation (11).

Generally, culture and antimicrobial susceptibility testing of *N. gonorrhoeae* isolates is time consuming and labor intensive. Therefore, polymerase chain reaction (PCR)-based tests, which are highly sensitive and specific, have been developed. However, the antimicrobial susceptibility is not generally included in these tests. Thus, we developed a PCR method for gonococcal identification and also differentiation of their antimicrobial resistance. A single-tube multiplex PCR specific for gonococci including a series of primers to detect penicillin, tetracycline, and fluoroquinolone-resistant genes was developed (12). Furthermore, our method also differentiates the specific types of plasmid-mediated resistance to penicillin (Asia, Africa, and Toronto), and tetracycline (American and Dutch). This method is suitable to be used in the routine laboratory to reduce the time-consuming and labor-intensive standard methods for the detection of gonococci and its antibiotic susceptibility.

Forty gonococci and 42 non-gonococcal isolates were used to validate both specificity and sensitivity of the identification and detection of antimicrobial resistance and were in both cases 100 %. The limit of detection for our method was 500 CFU/reaction, which could be reduced 100-fold by separating the reaction into two multiplex PCR reaction tubes, detecting the penicillin-tetracycline resistance in one tube and the gyrase and GFPuv genes in the other tube. The sensitivity in 145 tested isolates is 100 % and the specificity for the detection of PPNG, TRNG and decreased susceptibility to quinolones is 100, 98, and 100 %, respectively. Three non-tetracycline-resistant isolates, by phenotypic methods, show a positive amplicon of *tetM* gene. Therefore, this PCR approach could be of great benefit for the surveillance and monitor the gonococcal infection. Moreover, the epidemiological data from this method are very helpful for policy makers to establish the surveillance programs for prevention and control of the spread of gonococcal disease.

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## 2. Materials

### 2.1. DNA Template Preparation

1. Culture media: Chocolate agar, blood agar, Luria broth.
2. Bacterial isolates:
  - (a) *N. gonorrhoeae* (185 isolates).
  - (b) Three non-gonococcal *Neisseria* species: *N. meningitidis* (one isolate), *N. sicca* (one isolate), *N. mucosa* (one isolate).
  - (c) Thirty-two non-*Neisseria* species: *Haemophilus ducreyi* (six isolates), *H. influenzae* (three isolates), *Moraxella catarrhalis* (one isolate), *Micrococcus luteus* (one isolate),

*Staphylococcus aureus* (one isolate), *S. epidermidis* (one isolate), *Streptococcus pyogenes* (one isolate), *S. agalactiae* (one isolate), Group D Streptococcus (one isolate), *Enterococcus faecalis* (one isolate), *Bacillus subtilis* (one isolate), *Corynebacterium diphtheriae* (one isolate), *Listeria monocytogenes* (one isolate), *Escherichia coli* (one isolate), *Klebsiella pneumoniae* (one isolate), *Salmonella choleraesuis* (one isolate), *Serratia marcescens* (one isolate), *Shigella dysenteriae* (one isolate), *Citrobacter freundii* (one isolate), *Enterobacter cloacae* (one isolate), *Morganella morganii* (one isolate), *Yersinia enterocolitica* (one isolate), *Plesiomonas shigelloides* (one isolate), *Vibrio cholerae* (one isolate), *V. mimicus* (one isolate), *Aeromonas carvii* (one isolate), *A. hydrophila* (one isolate), *Achromobacter xylosoxidans* (one isolate), *Pseudomonas aeruginosa* (one isolate), *Burkholderia cepacia* (one isolate), *Stenotrophomonas maltophilia* (one isolate), and *Acinetobacter haemolyticus* (one isolate).

(d) *E. coli* isolate carrying the histidine6-green fluorescent protein plasmid (pH6GFPuv).

3. Distilled water.
4. 0.5 McFarland standard no. 1.
5. NucleoSpin® Plasmid kit: Buffer, NucleoSpin column, collection tube.
6. Hot plate.
7. Spectrophotometer.
8. General lab materials and equipments: Microcentrifuge tubes, pipette tips, pipettes, centrifuge, and disposable gloves.

## **2.2. Multiplex PCR Amplification**

1. *Taq* DNA polymerase: 10× PCR buffer with KCl, 25 mM MgCl<sub>2</sub>, 5 U/μL *Taq* polymerase.
2. 25 mM each dNTPs.
3. Synthetic nucleotide primers GC1F, GC2F, GC3R, GC4R, TetMF, TetMR, GyrAF, GyrAR, GFPuvF, and GFPuvR (see Table 1).
4. Histidine6-green fluorescent protein plasmid (pH6GFPuv) (see Note 1).
5. PCR amplification machine (iCycler).
6. Primer 3 program version 0.3.0, <http://fokker.wi.mit.edu/primer3/input.htm>.
7. Basic Local Alignment Search Tool (BLAST), National Institutes of Health <http://www.ncbi.nlm.nih.gov/BLAST/>.
8. General lab materials and equipments: PCR tubes, pipette tips, pipettes, centrifuge, and disposable gloves.

**Table 1**  
**Primer sequences, target genes and size of PCR amplicons before and after digestion with *Hinf*I restriction enzyme**

Names <sup>a</sup>	Sequences	$T_m$ (°C) <sup>b</sup>	Genes	Types	Regions of target gene <sup>c</sup>	Sizes of PCR products	Sizes of restriction products
GC1F	AACTCACGGACA AAATCAGCC	60.0	$\beta$ -Lactamase-producing plasmid	Africa	1,445–1,880 and 3,708–4,343	1,070	1,070
GC2F	CACCTATAAATCT CGCAAGCC	57.5		Asia	3,606–4,343	737	737
GC3R	AACGCAAGCAGG ACGAAATC	61.1		Toronto	3,606–3,802 and 6,075–6,314	435	435
GC4R	CCTCCACCTTCAT CCTCAGC	60.6					
TetMF	ACTGTTGAACCGA GYAAACCT	54.0–59.6	<i>tetM</i>	American	1,272–2,113	841	748+93
TetMR	TCTATCCGACTAT TTGGACGACG	61.1		Dutch		841	572+176+93
GyrAF	CGGCGCGTACTGT ACGCGAIGCA	74.2	<i>gyrA</i>	Ser-91Mutation	160–438	278	278
GyrAR	AATGTCTGCCAGCAT TTCATGTGAGA	66.6		Non-mutation		278	166+112
GFPuvF	GTCAGTGGAGAGGG TGAAGG	58.1	GFPuv	GFPuv	373–944	571	394+177
GFPuvR	ACCATGTGGTCAC GCTTTTC	59.4					

<sup>a</sup>F forward primer, R reverse primer

<sup>b</sup>Melting temperature ( $T_m$ , basic) is calculated using software available at <http://www6.appliedbiosystems.com/support/techtools/calc/>

<sup>c</sup>Target sites of designed primers correlated to GenBank accession numbers U20374 (Asia type), L12242, U08817 and U62636 for  $\beta$ -lactamase-producing plasmid, tetracycline resistance, gyrase A, and GFPuv plasmid, respectively

**2.3. Restriction  
Fragment Length  
Polymorphism**

1. *Hinf*I restriction enzyme: 10× Buffer R, 10 U/μL *Hinf*I restriction enzyme.
2. Water bath or heat box.
3. General lab materials and equipments: Microcentrifuge tubes, pipette tips, pipettes, centrifuge, and disposable gloves.

**2.4. Amplicon  
Detection**

1. Agarose.
2. 50× TAE buffer (2 M Tris–acetate, 50 mM EDTA) pH 8.0.
3. GeneRuler™ 100 bp ladder plus standard marker.
4. Gel electrophoresis set: Power supply, electrophoresis tray, chamber WEALTEC Elite 300 plus.
5. Ethidium bromide, 10 mg/mL.
6. Gel documentation.
7. General lab materials and equipments: Microcentrifuge tubes, pipette tips, pipettes, centrifuge, and disposable gloves.

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### 3. Methods

**3.1. Preparation  
of DNA Template**

Forty *N. gonorrhoeae* isolates, 3 other *Neisseria* species, and 36 other bacteria species are used as known control isolates for sensitivity and specificity detection of the PCR reactions. The histidine6-green fluorescent protein plasmid DNA (pH6GFPuv) is prepared (see Note 1) and added as gene control in the PCR reaction.

1. Bacterial colonies are suspended in sterile distilled water to a turbidity of 0.5 McFarland (ca.  $1.5 \times 10^8$  CFU/mL). Heat the bacterial cell suspension to 100 °C for 15 min and then centrifuge at 11,000×g for 1 min. Transfer the supernatant of each samples into a new labeled centrifuge tube and use as DNA template or store at –20 °C. The supernatant can be used as DNA template directly without further purification.
2. The pH6GFPuv is propagated in *E. coli* strain TG1 grown in 3 mL Luria broth with 3 μL of 100 mg/mL ampicillin. Harvest the overnight bacterial culture by centrifugation at 11,000×g 1 min and purify the plasmid from the cell pellet by NucleoSpin® Plasmid kit.
  - (a) Add 250 μL Buffer A1 (resuspension buffer) and mix well.
  - (b) Add 250 μL of Buffer A2 (lysis buffer) to the suspension. Mix gently by inverting the tube and leave at room temperature for 5 min.

- (c) Add 300  $\mu\text{L}$  of Buffer A3 (binding buffer) to the suspension. Mix gently by inverting the tube 6–8 times. Centrifuge the suspension at  $11,000\times g$  for 10 min at 4 °C.
- (d) Load the supernatant into the NucleoSpin column in a 2-mL collection tube. Centrifuge at  $11,000\times g$  for 1 min and discard the flow-through.
- (e) Wash the spin column with 500  $\mu\text{L}$  of 50 °C Buffer AW (wash buffer). Centrifuge at  $11,000\times g$  for 1 min and discard the flow-through.
- (f) Wash the spin column with 600  $\mu\text{L}$  of Buffer A4 with ethanol (wash buffer). Centrifuge at  $11,000\times g$  for 1 min and discard the flow-through. Recentrifuge at  $11,000\times g$  for 2 min to remove residual ethanol from the spin column.
- (g) Place the NucleoSpin column in a 1.5-mL microcentrifuge tube. Elute the plasmid DNA by adding 50  $\mu\text{L}$  of Buffer AE (elution buffer) to the column. Incubate 1 min at room temperature and centrifuge at  $11,000\times g$  for 1 min.
- (h) Measure the concentration of purified plasmid DNA at UV spectrophotometer (1 unit of OD 260 = 50  $\mu\text{g}/\text{mL}$ ). Dilute the solution to 2  $\text{ng}/\mu\text{L}$ .

### 3.2. Multiplex PCR System

The target gene sequences are retrieved from NCBI database and using BLAST program to identify the unique conserved regions. Then, the Primer 3 program version 0.3.0 software was used to design primers (see Note 2). All PCR reagents are added into a single tube and perform a reaction under the same conditions. The condition of multiplex PCR system is optimized as shown in Note 3. The specificity and the detection limit of test were also evaluated (see Notes 4 and 5). Reaction is prepared as followed.

1. Multiplex PCR is carried out in 20  $\mu\text{L}$  of a reaction mixture. A master mix for all reactions is prepared by adding the volumes of the required solutions to one tube: 2.0  $\mu\text{L}$  10 $\times$  PCR buffer with KCl, 1.2  $\mu\text{L}$   $\text{MgCl}_2$  (25 mM), 2.0  $\mu\text{L}$  dNTPs (2 mM each), 0.5  $\mu\text{L}$  GC1F primer (40 pmol/ $\mu\text{L}$ ), 0.4  $\mu\text{L}$  GC2F primer (5 pmol/ $\mu\text{L}$ ), 0.5  $\mu\text{L}$  GC3R primer (40 pmol/ $\mu\text{L}$ ), 0.4  $\mu\text{L}$  GC4R primer (5 pmol/ $\mu\text{L}$ ), 0.5  $\mu\text{L}$  TetMF primer (30 pmol/ $\mu\text{L}$ ), 0.5  $\mu\text{L}$  TetMR primer (30 pmol/ $\mu\text{L}$ ), 0.25  $\mu\text{L}$  GyrAF primer (10 pmol/ $\mu\text{L}$ ), 0.25  $\mu\text{L}$  GyrAR primer (10 pmol/ $\mu\text{L}$ ), 0.2  $\mu\text{L}$  GFPuvF primer (10 pmol/ $\mu\text{L}$ ), 0.2  $\mu\text{L}$  GFPuvR primer (10 pmol/ $\mu\text{L}$ ), 0.5  $\mu\text{L}$  pH6GFPuv template (2  $\text{ng}/\mu\text{L}$ ), and 0.3  $\mu\text{L}$  Taq polymerase (1 U/ $\mu\text{L}$ ). Solution is mixed gently by pipetting up and down.
2. Add 4.3  $\mu\text{L}$  of DNA templates (boiled bacterial suspension) to the bottom of labeled PCR tubes.

3. Aliquot 15.7  $\mu\text{L}$  of the master mix to each DNA template tubes to a final volume of 20  $\mu\text{L}$ . Centrifuge briefly to bring solution to the bottom of the tube.
4. Load tubes to iCycler PCR machine and perform PCR amplification by following cycle conditions: initial activation at 94 °C for 5 min, then 35 cycles at 94 °C for 30 s, 60 °C for 1 min, and 72 °C for 1 min 30 s, and a final elongation at 72 °C for 10 min. The hold temperature is 4 °C.

### **3.3. Restriction Fragment Length Polymorphism**

The products of PCR reaction solution are further digested with *HinfI* restriction enzyme.

1. A master mix for all enzyme reactions is prepared by adding the volumes of the required solutions to one tube: 1.0  $\mu\text{L}$  10 $\times$  buffer R, 0.5  $\mu\text{L}$  *HinfI* restriction enzyme (10 U/ $\mu\text{L}$ ), and 3.5  $\mu\text{L}$  distilled water.
2. Add 5  $\mu\text{L}$  each PCR reaction solution into new labeled microcentrifuge tubes.
3. Aliquot 5  $\mu\text{L}$  of this master mix to each microcentrifuge tubes (step 2). The reaction tube is centrifuged to bring solution to the bottom of the tube.
4. Transfer all tubes to a water bath and incubated at 37 °C for 1 h and then chill the reaction tube on ice.

### **3.4. Detection of Products**

After digestion with *HinfI*, the presence of PCR products is analyzed on 1.5 % (w/v) agarose gel electrophoresis.

1. Prepare gel at 1.5 % (w/v) agarose in 1 $\times$  TAE buffer pH 8.0.
2. Add 1 and 2  $\mu\text{L}$  of loading dye into 5  $\mu\text{L}$  of PCR reaction solution and digested reaction tube, respectively.
3. Both solutions from step 2 are loaded into the gel. The standard marker is also loaded into each gel.
4. The gel is run on electrophoresis (WEALTEC Elite 300 plus) at 100 V for 20 min.
5. Stained gel with 0.5  $\mu\text{g}/\text{mL}$  ethidium bromide for 5 min and destained in water for 10 min.
6. Gel is photographed in UV light under a gel documentation.
7. The product sizes are compared with known control sizes of standard marker.

### **3.5. Interpretation Guideline**

The products are used to identify the  $\beta$ -lactamase plasmid types in PPNG, *tetM* types in TRNG and the Ser-91 mutation of GyrA for decreased susceptible *N. gonorrhoeae* (Fig. 1).

1. The PPNG isolates carrying the Asia-, the Africa-, or the Toronto type  $\beta$ -lactamase-producing plasmids. Product size of isolates carrying the Asia, Africa, and the Toronto-type plasmid are 737,

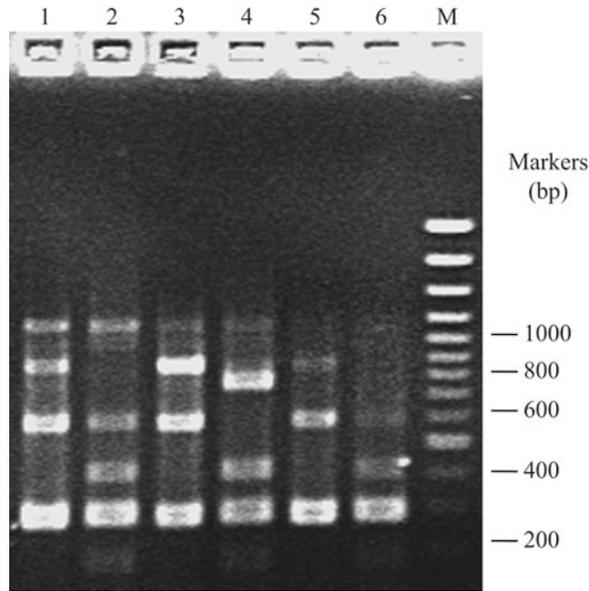


Fig. 1. Multiplex PCR amplicons of  $\beta$ -lactamase-producing genomic types, quinolone-resistant determining region (QRDR) within the *gyrA* gene from *Neisseria gonorrhoeae* isolates and green fluorescent protein (GFP) within the GFP carrying plasmid as an internal control. Lanes 1, 3, 5 are represented PCR products generated by multiplex PCR amplification and lanes 2, 4, and 6 are represented *HinfI* digestion of PCR products of lanes 1, 3 and 5, respectively. Lanes 1–2 are represented the penicillin resistance, tetracycline resistance and not susceptible to ciprofloxacin *N. gonorrhoeae* isolate by carrying Africa type and Dutch type plasmids and mutation at serine 91 of gyrase A. Lanes 3–4 are represented the penicillin resistance, tetracycline resistance and not susceptible to ciprofloxacin *N. gonorrhoeae* isolate by carrying Africa type and America type plasmids and mutation at serine 91 of gyrase A. Lanes 5–6 are represented the non-penicillinase producing, tetracycline resistance and not susceptible to ciprofloxacin *N. gonorrhoeae* isolate by carrying Dutch type plasmid and mutation at serine 91 of gyrase A. Lane M is standard control markers.

1,070, and 435 base pair (bp), respectively. These products do not carry any *HinfI* site, thus, no change in the product size is detected after restriction with the *HinfI* enzyme.

2. The TRNG isolates carrying the American or the Dutch type plasmids. These two plasmid types carry the *tetM* genes that only show minor differences in DNA sequence. Thus, a primer set binding at the same region for both plasmid types and produce an 841 bp product in both types. However, American type contains only one *HinfI* restriction site while the Dutch type contains two sites. After cut with *HinfI* enzyme, product sizes of the American type are 748 and 93 bp while the product sizes of Dutch type are 572, 176, and 93 bp.
3. The fluoroquinolone intermediate and resistant isolates possess a mutation at the Ser-91 of the GyrA and the *gyrA* gene naturally contains the *HinfI* site at serine 91. Thus, the amplification product of 278 bp is cut into 166 and 122 bp after digestion

with *HinfI* enzyme for quinolone susceptible *N. gonorrhoeae* (QSNNG) while a non-digested product (278 bp) is shown in quinolone decreased susceptible *N. gonorrhoeae*, respectively.

4. The amplification and digested products of GFPuv is used as an internal control for amplification and restriction digestion system. The amplicon of GFPuv (571 bp) is presented in all PCR reaction. The product contains one *HinfI* site, thus the product is separated into 394 and 177 bp after digestion with the *HinfI* enzyme.

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## 4. Notes

### 1. Preparation of H6GFPuv plasmid

- (a) The 3,334 bp plasmid of green fluorescent protein with six histidine in pGFPuv (pH6GFPuv) is constructed in our lab (13). The pH6GFPuv was generated by annealing oligonucleotides (5'-AGCTTACACCATCACCATCACCA TGCAGCTCTGCGGTAC-3') and (5'-CGCAGAGCTC GCATGGTGATGG TGATGG TGTA-3') encodings six histidine residues and containing an *SacI* site to derive a piece of double-stranded DNA which was inserted between the *HindIII* and *KpnI* sites of pGFPuv (Clontech Laboratories, USA). The pH6GFPuv is transformed into *E. coli* strain TG1.
- (b) The pH6GFPuv is purified from overnight culture of the pGFPuv in *E. coli* strain TG1 in 3 mL of Luria broth with 3  $\mu$ L of 100 mg/mL ampicillin. Cells are harvested by centrifugation at  $11,000 \times g$  1 min and used a NucleoSpin® Plasmid kit to purify the plasmid. Plasmid DNA is eluted in 50  $\mu$ L of Buffer AE (elution buffer) and diluted to get a concentration of 2 ng/ $\mu$ L before use.

### 2. Design of PCR primers

The NCBI database is used to find and retrieve the target gene sequences. The sequences are then compared using BLAST to identify the unique conserved regions. Primers are then designed using the Primer 3 program version 0.3.0 software. Each primer sets is designed to produce an amplicon under the same conditions and a melting temperature of all primers is selected at 60 °C for a high stringency condition of PCR reaction (11). Details of the PCR primers, target genes, and sizes of PCR amplicons are summarized in Table 1.

- (a) Three types of epidemic  $\beta$ -lactamase-producing plasmids (Asia, Africa, and Toronto types) from *N. gonorrhoeae* are used as target genes. Genes of the Africa type (the first

type) and the Toronto type (the second type) are deletion derivatives of the Asia type  $\beta$ -lactamase-producing plasmid pJD4 (GenBank accession numbers U20374) (the third type). The deletion regions of Africa and Toronto types correspond to nucleotides at 1,881–3,707 and 3,803–6,074 of Asia type, respectively. Based on different sizes and regions of these three  $\beta$ -lactamase-producing plasmids, four primers were designed. The product size of the Asia type is 737 bp which is equivalent to nucleotide position 3,606–4,343 bp while the Africa and the Toronto types give product sizes of 1,070 and 435 bp that are equivalent to pJD4 at nucleotide position 1,445–1,880 and 3,708–4,343 bp for the Africa type and 3,606–3,802 and 6,075–6,314 bp for the Toronto type, respectively.

- (b) The American- and the Dutch-type tetracycline resistance plasmid sequences can be found under the GenBank accession numbers L12241 and L12242, respectively. These two plasmid types carry the *tetM* genes that show minor differences in DNA sequence. Thus, a primer set is designed to bind at the same region and amplify a gene at nucleotide 1,272–2,113 bp (841 bp product) from both plasmid types (11).
- (c) The primers are designed to amplify the *gyrA* gene at nucleotide position 160–438 bp of the GenBank accession numbers U08817 that is cover the serine-91 of the quinolone resistance-determining region (QRDR) (9). Gyrase A gene presents a naturally *HinfI* site at nucleotide 269 of U08817 that is corresponded to the serine 91 of gyrase A. The mutation at serine 91 of QRDR is linked to fluoroquinolone resistance and detected by deletion of *HinfI* sites in QRDR.
- (d) GFPuv primers are designed to amplify the 571 bp of the green fluorescent protein (GFPuv) gene (equivalent to nucleotide position 373–944 of the GenBank accession numbers U62636). The product contains one *HinfI* restriction site and used as positive control (11). Thus, adding of the pH6GFPuv and GFPuv primers in the reaction is used as positive gene control for PCR amplification and *HinfI* restriction enzyme digestion system.

### 3. Multiplex PCR system

The condition of multiplex PCR system is optimized as follow.

- (a) An annealing temperature of 60 °C is chosen for the multiplex PCR reaction that makes PCR reaction can perform under high stringency conditions (11).
- (b) Prepare a PCR reaction by adding buffer, MgCl<sub>2</sub>, dNTPs, Taq polymerase, a set of primer for  $\beta$ -lactamase-producing

plasmids (GC1F, GC2F, GC3R, GC4R), and known control *N. gonorrhoeae* carrying each plasmid types (Asia, Africa or Toronto types). Check product sizes, 1,070 bp (Africa), 737 bp (Asia), 435 bp (Toronto) and thickness of band on gel. Change amounts to get different primer ratio in the reaction for optimization of a thickness of band product from all plasmid types.

- (c) Similar procedures of step 2 are performed with tetracycline, gyrase A and GFP primer sets and known control templates.
- (d) Perform a reaction of all primer sets ( $\beta$ -lactamase primers, tetracycline, gyrase A, and GFP) with selected concentration (steps 2 and 3) and known control template. Check the size and band thickness of the products. Change ratio of primer sets in reaction by increasing the amount of primer in sets that show weak band signal or decrease amount of primer sets that show very thick bands. Select the primer concentrations that give similar yield of all product sizes.

#### 4. *Specificity of the test*

The method must be tested with other bacteria in order to determine the specificity. We determined the specificity of the test using 40 *N. gonorrhoeae* isolates and 35 other bacterial species including 3 non-gonococcal *Neisseria* species, and 32 non-*Neisseria* species as templates. Three non-gonococcal *Neisseria* isolates are *N. meningitidis*, *N. sicca*, and *N. mucosa* which are commonly found in clinical specimens. Other non-*Neisseria* species are included both Gram positive and Gram negative bacteria.

#### 5. *Detection limit for multiplex PCR approach*

The detection limit is evaluated by determining the numbers of bacteria in a multiplex PCR system with a serial dilution.

- (a) The bacterial suspension of 0.5 McFarland standard (ca.  $1.5 \times 10^8$  CFU/mL) is diluted in a tenfold serial dilution and an aliquot of each dilution inoculated on an agar plate to determine the original number of bacteria in the suspension (CFU/mL).
- (b) An aliquot of each dilution is heated at 100 °C for 15 min and then centrifuged at  $10,000 \times g$  for 1 min. The supernatant is then used as DNA template in the PCR. The detection limit for *N. gonorrhoeae* isolates is defined by the minimum concentration of bacteria (CFU/PCR reaction) that can be amplified and detected on the 1.5 % agarose gel.

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## Protocol for Gene Expression Profiling Using DNA Microarrays in *Neisseria gonorrhoeae*

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### Abstract

Gene expression profiling using DNA microarrays has become commonplace in current molecular biology practices, and has dramatically enhanced our understanding of the biology of *Neisseria* spp., and the interaction of these organisms with the host. With the choice of microarray platforms offered for gene expression profiling and commercially available arrays, investigators must ask several central questions to make decisions based on their research focus. Are arrays on hand for their organism and if not then would it be cost-effective to design custom arrays. Other important considerations; what types of specialized equipment for array hybridization and signal detection are required and is the specificity and sensitivity of the array adequate for your application. Here, we describe the use of a custom 12K CombiMatrix ElectraSense™ oligonucleotide microarray format for assessing global gene expression profiles in *Neisseria* spp.

**Key words:** DNA microarray, Gene expression analysis, RNA purification, CombiMatrix, Electrochemical detection, *Neisseria* spp.

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### 1. Introduction

The last decade of study of the human pathogenic *Neisseria* spp. has been dramatically facilitated by the determination of the genome sequences of both *Neisseria gonorrhoeae* and *Neisseria meningitidis*. These achievements have supported the development of various microarray strategies that have been employed to perform gene expression profiling in *Neisseria* spp. (1–18), comparative genomics (19–26), multi-locus sequence typing (27), identification of potential vaccine antigens (28, 29), and the development of clinical assays (30–32) to identify these pathogens and their antibiotic resistance profiles. With the completion of the sequence of the human genome, microarrays also have been used to examine the host response to neisserial infection (33–39).

Thus, it is no understatement that microarray technology has been instrumental in the rapid progress that has been made in the last decade in the study of *N. meningitidis* and *N. gonorrhoeae*. Our own experience with microarrays has employed spotted DNA arrays (13), Affymetrix arrays (1, 2), and most recently CombiMatrix ElectraSense arrays (manuscript in preparation) for gene expression profiling in the gonococcus. Each microarray format has advantages and disadvantages. Spotted microarrays offer low cost and ease of use, but often suffer from a lack of printing uniformity between microarrays. The Affymetrix oligonucleotide-based microarray is an extremely robust platform, but requires the availability of costly dedicated equipment and, in our experience with custom arrays, proprietary concerns can significantly hamper the easy availability of this format. Affymetrix microarrays also are the most expensive of commercially available options. CombiMatrix oligonucleotide microarrays have many of the advantages of the Affymetrix format, but are less expensive and can be reused multiple times, cutting costs even further. The CombiMatrix 12K ElectraSense array is a sensor chip that is a specially modified semiconductor circuitry with over 12,000 individual microelectrodes (features). Following hybridization of the biotin-labeled target to the oligonucleotide probe, HRP-streptavidin conjugate binds to the biotin-labeled target. The resulting redox reaction occurring on each microelectrode that releases an electron is detected by the ElectraSense reader as a current proportional to the biotin-labeled target present, eliminating problems with spot uniformity present in other platforms and differences in array to array variability when using fluorescent detection. Using the ElectraSense process has additional advantages, including the elimination of the use of fluorescent dyes with concomitant problems including higher cost, photo bleaching, and the limited storage life of the labeled cDNA. Below, we describe the use of custom CombiMatrix ElectraSense microarrays for gene expression profiling in *N. gonorrhoeae*.

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## 2. Materials

### 2.1. *Gonococcal Growth Conditions*

1. Complete defined medium (CDM); iron-free CDM-0 and CDM-10 (10  $\mu$ M Ferric nitrate) (1, 2).
2. 10 mM Fe(NO<sub>3</sub>) pH 1.0.
3. *N. gonorrhoeae* FA1090.
4. GC agar supplemented with 2 % Isovitalex (Becton Dickinson).
5. 37 °C incubator. 5 % CO<sub>2</sub>.
6. 37 °C shaking incubator.
7. 500 mL side-arm flasks.

**2.2. Gonococcal Total RNA Extraction “Hot Phenol Method”**  
(See Note 1)

1. Water saturated phenol pH 7.9.
2. 100 % (v/v) Ethanol (store at  $-20^{\circ}\text{C}$ ).
3. TE Buffer pH 8.0: 0.1 M EDTA, 10 mM Tris-HCl, pH 8.0.
4. 10 % SDS.
5. 1 M sodium acetate pH 5.5.
6. 3 M sodium acetate pH 5.5.
7. 80 % (v/v) Ethanol.
8. Nuclease-free water.
9. 15 mL Falcon tubes.
10. RNaseZap RNase Decontamination Solution (Ambion/Applied Biosystems) (see Note 2).
11. Sterile 1.5 mL Eppendorf snap-cap microcentrifuge tubes (see Note 3).
12.  $65^{\circ}\text{C}$  water bath.
13. NanoDrop™ ND1000 (NanoDrop Products, Fisher Thermo Scientific).
14. Microlite RF Centrifuge (Thermo IEC).
15. Freezers ( $-20$  and  $-80^{\circ}\text{C}$ ).
16. 100 % (v/v) Chloroform.

**2.3. Assessment of RNA Quality**

1. Agilent 2100 Bioanalyzer.
2. RNA 6000 Nano Chip Kit (Agilent).

**2.4. DNase Treatment**

1. RQ1 RNase-Free DNase (1 U/ $\mu\text{L}$ ) with 10 $\times$  Reaction Buffer (Promega).
2. Tri Reagent: phenol and guanidine thiocyanate in a monophasic solution (Molecular Research, Inc.).
3. 100 % (v/v) Chloroform.
4. 100 % (v/v) Isopropanol.
5. Sterile 1.5 mL Eppendorf snap-cap microcentrifuge tubes (see Note 3).
6. Nuclease-free water (Ambion/Applied Biosystems).
7.  $37^{\circ}\text{C}$  heat block.
8. 80 % (v/v) Ethanol (store at  $4^{\circ}\text{C}$ ).
9. Freezer ( $-80^{\circ}\text{C}$ ).
10. NanoDrop™ ND1000 (NanoDrop Products, Fisher Thermo Scientific).
11. Microliter centrifuge.

**2.5. Reverse  
Transcriptase  
and cDNA Labeling**

1. High-Capacity cDNA Archive Kit (Applied Biosystems).
2. *Arabidopsis thaliana* mRNA positive controls (Stratagene):  
SpotReport® mRNA Spike 1, Cab 10 ng/μL (store at -80 °C).  
SpotReport® mRNA Spike 2, RCA 10 ng/μL (store at -80 °C).  
SpotReport® mRNA Spike 3, RBCL 10 ng/μL (store at -80 °C).
3. Label IT® μArray® Biotin Labeling Kit (Mirus).
4. 0.5 M EDTA, pH 8.0.
5. MicroAmp® Optical 96-well reaction plate (Applied Biosystems).
6. MicroAmp® 96-well clear adhesive film (Applied Biosystems).
7. QIAquick® PCR Purification Kit (Qiagen).
8. GeneAmp® 9700 thermal cycler (Applied Biosystems).
9. Nuclease-free water.
10. Sterile 1.5 mL Eppendorf snap-cap microcentrifuge tubes (see Note 3).
11. 37 °C heat block.
12. Centrifuge with 96-well plate carriers.

**2.6. CombiMatrix  
Arrays: Hybridization  
and Data Collection**

1. *N. gonorrhoeae* FA1090 CombiMatrix 12K ElectraSense™ Custom Array.
2. 12K ElectraSense™ Hybridization Chamber (CombiMatrix).
3. CustomArray™ holders for rotisserie (CombiMatrix).
4. Hybridization oven with rotisserie (e.g., Fisher Thermo Scientific).
5. Nuclease-free water.
6. Pre-Hybridization Buffer (50 μL): 5 μL 2× Hyb Solution Stock, 34 μL Nuclease-free water, 10 μL 50× Denhardt's Solution, 1 μL Sonicated Salmon sperm DNA (10 mg/mL). Before adding to the Pre-Hybridization Buffer, heat the Salmon sperm DNA for 5 min at 95 °C followed by 1 min on ice.
7. 2× Hyb Solution Stock (10 mL): 6 mL 20× SSPE, 100 μL 10 %/v/v Tween-20, 560 μL 0.5 M EDTA, 3.34 mL Nuclease-free water.
8. 50× Denhardt's Solution (Sigma-Aldrich).
9. Sonicated Salmon sperm DNA (Ambion/Applied Biosystems).
10. 10 % (v/v) Tween-20.
11. 0.5 M EDTA, pH 8.0.
12. 95 °C heat block.
13. Ice and ice bucket.

14. 20× SSPE Buffer: 3 M NaCl, 0.2 M Na<sub>2</sub>PO<sub>4</sub>, 0.020 M EDTA.
15. Hybridization Buffer (100 μL): 50 μL Hyb Solution Stock, 1 μL Salmon sperm DNA heat denatured as listed for Pre-Hybridization Buffer, 4 μL 1 % SDS, biotin-labeled cDNA up to 45 μL, Nuclease-free water to bring the volume to 100 μL.
16. Biotinylated cDNA (see Subheading 2.5).
17. 3× SSPET Wash Solution (100 mL): 15 mL 20× SSPE, 500 μL 10 % Tween-20, 84.5 mL Nuclease-free water.
18. 0.5× SSPET Wash Solution (100 mL): 2.5 mL 20× SSPE, 500 μL 10 % Tween-20, 97 mL Nuclease-free water.
19. PBST Wash Solution (100 mL): 20 mL 10× PBS, 1 mL 10 % Tween-20, 79 mL Nuclease-free water.
20. 10× Phosphate Buffered Saline (PBS): 1.37 M NaCl, 0.08 M Na<sub>2</sub>PO<sub>4</sub>, 0.02 M KH<sub>2</sub>PO<sub>4</sub>, 0.027 M KCl pH 7.4.
21. CombiMatrix ElectraSense Detection Kit™ (CombiMatrix).
22. CombiMatrix ElectraSense™ reader (CombiMatrix).

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### 3. Methods

#### 3.1. *Gonococcal Growth Conditions*

1. Growth in iron minus (CDM-0) and iron plus (CDM-10) medium was carried out as previously reported (1, 2). *N. gonorrhoeae* strain FA1090 was grown overnight from glycerol stocks on GC agar supplemented with 2 % IsoVitalax at 37 °C and 5 % CO<sub>2</sub>. Non-piliated organisms were selected and inoculated to another GC agar plate and incubated overnight for growth. Piliated and non-piliated colonies will grow as two distinct colony sizes with non-piliated colonies being larger than piliated colonies on GC agar. Growth of colonies from piliated *N. gonorrhoeae* causes significant clumping in broth making O.D. determinations difficult.
2. Non-piliated cells were inoculated into a 500 mL flask of CDM-0 to an absorbance of 0.100 at 600 nm. The flask was incubated at 37 °C (150 rpm) and the cells were allowed to double to an absorbance of 0.200 without the addition of ferric nitrate to deplete internal iron stores. Doubling time typically takes between 1.5 and 2 h.
3. The iron-depleted cells were then split and diluted twofold into 500 mL flasks containing either CDM-0 or CDM-10. 10 mM Fe(NO<sub>3</sub>)<sub>3</sub> pH 1.0 was added to the CDM-10 to a final concentration of 10 μM.
4. The two flasks were returned to the 37 °C (150 rpm) incubator. Cells were collected at 3 h time points for RNA isolation.

**3.2. *Gonococcal Total RNA Extraction "Hot Phenol Method"***

1. Add 5 mL of a 3 h culture (0.600 O.D.) into a 15 mL plastic conical Falcon tube.
2. Add 1:10 v/v of water saturated phenol pH 7.9 to ice cold 100 % ethanol to 5 mL of culture. (500  $\mu$ L water saturated phenol and 4.5 mL 100 % ethanol) (see Note 4).
3. Centrifuge for 10 min at  $5,000\times g$  and 4 °C to pellet the cells.
4. Aspirate off the supernatant leaving as little medium behind as possible.
5. At this point, cell pellets maybe snap frozen using dry ice or liquid nitrogen and stored at -80 °C for up to a week.
6. Start the process of lysing the cells by adding 700  $\mu$ L Buffer TE, pH 8.0, and transfer to a 1.5 mL sterile microcentrifuge tube.
7. Add 7  $\mu$ L of 10 % SDS, invert several times, and put in a 65 °C water bath for 1–2 min to lyse cells.
8. Add 77  $\mu$ L of 1 M sodium acetate, pH 5.5, and 850  $\mu$ L of hot (65 °C) water saturated phenol and invert ten times (see Note 4).
9. Place the tubes in a 65 °C water bath for 6 min. Invert tubes every 60 s.
10. Centrifuge 15 min at 4 °C,  $18,000\times g$ .
11. There will be two apparent phases after centrifugation. Aspirate off the top layer into a new sterile 1.5 mL microcentrifuge tube containing 850  $\mu$ L 100 % chloroform and invert several times.
12. Centrifuge 15 min at 4 °C,  $18,000\times g$ .
13. Pull off the top aqueous layer being careful not to disturb the lower organic layer. Split the aqueous layer that carries the RNA into two sterile 1.5 mL microcentrifuge tubes (see Note 5).
14. Precipitate the RNA by adding 800  $\mu$ L of cold ethanol (100 %) and 35  $\mu$ L of 3 M sodium acetate to each tube.
15. Place the tubes at -80 °C for at least 2 h (see Note 6).
16. Centrifuge tubes for 25 min at 4 °C,  $18,000\times g$ .
17. Decant the supernatant leaving the RNA pellet which should appear as white or translucent (see Note 7).
18. Wash the RNA pellet by adding 1 mL of cold 80 % ethanol and vortex gently
19. Centrifuge for 15 min at 4 °C,  $18,000\times g$ .
20. Repeat ethanol wash steps 18 and 19.
21. Remove the last ethanol wash carefully and allow the RNA samples to air-dry for 10–15 min and then resuspend the pellets in Nuclease-free water (see Note 8). For our application, the desired RNA concentration is 1–2  $\mu$ g/ $\mu$ L.
22. Determine the RNA concentration of the samples using the NanoDrop 1000 (NanoDrop Products, Fisher Thermo Scientific) and store all samples at -80 °C until DNase treatment.

### 3.3. Assessment of RNA Quality

Isolation of high quality RNA is essential for many downstream applications including generation of cDNA for microarrays and qRT-PCR, making it crucial to assess the RNA integrity prior to investing additional time and resources. A 260/280 ratio of 1.8 or greater indicates the purity of the RNA, but does not address the status of RNA degradation. We assess the quality of RNA samples using the Agilent Bioanalyzer. RNA samples were analyzed with the Agilent RNA 6000 Nano Chip Kit. The Bioanalyzer software generates an RNA Integrity Number (RIN) taking into account any degradation along the entire electropherogram and not just the 16S and 23S ribosomal RNA peaks. As seen in the electropherogram example in Fig. 1, the peaks for 16S and 23S ribosomal RNA are sharp with little degradation. The RIN score for this RNA sample was 9.2. An RIN of 10 is ideal indicating the least amount of degradation. Figure 2 shows an RNA sample with significant degradation. Note the 16S and 23S peaks are one broad peak and the increase in size of the 5S ribosomal RNA peak. The RIN was 4.2 for this sample, however, the 260/280 ratio was 2.0 (see Notes 9 and 10).

### 3.4. DNase Treatment

1. Add 1 U of Promega RQ1 RNase-Free DNase to 1  $\mu$ g total RNA into a sterile 1.5 mL microcentrifuge tube.
2. Add 1/10 volume RQ1 RNase-Free DNase 10 $\times$  Reaction Buffer and Nuclease-free water to the appropriate final reaction volume taking into account the volume of the RNA and RQ1 RNase-free DNase (see Note 11).
3. Incubate the tube in a heat block at 37  $^{\circ}$ C for 30 min.

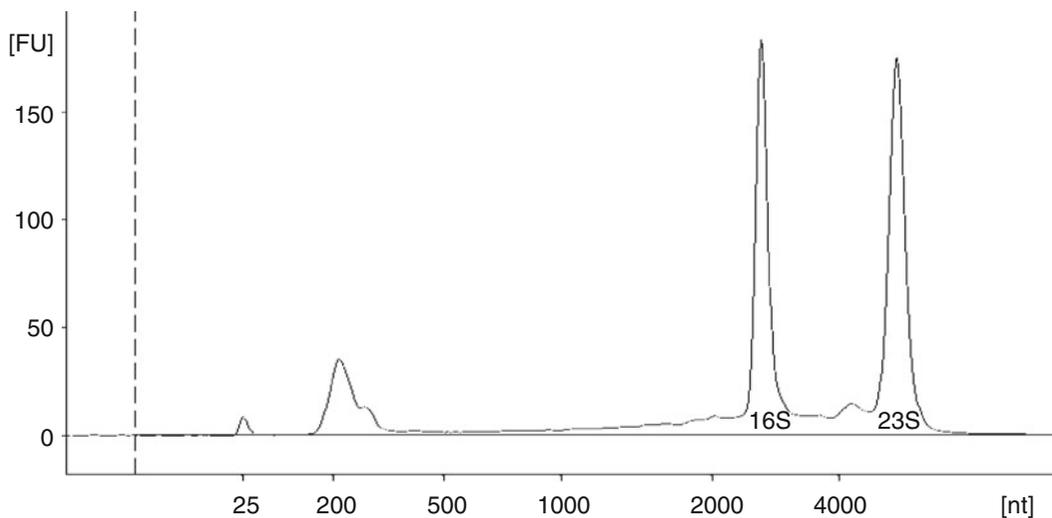


Fig. 1. Electropherogram of RNA isolated from *N. gonorrhoeae* FA1090 grown in CDM in iron minus conditions. The smallest peak is 5S ribosomal RNA. The peak at 25 nucleotides is the internal sizing standard. nt indicates nucleotide size. FU indicates Fluorescent Units (see Note 10).

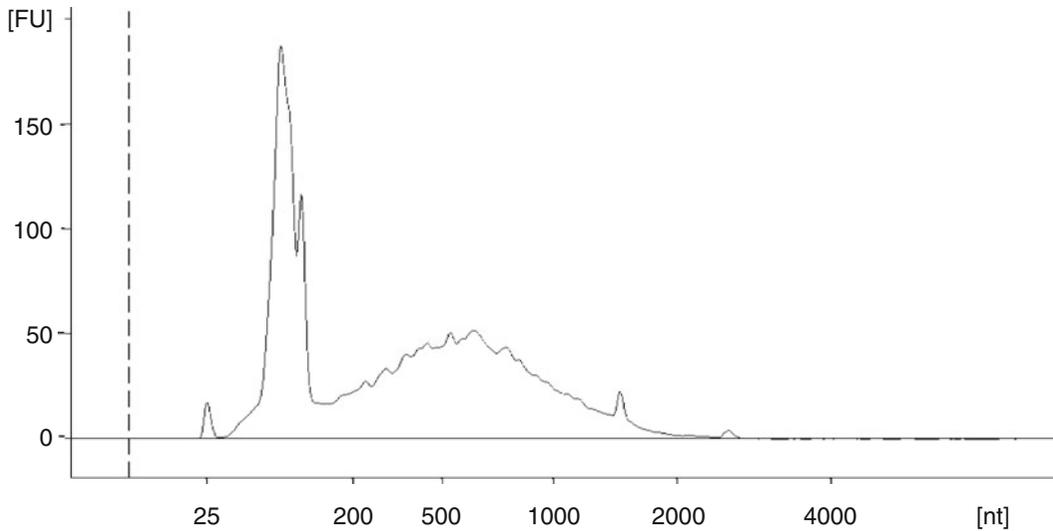


Fig. 2. Electropherogram of RNA isolated from *N. gonorrhoeae* FA1090 grown in CDM in iron minus conditions showing significant RNA degradation. The peak at 25 nucleotides is the internal sizing standard. nt indicates nucleotide size. FU indicates Fluorescent Units.

4. Add 1 mL of TriReagent to the tube and proceed to RNA clean-up as detailed in step 5 below (see Note 12).
5. Add 200  $\mu\text{L}$  chloroform, vortex, and centrifuge  $12,000 \times g$  for 15 min,  $4^\circ\text{C}$ .
6. Carefully pull off the top aqueous layer containing the RNA into a new sterile 1.5 mL microcentrifuge tube.
7. Precipitate the RNA by adding 250  $\mu\text{L}$  100 % isopropanol, followed by a 15 min centrifugation at  $4^\circ\text{C}$ ,  $12,000 \times g$ .
8. Wash the RNA pellet by adding 1 mL cold 80 % ethanol. Centrifuge at  $4^\circ\text{C}$  for 10 min,  $12,000 \times g$ .
9. Remove the supernatant and allow the pellet to air-dry for 10–15 min to remove any residual ethanol (see Note 8).
10. Resuspend all pellets in Nuclease-free water and determine the RNA concentration in the samples using the NanoDrop 1000 (NanoDrop Products, Fisher Thermo Scientific) and assess RNA quality as in Subheading 3.3. Store all samples at  $-80^\circ\text{C}$ .

### 3.5. Reverse Transcription and cDNA Labeling

1. To generate the cDNA, reverse transcribe 10  $\mu\text{g}$  of DNase-treated total RNA in a final volume of 100  $\mu\text{L}$  using Applied Biosystems High-Capacity cDNA Archive Kit (see Note 13):
2. Prepare the MicroAmp 96-well reaction plate by adding 50  $\mu\text{L}$  of a  $2\times$  Reverse Transcription (RT) master mix to the appropriate number of wells. Each 50  $\mu\text{L}$  aliquot of  $2\times$  RT master mix contains, from the High-Capacity cDNA Archive Kit: 10  $\mu\text{L}$   $10\times$  RT Buffer, 4  $\mu\text{L}$   $25\times$  100  $\mu\text{M}$  dNTP, 10  $\mu\text{L}$  Random

Primer mix, 5  $\mu\text{L}$  Multiscribe™ Reverse Transcriptase (50 U/ $\mu\text{L}$ ), 21  $\mu\text{L}$  Nuclease-free water.

3. Add 50  $\mu\text{L}$  of RNA sample to each well containing the RT master mix and pipet up and down to mix. The 50  $\mu\text{L}$  RNA sample will have 10  $\mu\text{g}$  of DNase-treated RNA and 1  $\mu\text{L}$  each of the three SpotReport mRNA positive control spikes diluted to 1 ng/ $\mu\text{L}$ . Add the appropriate amount of Nuclease-free water to bring the total volume to 50  $\mu\text{L}$  (see Note 14).
4. Seal the plate with MicroAmp 96-well clear adhesive film. Briefly centrifuge the plate to spin down the contents and remove any air bubbles present.
5. Set the thermal cycler conditions: 25 °C for 10 min followed by 37 °C for 120 min and load the plate containing the RT reactions. The cDNA is now ready for labeling.
6. Following the Mirus cDNA labeling protocol, hydrolyze the RNA template by adding 0.3 volumes 0.5 M EDTA and 0.1 volume. Reagent D1 to the completed RT reaction in the 96-well plate.
7. Set the thermal cycler at 65 °C for 30 min and allow samples to cool to room temperature before continuing to the next step.
8. Neutralize samples by adding 0.125 volume (according to the original reaction volume) of Neutralization Buffer N1.
9. Purify the cDNA using QIAquick PCR Purification method. Add 5 volumes of Buffer PB to 1 volume of the biotin-labeled cDNA.
10. Bind the cDNA to the column by applying the sample to a QIAquick column and spin for 30 s. Discard the flow through (see Note 15).
11. Wash the column by adding 750  $\mu\text{L}$  Buffer PE (see Note 16) and centrifuge for 30 s. Discard the flow through and centrifuge again for an additional 1 min to remove any residual ethanol.
12. Place the column in a new 1.5 mL microcentrifuge tube and elute by carefully adding 30  $\mu\text{L}$  of Nuclease-free water directly to the column membrane. Allow the column to set for 1–2 min. Centrifuge the column for 2 min.
13. Measure the cDNA concentration on the NanoDrop 1000, choosing ssDNA-33 as sample type.
14. Label 3  $\mu\text{g}$  of purified cDNA in a 100  $\mu\text{L}$  reaction volume according to the Mirus kit protocol: in a sterile 0.5  $\mu\text{L}$  microcentrifuge tube, add the appropriate volume of cDNA sample up to 86  $\mu\text{L}$ , 10  $\mu\text{L}$  10 $\times$  Labeling Buffer and Nuclease-free water to bring the volume to 96  $\mu\text{L}$ , then add 4  $\mu\text{L}$  *Label IT*  $\mu\text{Array}$  Biotin Reagent (see Note 17).

15. Incubate the labeling mixture at 37 °C for 1 h in a heat block.
16. Add 0.1 volume Reagent D1 and incubate at room temperature for 5 min, followed by addition of 0.1 volume Neutralization Buffer N1, incubate on ice for 5 min.
17. Clean up the biotin-labeled cDNA using the QIAquick PCR Purification method as described previously in steps 9–13. The biotin-labeled cDNA is now ready to proceed to hybridization (see Note 18).

### 3.6. CombiMatrix Arrays: Hybridization and Data Collection

Our laboratory focuses on iron regulation in the human pathogen *N. gonorrhoeae*. We are currently interested in the role small RNAs play in the gonococcal iron regulon by assessing the global transcriptional profile in iron minus and iron plus growth conditions. Our custom 12K gene expression array was designed by CombiMatrix's bioinformatics team. The in situ technology uses an electrochemical process to synthesize probes/features (30–40-mers) directly onto the chip. Included in the custom array are factory-built negative controls and “no-synthesis” (quality controls) positioned in areas throughout the chip. *A. thaliana* genes were added as positive controls. We also included intergenic regions meeting bioinformatic criteria for small RNA expression. These intergenic features were designed for both the sense and antisense strands. If possible, all annotated genes had five to six features spanning the entire open reading frame. Figure 3 shows a typical gonococcal gene expression microarray. Each feature is visually represented as a square. Since the ElectraSense is an electrochemical detection method, the read out will be a direct numeric quantification of the hybridization signal. Grid alignment or spot finding is not necessary.

1. Place the array in the 12K ElectraSense hybridization chamber, add Nuclease-free water, and incubate at 65 °C for 10 min. Unless otherwise stated, all arrays are rotated during incubations using the custom array holders for the rotisserie. After

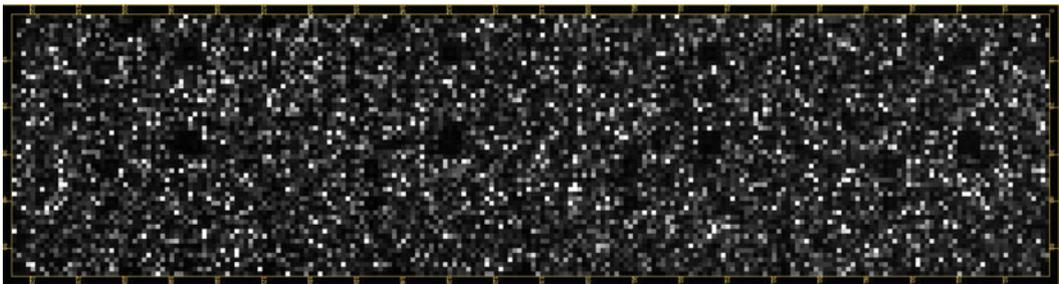


Fig. 3. Biotinylated cDNA from *N. gonorrhoeae* FA1090 grown in CDM in iron minus conditions was hybridized to a 12K CombiMatrix ElectraSense™ CustomArray. The array was scanned and electrochemical detection read on the CombiMatrix ElectraSense™ reader. Signal intensity values and annotation information for each feature were exported for analysis in GeneSpring GX 11.

the 65 °C incubation step, bring the hybridization chamber to room temperature and refill with Pre-Hybridization Buffer. Incubate at 50 °C for 30 min.

2. Denature the hybridization solution containing the biotin-labeled cDNA by heating for 3 min at 95 °C and 1 min on ice.
3. Add the denatured biotin-labeled cDNA to the hybridization chamber and incubate for 16 h at 50 °C .
4. Rinse the array by filling the hybridization chamber with 3× SSPET pre-warmed to 50 °C. Pull off the rinse, add fresh 3× SSPET, and incubate for 5 min.
5. Rinse the array with 0.5× SSPET and again add fresh 0.5× SSPET and incubate at room temperature. All room temperature steps are done on the bench top.
6. Rinse the hybridization chamber with PBST Wash Solution and refill the chamber. Incubate at room temperature for 2 min. The array is now ready for post-hybridization labeling. Bring the ElectraSense Detection Kit™ to room temperature.
7. Remove the PBST Wash Solution and add the Blocking Buffer from the ElectraSense Detection Kit. Incubate at room temperature for 15 min.
8. Remove the Blocking Buffer and add the Biotin Labeling Solution to the hybridization chamber. Incubate at room temperature for 30 min.
9. Rinse the hybridization chamber with Biotin Wash Solution and refill with the Wash Solution. Incubate for 5 min at room temperature.
10. Repeat the Biotin Wash Solution step two more times.
11. Remove the Biotin Wash Solution and rinse with TMB Rinse Solution of the ElectraSense detection kit. Quickly remove the TMB Rinse Solution and add TMB Substrate Solution of the ElectraSense detection kit.
12. Place the hybridization chamber in the ElectraSense reader and scan the array within 1 min. Arrays can be rescanned by taking out the TMB Substrate Solution, rinsing first with Biotin Wash and then with TMB Rinse Solution before adding fresh TMB Substrate (see Note 19).

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## 4. Notes

1. Isolation of RNA requires the researcher to take precautions to minimize the introduction of nucleases by treating the bench-top, pipettes, and any equipment with commercial products that eliminate RNases. We routinely use RNaseZap RNase

Decontamination Solution. RNase-free buffers and reagents are available from Ambion. Gloves must always be worn to prevent contamination from nucleases present on the skin. Equally important is the use of barrier pipette tips. All dilutions of stock solutions listed in the Subheading 2 were done with Nuclease-free water.

2. Surfaces and equipment are decontaminated by spraying RNaseZap followed by rinsing with Nuclease-free water.
3. Nuclease-free microcentrifuge tubes are available through Ambion. In our experience, sterilizing the microcentrifuge tubes by autoclaving is a cost-efficient alternative.
4. Ensure that the water-saturated phenol is mixed well prior to use. The aqueous layer will separate out quickly.
5. We find it advantageous to leave a small amount of the aqueous layer to prevent DNA and protein contamination, however; if the RNA quantity will be low, recover as much of the aqueous layer as possible.
6. 2 h is the minimum time to precipitate the RNA. We typically leave RNA samples overnight at  $-80^{\circ}\text{C}$  to increase RNA yields.
7. If the RNA pellet does not adhere to the side of the tube after centrifugation, aspirate off the supernatant being careful not to disturb the RNA pellet.
8. Remove any residual ethanol from the sides of the tube with a pipette. We find it easier to remove small drops of ethanol using 10  $\mu\text{L}$  barrier pipette tips. Do not overdry the RNA pellet as this makes the RNA less soluble.
9. An RIN number of 8.5 or greater generally indicates good quality RNA with modest degradation; however, this number will vary for different cells and/or tissues and different experimental conditions. Lower RIN values are acceptable if the downstream application is tested and validated by other methods.
10. The 5S ribosomal peak will not be present if using Qiagen RNeasy columns to isolate the RNA. Nucleic acids smaller than 200 bp pass through the column. Many microarrays are now designed to detect small RNAs transcripts and may be missed using a column.
11. The following is an example of a 100  $\mu\text{L}$  DNase treatment reaction using Promega DNase; 25  $\mu\text{L}$  RNA at a concentration of 2.0  $\mu\text{g}/\mu\text{L}$ , 10  $\mu\text{L}$  RQ1 RNase-Free DNase 10 $\times$  Reaction Buffer, 50  $\mu\text{L}$  RQ1 RNase-Free DNase (1 U/ $\mu\text{L}$ ), 15  $\mu\text{L}$  Nuclease-free water.
12. Total volume of the DNase reaction to which 1 mL of Tri Reagent is added should not exceed 200  $\mu\text{L}$ .
13. The following is an example of a 100  $\mu\text{L}$  RT reaction using the High-Capacity cDNA Archive Kit (Applied Biosystems) with an RNA sample at a concentration of 2.0  $\mu\text{g}/\mu\text{L}$ ; 10  $\mu\text{L}$

10× RT Buffer, 4 μl 25× dNTP, 10 μL Random Primers, 5 μL Multiscribe™ Reverse Transcriptase (50 U/μL), 5 μL DNase-treated RNA (2.0 μg/μL), 1 μL SpotReport® mRNA Spike 1 (1 ng/μL), 1 μL SpotReport® mRNA Spike 2 (1 ng/μL), 1 μL SpotReport® mRNA Spike 3 (1 ng/μL), 63 μL Nuclease-free water.

14. All reagents and 96-well plates should be placed on ice when adding RT master mix and RNA samples.
15. All steps in purification process of the biotinylated cDNA and centrifugation (14,000×g) are done at room temperature.
16. Ensure that the appropriate amount of 100 % ethanol has been added to the Buffer PE.
17. The micrograms of cDNA that are biotin-labeled should be empirically determined for each organism and/or sets of experimental conditions. We found that 3 μg of biotin-labeled cDNA gave an optimal hybridization signal on our custom array.
18. We have used biotin-labeled cDNA that was stored at -20 °C for up to 2 days on our arrays without significant loss of signal.
19. A common scan error message is failure of some of the electrodes to read. This is usually the result of the electrical contact pads on the array slide not being clean. When this occurs, the contacts are cleaned and the hybridization chamber is washed first with Biotin Wash and then TMB Biotin Rinse, followed by fresh TMB substrate, and the slide rescanned. The ElectraSense arrays have the advantage of being able to be scanned multiple times, but in our hands most arrays were scanned at least twice and occasionally three to four times to get a good read.

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## Protocol for Analyzing Human Leukocyte Antigen Variants and Sexually Transmitted Infections: From Genotyping to Immunoassays

Jianming Tang and Anju Bansal

### Abstract

This chapter describes experimental and analytical procedures that can be used to decipher the specific role of human leukocyte antigen (HLA) variants in infectious diseases. The techniques are distilled from more than one decade of active immunogenetics research, primarily on sexually transmitted infections (STIs) caused by viral and bacterial pathogens, including human immunodeficiency virus type 1 (HIV-1), hepatitis B virus (HBV), and *Chlamydia trachomatis*. The specific approaches cover (1) sequence-specific oligonucleotide (SSO) probe hybridization for low-resolution genotyping, (2) sequencing-based typing (SBT) for high-resolution, (3) statistical methods for testing associations between HLA variants and phenotypic traits, and (4) enzyme-linked immunospot (ELISpot) assay for enumerating HLA-restricted and epitope-specific T-lymphocyte responses. Proper application of these mature and robust techniques should help establish the importance of individual HLA alleles, haplotypes, and supertypes to host–pathogen interactions.

**Key words:** Human leukocyte antigen (HLA), Immunogenetics, ELISpot, PCR-SSO, SBT

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### 1. Introduction

As documented in the dbMHC database and other valuable resources (see Appendix 1), HLA genes within the human major histocompatibility complex (chromosome 6p23) are characterized by extensive allelic and haplotypic variations (1). Within a given population, the number of common and well-documented (CWD) alleles is often limited (2), so the importance of CWD alleles and their haplotypes to STIs can be assessed effectively in properly designed studies, even with a modest sample size.

Method for HLA typing depends heavily on availability of resources, the desired resolution and types of biological materials.

Both serologic and molecular (DNA-based) methods are now readily available (Table 1). Here, we focus on the analysis of *HLA-B* gene, which has over 800 individual alleles assigned officially by the World Health Organization Committee for the Nomenclature of HLA Factors (1). The two highlighted genotyping techniques, SSO and SBT, are suitable for low- and high-resolution genotyping, respectively. These techniques are equally suitable for other HLA genes (class I or class II), but readers may need to consult several review articles (3–5) in order to fully grasp the complexity and evolution of serologically and molecularly defined HLA variants.

Once HLA typing results are gathered from a study population, association of individual HLA variants with phenotypic traits can be assessed in univariate and multivariable models. We attempt to touch base on (1) computing software, (2) analysis of genetic heterogeneity, (3) genetic association tests, (4) issues related to false discoveries, and (5) cautionary interpretation in the context of local and extended haplotypes.

The ELISpot assay described in this chapter tests HIV-1 Gag epitope recognized by HLA-B\*57:01. This technique is based on the principle of ELISA and originally developed by Cecil Czerkinsky in 1983 to detect antibody-secreting B cells (6). It has evolved to become a widely used and sensitive assay for measuring the function of T- and B-cell-mediated immune responses to specific antigens. The versatility of this assay allows for rapid detection or mapping of antigen-specific response profile at a single-cell level. Overall, the assay is relatively easy to perform even in resource-poor settings and it works well with both fresh and frozen cells, with the potential for high-throughput. ELISpot also provides a cost-effective approach to screening peptides covering an entire pathogen. Numerous applications of ELISpot have dealt with the analysis of  $T_H1/T_H2$  responses in the context of allergy, autoimmune disorders, cancer, infectious diseases, and vaccine development. In our studies, the IFN- $\gamma$  ELISpot assay has proved useful in detecting HIV-1-specific T-cell responses (7, 8).

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## 2. Materials

### **2.1. Materials for Running PCR-SSO Assays (The “INNO-LiPA HLA-B Update Plus” Kit from Innogenetics)**

1. PCR amplification pack stored at  $-20\text{ }^{\circ}\text{C}$ , with three components: (1) amplification buffer; (2) *HLA-B* multiplex primers; and (3) Lipa-Taq (DNA polymerase) at  $2\text{ U}/\mu\text{L}$  (see Notes 1–2).
2. Autoclaved PCR grade water (e.g., Tri-L-15NH system, with K00173 and K00174 filters from Manitowoc Ice, Inc.) stored at room temperature.
3. 0.2 mL Tubes or 96-well plates and sealing film suitable for PCR.

**Table 1**  
**PCR-based methods for HLA class I genotyping, as tested in our own research**

Genotyping methods and resolution <sup>a</sup>						
Locus	PCR-SSO <sup>b</sup>		PCR-SSP <sup>c</sup>		SBT <sup>d</sup>	
	Probes	Resolution	Reactions	Resolution	Exons	Resolution
<i>HLA-A</i>	44	Low/medium	24	Low/medium	2–4 (bidirectional)	High
<i>HLA-B</i>	67	Low/medium	48	Low/medium	2–4 (bidirectional)	Medium/high
<i>HLA-C</i>	28	Low/medium	23	Low/medium	2–4 (bidirectional)	High

<sup>a</sup>Two techniques (PCR-SSO and SBT) are highlighted in this chapter. With rare exceptions, low/medium resolution defines mostly 2-digit specificities (allele groups), while high resolution defines at least 4-digit alleles (not counting synonymous SNPs and SNPs outside of the open reading frame). SSP, sequence-specific primers; SSO, sequence-specific oligonucleotides; SBT, sequencing-based typing

<sup>b</sup>From Innogenetics (Alpharetta, Georgia)

<sup>c</sup>From Dynal/Invitrogen (Brown Deer, Wisconsin)

<sup>d</sup>From Abbott Molecular (Des Plaines, Illinois); HLA database used for individual allele assignments corresponds to the 17 July 2007 IMGT updates (see Appendix 1)

4. Agarose gel electrophoresis system and supplies, including: (1) molecular biology grade agarose, 500 g powder stored at room temperature; (2) 10× TBE buffer (324.0 g Tris base, 165 g boric acid, and 22.32 g Na<sub>2</sub>EDTA in one liter of distilled water); (3) microwave oven to melt agarose; (4) Quick-Load 100-bp DNA Ladder (50 µg/mL) stored at 4 °C; (5) agarose gel electrophoresis unit and power supply; (6) 1.0 % ethidium bromide solution, 10 mL stored at room temperature and diluted 1:1,000 before use; (7) a shaker and container for staining agarose gels; (8) digital imager (e.g., Gel Doc XR, BioRad) for scanning agarose gels under UV illumination; (9) thermo printer for printing digital gel images; and (10) proper waste disposal bags and containers.
5. SSO probes stored at 4 °C.
6. DNA hybridization trays stored at room temperature.
7. Hybridization Pack (5× SSPE stored at 4 °C) with three components: (1) denaturation solution, 1.0 mL; (2) hybridization solution, 80 mL; and (3) stringent wash solution, 200 mL.
8. Color development pack stored at 4 °C, with five components: (1) rinse solution, 5 × 150 mL; (2) conjugate, 100 × 1.5 mL; (3) conjugate diluent, 150 mL; (4) substrate buffer, 235 mL; and (5) substrate, 100 × 1.5 mL.
9. LiPA-scan reading template stored at room temperature.
10. Fixed-angle microcentrifuge (e.g., SFA13K, Savant).
11. Swing-bucket centrifuge (e.g., Model 5804, Eppendorf) with adaptors for 96-well plates.

12. 96-Well thermocycler (e.g., ABI 2720 Thermal Cycler) for PCR.
13. Equipment (e.g., Auto LiPA 30) for automated DNA hybridization.
14. Digital scanner (e.g., Epson Perfection 1660 PHOTO) and desktop PC for allele assignment.

### **2.2. Materials for SBT**

1. Agarose gel electrophoresis system and supplies, as listed under Subheading 2.1.
2. AlleleSEQR HLA core reagent pack from Atria Genetics (distributed by Abbott Molecular), including AmpliTaq Gold stored at  $-20^{\circ}\text{C}$  and NaOAc/EDTA stored at  $4^{\circ}\text{C}$ .
3. AlleleSEQR HLA-B SBT pack from Atria Genetics, which contains PCR mix and sequencing primers (B2F, B2R, B3F, B3R, B4F, and B4R) stored at  $-20^{\circ}\text{C}$ .
4. 96-Well plate and sealing film from Applied Biosystems and stored at room temperature.
5. Absolute (100 %) alcohol (Decon Labs Inc.) stored at room temperature.
6. Hi-Di Formamide (Applied Biosystems) stored at  $-20^{\circ}\text{C}$ .
7. Capillary electrophoresis consumables (Applied Biosystems) stored at  $4^{\circ}\text{C}$  (for the ABI 3130xl DNA Analyzer), including (1) POP-6 polymer, (2) running buffer (10 $\times$ ) with EDTA, and (3) 16-channel capillary.
8. Fixed-angle microcentrifuge and swing-bucket centrifuge as described under Subheading 2.1.
9. 96-Well thermocycler (ABI 2720 Thermal Cycler) for PCR, cycle sequencing, and DNA denaturation.
10. DNA sequencer (e.g., ABI 3130xl DNA Analyzer, Applied Biosystems) with proper software for allele assignment.

### **2.3. Materials for Association Analysis**

1. HLA genotyping results from a study population with adequate sample size.
2. Well-defined outcome measures, quantitative or categorical.
3. Computer with appropriate software (e.g., SAS and SAS Genetics).
4. Reference articles that describe HLA associations of interest.
5. References that report on distribution of HLA variants in relevant populations.

### **2.4. Materials for ELISpot Assay**

1. Cell culture (R-10) medium store at  $4^{\circ}\text{C}$ —prepare fresh medium each time using the recipe below:  
 RPMI 1640 medium (Invitrogen), 90 mL.  
 Human AB serum (Sigma), 10 mL.  
 HEPES buffer (Invitrogen), 2.5 mL.

Pencillin/Streptomycin (10,000 U/mL and 10 mg/mL, Mediatech), 0.5 mL.

L-Glutamine (200 mM, Mediatech), 1.0 mL.

2. Peripheral blood mononuclear cells (PBMCs): fresh PBMCs (heparinized blood separated over a Ficoll/Histopaque gradient) or PBMCs stored in liquid nitrogen.
3. Benzonase nuclease (250 U/ $\mu$ L, Semba Biosciences).
4. R-10 plus benzonase: dilute benzonase to a final concentration of 50 U/mL using R-10 media.
5. 37 °C water bath.
6. Biosafety hood for handling cells under sterile conditions.
7. Antibodies and substrate for visualizing cells forming spots, including (1) coating antibody (1 mg/mL, anti-human mAb 1-D-1K, Mabtech) diluted 1:200 (5  $\mu$ g/mL) in sterile PBS, pH 7.2; (2) biotinylated detection antibody (1 mg/mL, clone 7-B6-1, Mabtech), diluted 1:1,000 (1  $\mu$ g/mL) in antibody dilution buffer; (3) streptavidin and alkaline phosphatase conjugate (1.0 mL, Southern Biotechnology), diluted 1:500 in antibody dilution buffer; and (4) BCIP/NBT (5-bromo-4 chloro-3-indolyl phosphate/nitroblue tetrazolium, Southern Biotechnology).
8. Antibody dilution buffer (1 $\times$  PBS with 1 % fetal bovine serum and 0.01 % Tween-20).
9. Washing buffers, either 1 $\times$  PBS (PBS alone) or 1 $\times$  PBS plus 0.01 % (v/v) Tween 20 (PBS and Tween).
10. Peptide antigens—lyophilized 9-11mer peptides are reconstituted at 20 mg/mL in 100 % DMSO (stock solution stored at -80 °C) and used at a final concentration of 2–10  $\mu$ g/mL.
11. Phytohemagglutinin (PHA) for positive control, at 5  $\mu$ g/mL.
12. ELISpot plates and seals, including 96-well MultiScreen<sub>HTS</sub> HA 0.45  $\mu$ m filter plates, sterile and clear (Millipore) and 96-well MultiScreen IP (Immobilon P or hydrophobic PVDF) membrane (Millipore).
13. Miscellaneous supplies for cell counting and plating: (1) hemocytometer; (2) 5, 10, and 25 mL graded sterile serological pipettes; (3) sterile pipette tips (20–1,000  $\mu$ L); (4) multichannel pipette (200  $\mu$ L); (5) repeater Pipette; (6) sterile syringe filters (low protein binding 0.22  $\mu$ m pore size); (7) 20–50 mL sterile syringe; (8) 15 and 50 mL sterile polypropylene tubes; (9) absorbent paper towels; (10) sterile polystyrene basins; (11) 0.4 % Trypan blue (Sigma-Aldrich Co.).
14. Spot-counting equipment—stereo microscope (e.g., Leica gz7) or automated ELISpot reader (e.g., ImmunoSpot Analyzer, Cellular technology Ltd.).
15. ELI-Puncher Kit for removing plate covers (optional).

### 3. Methods

#### 3.1. HLA-B Genotyping Using SSO (See Note 3)

##### 3.1.1. Part I: Set up and Run PCR (See Note 4)

1. Thaw reagents listed below and vortex briefly before making a PCR master mix using one of the recipes given below (for 10 or 20 reactions).

Number of PCR reactions	10	20
PCR grade water ( $\mu\text{L}$ )	120	240
Amplification Buffer ( $\mu\text{L}$ )	50	100
Primer Solution ( $\mu\text{L}$ )	50	100
Lipa-Taq ( $\mu\text{L}$ )	6.5	13

2. Vortex the PCR master mix and then aliquot 22.5  $\mu\text{L}$  into each PCR tube (or 96-well plate).
3. Add 2.5  $\mu\text{L}$  of target DNA (adjust concentration to  $\sim 50 \text{ ng}/\mu\text{L}$ ) to the PCR master mix in each tube (see Note 5).
4. Cap PCR tubes and vortex to mix contents.
5. Centrifuge the reaction tubes at high speed ( $13,000 \times g$ ) for 10 s and make sure that all the volume (25  $\mu\text{L}$  total) is at the bottom of the tube.
6. Place PCR tubes or plate in a thermocycler and run the following cycle

1 cycle	5 min at 96 °C
5 cycles	30 s at 96 °C $\rightarrow$ 50 s at 64 °C $\rightarrow$ 50 s at 72 °C
5 cycles	30 s at 96 °C $\rightarrow$ 50 s at 62 °C $\rightarrow$ 50 s at 72 °C
12 cycles	30 s at 96 °C $\rightarrow$ 50 s at 60 °C $\rightarrow$ 50 s at 72 °C
18 cycles	30 s at 96 °C $\rightarrow$ 50 s at 55 °C $\rightarrow$ 50 s at 72 °C
1 cycle	10 min at 72 °C
Hold	$\infty$ at 4 °C

##### 3.1.2. Part II: Verifying PCR Amplicons

1. Prepare a 1.3 % agarose gel in 1 $\times$  TBE buffer: weigh 2 g of agarose powder and add to 150 mL of 1 $\times$  TBE in a 250 mL flask. Heat the mixture in a microwave oven at 1 min increments until agarose is fully melted into solution. Leave melted agarose at room temperature for 10 min and pour enough into a large gel cassette. Alternatively, melted agarose is sealed in the flask and kept in a 60 °C water bath for a few hours until use.
2. Mix 2  $\mu\text{L}$  (4 %) of PCR reaction with 6  $\mu\text{L}$  of 2 $\times$  DNA loading dye and load the sample to the agarose gel immersed in 1 $\times$  TBE buffer.
3. Load 5  $\mu\text{L}$  DNA ladder to each section of the agarose gel.

4. Connect electrophoresis unit to a power supply and run at 140 V constant power for 30 min.
5. Stain gel in ethidium bromide solution (20 ng/mL) for 10 min and transfer gel to a large container filled with ample amount of distilled water.
6. Place gel in a digital imager with UV illumination and CCD camera.
7. Print a picture of the gel for evaluation of PCR quality.
8. Proceed to Part III below if PCR amplicons are detected at the expected sizes (see Notes 6–7).

**3.1.3. Part III: DNA Hybridization Using the Auto LiPA 30 System (Tecan US, Research Triangle Park, NC)**

1. Follow the standard operation manual provided by the manufacturer (see Notes 8–10 and Appendix 2).
2. At the completion of all automated procedures, allow the SSO strips to air dry for 10–20 min in the hybridization tray (see Note 11).
3. Use plastic tweezers to pick up each SSO strip and apply it to the LiPA-Scan Reading Template.
4. Align the control line (the second line on each strip, counting from the top) on each strip with the marks on the template sheet.
5. Label each sheet with the study code, target locus (*HLA-B*) and date of assay.
6. Correctly and clearly label each strip with the sample ID.

**3.1.4. Part IV: Digital Imaging and Computer-Assisted Allele Assignment**

1. Open the LiPA HLA V5.0 program installed on a desktop PC connected to a scanner.
2. Follow program-specific instructions to scan strips and assign alleles (see Fig. 1 and Notes 12–13).
3. Record results in an Excel file or other designated data files.

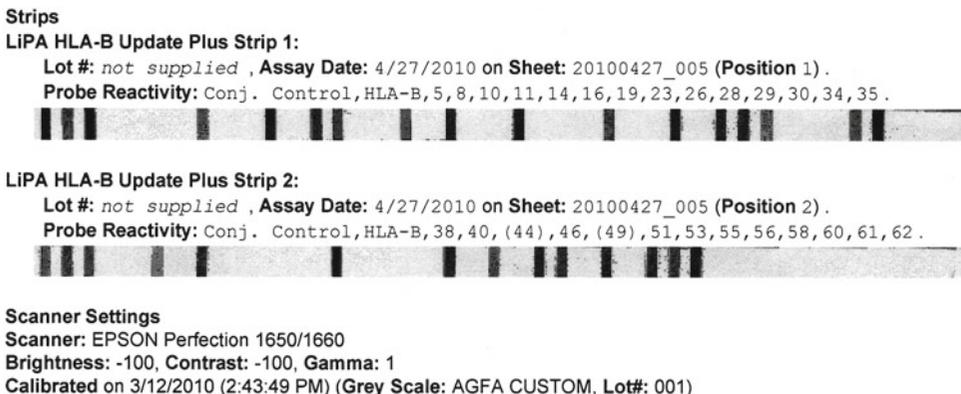


Fig. 1. *HLA-B* genotyping using sequence-specific oligonucleotide (SSO) probe hybridization. The two SSO strips (1 and 2) contain 67 sequence-specific oligonucleotide probes. After digital scanning, the first three bands (*lanes on the left*) are for internal controls (e.g., test of conjugate and orientation). This particular test reveals 14 positive bands on Strip 1 (probes 5 to 35) and 11 more positive bands on Strip 2 (probes 38 to 62). Alleles assigned to the test sample are B\*08 (Bw6) and B\*57:02 (Bw4).

4. Print hard copies for filing if necessary.
5. Evaluate results in the context of known/expected allele frequencies and patterns of linkage disequilibrium (see Notes 14–17).
6. Run confirmatory assays whenever necessary.
7. Resolve ambiguities through SBT (Subheading 3.2 below).

### 3.2. Sequencing-Based Typing (See Note 17)

#### 3.2.1. Part I: PCR Set-up and Cycling Conditions (See Notes 18–19)

1. Prepare a fresh PCR mix using proper amounts of AmpliTaq Gold and the PCR premix from the *HLA-B* SBT kit (see Notes 18–19):

No. of PCR reactions	PCR premix (from kit)	AmpliTaq Gold (5 U/ $\mu$ L)
10	80 $\mu$ L	1.0 $\mu$ L
25	200 $\mu$ L	2.5 $\mu$ L
40	320 $\mu$ L	4.0 $\mu$ L

2. Transfer 8.1  $\mu$ L of PCR mix from step 1 to each reaction tube.
3. Add 1.5  $\mu$ L of genomic DNA (20 ng/ $\mu$ L) or sterile water (negative control) to each reaction tube containing the PCR mix.
4. Label reaction tube with sample ID and date and briefly mix the contents by tapping.
5. Centrifuge the reaction tubes at high speed for 10 s and make sure that all the volume (10  $\mu$ L total) is at the bottom of tube.
6. Place tubes in a thermocycler with a preheated lid (96 °C) and proceed with the following cycling profile:

Step	Number of cycles	Temperature (°C)	Time
Start	1	95	10 min
		96	20 s
Cycling	36	60	30 s
		72	180 s
End	1	4	$\infty$

7. Run 2.5  $\mu$ L of PCR product on a checking gel (1.3 % agarose in 1 $\times$  TBE buffer, as in Subheading 3.1) (see Note 20).

#### 3.2.2. Part II: Purification of PCR Amplicons (See Note 21)

1. Add 3  $\mu$ L of ExoSAP-IT enzyme mix (from kit) to each reaction tube and centrifuge briefly to combine enzyme and PCR amplicon.
2. Incubate samples in a thermocycler with the following cycling profile:

Step	Number of Cycles	Temperature (C)	Time
Incubation	1	37	30 min
		80	15 min
Stop	1	4	$\infty$

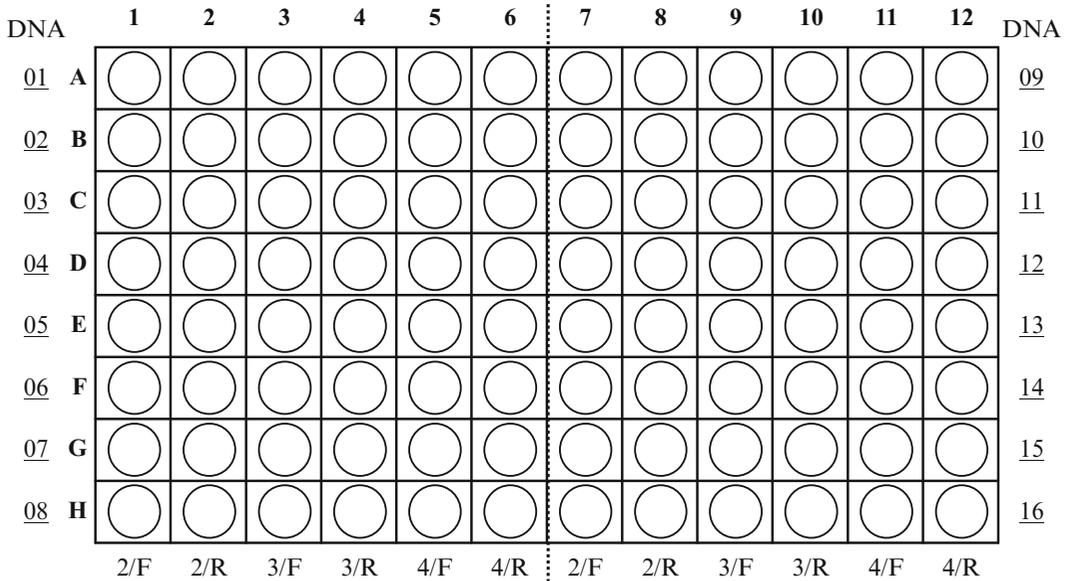


Fig. 2. Layout of a 96-well plate used for PCR cycle sequencing of *HLA-B* alleles. Orientation for rows (A–H) and columns (1–12) are shown in *bold*. Each plate is divided into two halves (*dotted line*) and each half can accommodate reactions for up to eight DNA samples (*underlined*, 01–08 on the *left* and 09–16 on the *right*). For each test sample, its locus-specific PCR amplicons are sequenced in forward (F) and reverse (R) direction to yield the sequences for three exons (from exon 2 to exon 4). Partial intronic sequences can be read after sequencing. However, they are not used for allele assignments because few alleles in the public database (e.g., IMGT) have defined intron sequences.

3. Dilute treated PCR product with 1× TE buffer or sterile deionized water (2 volumes of diluent plus 1 volume of purified PCR product).
4. Mix diluted PCR product by pipetting when ready for cycle sequencing (Part III below).

3.2.3. Part III: Cycle Sequencing Using Big Dye Chemistry (Applied Biosystems, Inc.)

1. Plan experiments for up to 16 samples in a single 96-well plate (Fig. 1).
2. Thaw sequencing mix and briefly vortex the tube.
3. Centrifuge sequencing mix at high speed for 10 s before use.
4. Aliquot 3 μL of ExoSAP-IT treated PCR product (diluted, see Part II in this section) to each well on a properly labeled and correctly oriented plate (Fig. 2).
5. Add 7 μL of sequencing mix to each of the wells containing diluted PCR product (see Note 22).
6. Place the 96-well plate in a desktop (swing bucket) centrifuge with proper adaptors and spin plate at 500×g for 30 s (to collect all volume at the bottom of each well).
7. Place the sequencing reactions in a thermocycler and cover plate with a rubber seal designated for the machine.
8. Proceed with the following cycling profile:

Step	No. of cycles	Temperature (°C)	Time
Cycling	25	96	20 s
		50	30 s
		60	2 min
Holding	1	4	∞

*3.2.4. Part IV: Purification of Sequencing (Extension/Termination) Products (See Note 23)*

1. Add 2.5  $\mu\text{L}$  of NaOAc/EDTA Buffer (from kit) to each 10  $\mu\text{L}$  sequencing reaction.
2. Seal the 96-well plate with an optical adhesive cover.
3. Spin plate in a desktop (swing bucket) centrifuge with proper adaptors, at  $500\times g$  for 30 s to collect the whole volume at the bottom of each well.
4. Add 25  $\mu\text{L}$  of absolute ethanol (EtOH) to each sequencing reaction and seal the whole plate again.
5. Vortex the plate vigorously for 1 min.
6. Centrifuge plate at  $2,000\times g$  for 8 min to precipitate DNA.
7. Remove the supernatant by inverting the plate onto a paper towel covered with Kimwipes (Kimtech Science,  $11\times 21$  cm).
8. Centrifuging plate again at  $100\times g$  for 10 s and add 50  $\mu\text{L}$  of 80 % EtOH to each well.
9. Centrifuge plate at  $2,000\times g$  for 8 min.
10. Repeat step 8 to decant supernatant.

*3.2.5. Part V: Capillary Electrophoresis and Data Collection Using ABI 3130xl DNA Analyzer (Applied Biosystems)*

1. Add 15  $\mu\text{L}$  HiDi formamide to each well.
2. Denature purified sequencing products in a thermocycler—a single cycle at 95 °C for 3 min.
3. Open the “Foundation Data Collection” software and design a “sample sheet” for each run (mapping out individual samples and reactions tested in each plate).
4. At the completion of electrophoresis, open the “Assign 3.5” program (see Note 24).
5. Import raw sequencing results from ABI 3130xl.
6. Use the “Assign 3.5” software for allele assignments against current HLA allele database (see Notes 25–26).
7. Consider use of alternative techniques to validate typing results or resolve ambiguities (see Note 27).

*3.2.6. Data Analyses to Identify Individual HLA Variants Associated with Phenotypic Traits*

1. Merge clinical data with genotyping results, using software like SAS (see Notes 28–29).
2. Tabulate HLA allele and local haplotype frequencies in the overall study population and assess genetic heterogeneity between subgroups defined by outcome measures (e.g., cases versus controls).

3. Evaluate Hardy-Weinberg equilibrium (HWE) (observed vs. expected frequencies of specific genotypes—homozygous and heterozygous combinations).
4. Calculate population (marker) frequency for common variants (see Note 30).
5. Proceed with univariate association analyses by logistic regression if phenotypic traits are categorical variables (see Notes 31–32).
6. Apply nongenetic factors (e.g., age, gender, and ethnicity) as covariates for adjusted odds ratio and 95 % confidence intervals.
7. Obtain *P* value from each test using Mantel-Haenszel  $\chi^2$  or Fisher exact test.
8. When the phenotypic traits are continuous variables, apply the generalized linear regression model (GLM) statistics to obtain (1) mean parameter (beta) estimate, (2) standard error of the mean, and (3) *p* value.
9. Apply mixed models if outcomes are quantitative measures gathered regularly over a study period.
10. Run multivariable association models when several factors show associations with the same outcome measure (see Note 33).
11. Eliminate factors with adjusted *p* values >0.05 until a reduced model is established
12. Separate novel (new) from confirmatory findings whenever possible (see Note 34)
13. Check false discovery rates for “novel” associations.
14. Consider other alternative hypotheses, including: (1) heterozygosity advantage or homozygosity disadvantage (9), (2) classification of HLA by supertype (see Note 35)
15. Verify key findings with another biostatistician or SAS programmer.

### **3.3. ELISpot Assay to Test Epitope- Specific Immune Responses**

#### *3.3.1. Part I: Coating Plates (See Notes 36–37)*

1. Under sterile conditions, add 100  $\mu$ L/well of mouse anti-human IFN- $\gamma$  antibody in 96-well ELISpot plates.
2. Incubate the plates overnight at 4 °C.
3. Use plate for procedures listed in Part IV below.

#### *3.3.2. Part II: Preparing Culture Medium and Cells (See Notes 38–39)*

1. Prepare fresh R-10 plus benzonase medium before each assay.
2. Prewarm the medium to 37 °C.
3. Transfer the PBMCs in cryovial from liquid nitrogen directly to a 37 °C water bath.
4. Wait until only a small amount of ice remains in the cryovial.

5. Remove the cryovial from water bath, wipe it clean with 70 % ethanol, and then transfer it to a biosafety hood.
6. Slowly add 1.0 mL of pre-warmed R10-Benzonase into the cryovial containing PBMCs.
7. Transfer the cell suspension to a 15 mL polypropylene conical bottom centrifuge containing 8 mL of pre-warmed R10-Benzonase media per vial of cells thawed.
8. Centrifuge the cells at  $250 \times g$  for 8 min.
9. Decant the media before adding 10 mL R-10.
10. Centrifuge again at  $250 \times g$  for 8 min.
11. Resuspend cells at 2 million per/mL and plate the cells in a sterile 6-well plate or T-25 cm<sup>2</sup> flask.
12. Leave cells to rest at 37 °C overnight.

*3.3.3. Part III: Assessing Cell Number and Viability (See Note 40)*

1. Stain dead cells with Trypan blue.
2. Count live cells using a hemocytometer.
3. Resuspend live cells in R10 medium at  $2 \times 10^6$  cells/mL.

*3.3.4. Part IV: Blocking the Antibody-Coated Plate (See Note 41)*

1. Discard the coating antibody from 96-well plate.
2. Wash the plate 6 times (6 $\times$ ) with 200  $\mu$ L sterile PBS (5 min for each wash).
3. Add 200  $\mu$ L of R-10 medium per well and incubate the plates for 2 h at 37 °C (with 5 % CO<sub>2</sub>).
4. Proceed to Part V.

*3.3.5. Part V: Antigenic Stimulation of PBMC (See Notes 42–45)*

1. Discard the blocking solution from 96-well plate.
2. Gently plate 50  $\mu$ L of cells in duplicate wells (100,000 cells/well).
3. Add the antigen in another 50  $\mu$ L volume—total cell + antigen = 100  $\mu$ L.
4. Incubate at 37 °C in 5 % CO<sub>2</sub> overnight (16–18 h).

*3.3.6. Part VI: Preparing and Applying Secondary Antibody (See Notes 46–47)*

1. Wash plate with 6 $\times$  with 1 $\times$  PBS + 0.01 % Tween-20, allowing buffer to sit in plate for 5 min each time.
2. Wash plate 2 $\times$  with deionized water, leaving 5 min between the washes.
3. Dilute anti-human IFN- $\gamma$ -biotinylated (7B61 from Mabtech)—1–1,000 dilution in antibody dilution buffer, at a final concentration of 1.0  $\mu$ g/mL.
4. Pass diluted antibody through a 0.22  $\mu$ m, low protein binding syringe filter.
5. Add 100  $\mu$ L diluted antibody to each well and incubate the plates for 2 h at room temperature.

3.3.7. *Part VII: Enzyme Conjugate*

1. Wash plates 6× with 1× PBS + 0.01 % Tween-20, leaving buffer in plates for 5 min between washes
2. Add alkaline phosphatase-conjugated streptavidin (SA-AP), diluted 1:500 in antibody dilution buffer.
3. Add 100 μL of diluted SA-AP to each well.
4. Incubate plate for 45 min to 1 h at room temperature.

3.3.8. *Part VIII: Development of Spots (See Notes 48–50)*

1. Wash plates 3× with 1× PBS + 0.01 % Tween-20, as in Part VII.
2. Wash plates 3× with 1× PBS alone.
3. Add 100 μL of substrate (NBT/BCIP) to each well.
4. Wait 5–10 min or until spots are maximally developed.
5. Wash plates with deionized H<sub>2</sub>O.
6. Remove the underdrain from each plate.
7. Rinse the backside of each plate with water.
8. Blot dry plate with absorbent towels.
9. Air dry plate at room temperature.

3.3.9. *Part IX: Visualizing and Counting Spot (See Fig. 3 and Notes 51–55)*

1. Set stereo microscope (e.g., Leica gz7) at ×40 magnification.
2. Connect microscope to a computer-assisted video image analysis system.
3. Count spots in each well.
4. Convert counts to spot-forming cells (SFC) per million PBMCs.
5. Enter results into a spreadsheet.
6. Analyze results using desired statistical software package.

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## 4. Notes

### SSO Assays

1. The reagents and supplies used for SSO are mostly from commercial sources. Only self-made solutions are accompanied by detailed recipes.
2. The primers used for SSO are considered proprietary. The manufacturer does not disclose the exact primer sequences, but partial information (e.g., codon specificity for individual SSO probes) can be obtained.
3. The “INNO-LiPA HLA-B Update Plus” kit (Innogenetics) provides a line probe assay that can resolve *HLA-B* alleles at the allele group level (corresponding to a 2-digit number). Similar kits can be obtained from other vendors.

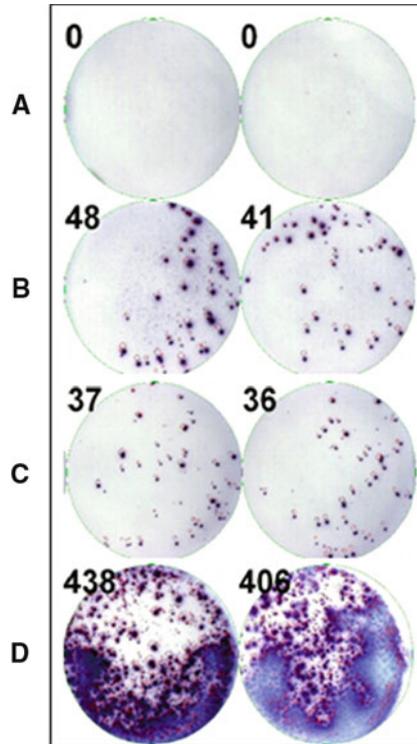


Fig. 3. A representative IFN- $\gamma$  ELISpot assay that tests responses of PBMCs (in duplicates of 10,000 cells per treatment) to: (1) no specific antigen (row A), (2) HLA-B\*57:01-restricted epitope, KAFSPEVIPMF derived from HIV-1 capsid protein Gag (row B), (3) another HLA-B\*57:01-restricted epitope, TSTLQEQIGWF derived from HIV-1 Gag (row C), and (4) the PHA mitogen (row D). The numbers next to each well correspond to the spot forming cells captured by the ImmunoSpot analyser (C.T.L.) software.

4. The PCR amplification pack can be used for all three HLA-I genes (*HLA-A*, *HLA-B*, and *HLA-C*). The PCR reaction volume (25  $\mu$ L) cited in Subheading 3.1 is half of what is recommended by the manufacturer and there is no compromise in assay quality. To allow minor pipetting errors and loss of volume in the mixed solutions, each PCR master mix should be enough for  $n+1$  reactions, where  $n$  is the desired number of tests.
5. DNA quality and quantity are critical. High molecular weight genomic DNA can be extracted from fresh or frozen peripheral blood, buffy coat, PBMC, and cervicovaginal lavage samples using the QIAamp Blood Kit and protocols recommended by the manufacturer (QIAGEN Inc., Valencia, California, USA). Whole genome amplification can be used if DNA quantity is a limiting factor and at least 10 ng of high-quality genomic DNA is available as the template for WGA (10).
6. When *HLA-B* amplicons are visualized on agarose gels, there should be three distinct bands to represent exons 2 (555 bp), 3 (436 bp), and 4 (323 bp), respectively.

7. Occasionally, locus-specific PCR amplification may yield nonspecific bands or smeared products. Use of a heat-activated *Taq* polymerase (e.g., Platinum Taq from Life Technologies) can reduce such problems.
8. SSO assays described here require specialized equipment for automated DNA hybridization (Auto LiPA 30). While individual laboratories may find these platforms unaffordable, access to core facilities can usually solve the problem.
9. For auto cleaning of the Auto LiPA 30 system, we recommend the use of autoclaved and distilled water; all beakers should be cleaned periodically to reduce chemical build-up (precipitates).
10. *HLA-B* typing requires 67 sequence-specific probe lines and 2 (positive and negative) control lines captured on two membrane strips (orientation is important).
11. SSO strips left in the hybridization tray should not be dried for a prolonged period of time as they tend to stick together. It is always safe to skip the last aspiration step because washing solution can be emptied manually.
12. In addition to allele assignments based on combinations of positive SSO probes, the individual reactivity can be captured to facilitate analysis of *HLA-B* sequence motifs (corresponding to polymorphic codon positions). Automatic export is possible with a slight modification of the LiRAS™ interpretation software (11).
13. If scanned SSO strips fail to produce any specific alleles, nonspecific bands may have been detected or there may be a problem with contamination or degradation of the DNA sample. As a precaution, results must be verified by two individuals in a timely manner (part of quality assurance—see Note 17 in this section).
14. Starting from April 2010, *HLA* allele names have taken new forms—semicolons are used to separate 2-digit allele groups from the next 2-digit allele names (1). Results from old assays do not have the semicolons.
15. As shown elsewhere (12), SSO can be used to define common alleles in *HLA-II* genes. In addition, following resolution of individual alleles at each locus, *DRBI-DQAI-DQBI* haplotypes can be reliably assigned according to known patterns of linkage disequilibria observed in various populations (13, 14).
16. *HLA* genotyping requires close attention to quality assurance. The standards of quality control set forth by the American Society of Histocompatibility and Immunogenetics (ASHI) are recommended. Briefly, areas for DNA extraction and PCR set-up are physically separated from areas for PCR amplification and electrophoresis. Genotyping results are read by two individuals (e.g., ASHI-certified specialists or well-trained technicians) before acceptance and recording in Excel spreadsheets.

Genotyping data can be readily compared with those reported in the vast literature for populations of similar ethnicity and geography. Alternatively, select samples can be typed by different laboratories or by different techniques in the same laboratory for consistent results. A further step toward minimizing genotyping errors is the use of randomly generated sample duplicates for testing at different time intervals or by different laboratory technicians. Matching results (100 % concordance) for duplicated specimens are often indicative of correct sample handling and data recording.

ASHI also recommends participation in the College of American Pathologists (CAP) quality control program, in which specimens of unknown genotypes are intermittently distributed for testing by multiple laboratories. Demonstration of proficiency with appropriate accuracy and resolution in typing HLA genes is necessary for laboratories that have just started doing HLA typing. Campus-wide CAP membership may reduce the CAP service fees. The less expensive (about \$700/year) ASHI Proficiency Testing is also a valid alternative.

### SBT

17. SBT is ideal for high-resolution HLA typing. The procedures described here are applicable to the use of *HLA-B* SBT kits from Conexio Genomics (distributed by Abbott Molecular). When the ABI 3031xl DNA Analyzer (Applied Biosystems, Inc.) is used for capillary electrophoresis, up to two 96-well plates can be tested at the same time and each plate can accommodate 16 samples.
18. Several notes cited in “SSO Assays,” especially Notes 1, 2, 5, and 7 under Subheading 4, apply to the SBT protocol. For example, the commercially available primers for SBT are also considered proprietary.
19. The PCR premix needs brief vortexing after retrieval from a  $-20^{\circ}\text{C}$  freezer.
20. There are two PCR amplicons (1.2 kb and 1.4 kb, respectively) for each test and both should be visible after electrophoresis (with ethidium bromide staining and UV illumination).
21. Before cycle sequencing, the *HLA-B* PCR amplicons are treated with ExoSAP-IT to eliminate unincorporated primers and excess dNTPs in the PCR mix.
22. The PCR cycle sequencing reactions target three informative exons (2–4) in both forward and reverse directions, so each test sample requires six individual sequencing reactions.
23. After cycle sequencing, DNA extension/termination products and their templates (PCR amplicons) are precipitated by ethanol; excess nucleotides and salts are washed off before capillary electrophoresis. This is a critical step, as purity is essential to high-quality sequencing results.

24. The “Assign 3.5” software (Conexio Genomics) is protected by a key code that requires annual renewal (free with SBT kits).
25. SBT may occasionally detect novel (new) alleles not found in any official database. These rare variants are most likely accompanied by other common alleles, i.e., in heterozygous samples. Their identity can be resolved by cloning, by allele-specific amplification and sequencing, or by single-genome analysis (protocols and recommendations available upon request).
26. For *HLA-B* allele assignments, the IMGT/HLA 2.26 release (July 2009) (also see Appendix 1) has been used in our recent work.
27. PCR with sequence-specific primers (SSP) can be an alternative technique. Use of PCR-SSP is attractive when (1) DNA quantity is not a limiting factor; (2) high-resolution typing is not necessary (e.g., for initial screening); and (3) laboratory is equipped with 96-well thermocyclers (for PCR), large-size electrophoresis units (for agarose gels), and digital imaging system (for photography of gel images).

#### Association Analysis

28. Computer software is rarely standardized, but we recommend the programs in SAS version 9.2 (including SAS Genetics).
29. When  $2N$  (number of chromosomes) is used as the denominator for allele/haplotype frequencies, the combined (total) frequency at a given locus should be 1.00 (100 %). At this point, overall locus-wide tests (Fisher exact or random simulations) can reveal genetic heterogeneity between comparison groups.
30. For calculating marker/carriage frequencies, the denominator is  $n$  (number of subjects for each comparison group); association analysis should be based on marker frequencies, with proper statistical adjustments for nongenetic factors (age, sex, ethnicity, etc.).
31. Unlike single nucleotide polymorphisms (SNPs), HLA variants resolved by SSO or SBT assays represent units encoded by the entire gene, with a string of SNPs. Interpretation of HLA associations can be misleading if results are not analyzed properly and consistently.
32. Hypothesis-free comparisons are bound to have high false discovery rates if a nominal  $p$  value  $\leq 0.050$  is considered statistically significant. Tests need to focus on HLA variants that are frequent enough (e.g.,  $\geq 4\text{--}5\%$ ) in the study population. Genetic heterogeneity revealed by global comparisons may offer some useful clues.
33. In a typical multivariable model, HLA and nongenetic factors associated with specific outcome measures are treated as independent factors. However, HLA factors with strong linkage disequilibrium (e.g., B\*14 and C\*08 or DRB1\*15:01 and

DQB1\*06:02) should not be treated as separate entities although they may have distinct function.

34. Whenever possible, alternative models can be tested in order to sort out the major from minor contributors.
35. Individual HLA alleles with similar peptide motif preferences can be grouped as supertypes (15). These are defined primarily by amino acid residues that form the B and F pockets in the peptide-binding groove. In the context of antigen presentation, association analyses may benefit from the testing of supertypes.

#### IFN- $\gamma$ ELISpot Assay

36. HA or Immobilon P plates can vary in sensitivity; they should be optimized based on low background readings for cells exposed to media alone.
37. ELISpot assay can give negative reading if they have incomplete coating with anti-IFN- $\gamma$  antibody.
38. For the human AB sera, avoid switching batches between experiments as background readings can change.
39. PBMCs stored in cryovial should be thawed properly (no shaking). The cells can be left to rest overnight for two reasons: (1) thawing of cells release intracytoplasmic compounds, which can increase background signals; (2) dead cells can generate false positive results.
40. PBMC samples with less than 60 % viability should not be used for testing.
41. Plates should never be allowed to dry out during the experiment.
42. PBMC concentration should not exceed 200,000 cells/well (to avoid multilayers).
43. Cells in each well should be evenly distributed.
44. Once antigens are added, the plates should remain still during incubation (to avoid smears/streaks of spots).
45. During incubation, the plates should not be stacked on top of each other (to avoid uneven temperature across the plate).
46. In washing buffer, Tween-20 concentration should not exceed 0.01 %.
47. Filtration of secondary antibody helps minimize nonspecific spot formation caused by protein aggregates.
48. Before color development, final washes with PBS are very important, as Tween-20 interferes with spot development.
49. Several reagents should be handled with care: (1) PHA is a mitogen that causes irritation on human skin; (2) undiluted Tween-20 is highly viscous; (3) NBT is a skin irritant and

- harmful if ingested; a fine black precipitate may develop during storage (no impact on product performance).
50. Extended incubation with antigens, enzyme conjugate, and color development solutions can ruin an experiment by increasing background readings.
  51. The spots may become sharper if the plates are stored at 4 °C overnight prior to reading.
  52. Spots produced by activated cells have a dark center with fuzzy borders, while artifacts can be developed by dead cells, antibody aggregation, or inadequate removal of unbound cells from the plate.
  53. After the experiment, plates can be kept in the dark for long-term storage.
  54. A cut-off for negative response can be established after testing the spontaneous secretion of IFN- $\gamma$  from PBMCs in the absence of antigen or in the presence of irrelevant antigens. Cells stimulated with a mitogen (phytohemagglutinin) are always included as a positive control to gauge the overall functionality of T-cells. Based on several assay validation studies, a response is considered positive if the SFC unit is (1) greater than the mean values of the unstimulated cells plus 3 standard deviations and (2) at least 55 SFC units per million PBMCs are counted (16).
  55. Before mass testing, each user needs to determine the optimal cell number, antibody concentration, peptide concentration, and duration of incubation suitable for each ELISpot assay.

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## Acknowledgments

The authors are grateful to their colleagues for valuable insights. The protocols for SBT and SSO have benefited greatly from Drs. Carla M. Wirtz and Angela Alexander, respectively.

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## Appendix 1: List of Useful Online Resources Related to HLA (more links can be found at <http://www.ashi-hla.org/links/#mhc>)

Name of database	Web site
The dbMHC database	<a href="http://www.ncbi.nlm.nih.gov/gv/mhc/main.cgi?cmd=init">http://www.ncbi.nlm.nih.gov/gv/mhc/main.cgi?cmd=init</a>
IMGT/HLA database	<a href="http://www.ebi.ac.uk/imgt/hla">www.ebi.ac.uk/imgt/hla</a>
IMGT: HLA allele summary	<a href="http://www.ebi.ac.uk/imgt/hla/stats.html">http://www.ebi.ac.uk/imgt/hla/stats.html</a>

(continued)

Name of database	Web site
The immunogenomics data analysis working group (IDAWG)	<a href="http://www.igdawg.org/">http://www.igdawg.org/</a>
NIH Genetic Association Database	<a href="http://geneticassociationdb.nih.gov/">http://geneticassociationdb.nih.gov/</a>
The Immune Epitope Database (IEDB)	<a href="http://www.immuneepitope.org/">http://www.immuneepitope.org/</a>
HIV Molecular Immunology Database	<a href="http://hiv-web.lanl.gov/immunology/index.html">http://hiv-web.lanl.gov/immunology/index.html</a>

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**Appendix 2:  
Standard  
Operation Manual  
for Semi-  
automated DNA  
Hybridization (Part  
of SSO Assay): 30  
Tests per Run**

1. Turn on Auto LiPA 30 system by pressing the switch button, which is located in the back of machine.
2. Verify that there is distilled water in the three beakers for automated rinsing and cleaning.
3. Empty all waste bottles at this point.
4. Prepare the machine for running a new program: (1) select “Run program;” (2) click on “<” and “>” to until finding “liquid prep” and then press “yes;” (3) choose “auto clean” and press “yes;” (4) after a few seconds, press “yes” sequentially for distilled water and tub cleaning; (5) remove water and press “yes” to start a new assay.
5. While the machine is warming up, locate two kits labeled Color dye 40 and Hybridization solution 5×SSPE.
6. Place Hybridization solutions and stringent wash solution in a 60 °C water bath until use.
7. Prepare the following reagents for one full tray (30 tests): (1) rinse solution (in 1-L glass bottle), consisting of 300 mL ddH<sub>2</sub>O and 75 mL concentrated rinse solution (5×); (2) conjugate diluent (in bottle from supplier) containing 70 mL conjugate diluent and 700 μL 100× conjugate; (3) substrate solution with 70 mL substrate buffer and 700 μL substrate (100×).
8. When reagents are ready and the auto cleaning function is complete, remove water beakers and replace with reagents from step 7 above.
9. Verify that the reagents bottles match seven sets of colored tubes: (1) brown (1 tube) for hybridization solution (kept at 60 °C); (2) red (3 tubes) for stringent wash (was kept at 60 °C); (3) orange (3 tubes) for wash solution (in glass bottle); (4) yellow (1 tube) for diluted conjugate; (6) green (1 tube) for substrate solution; and (7) blue (1 tube) for diluted substrate.

10. Set the desired SSO temperatures at 56 °C.
11. Press any key to continue and press start button to run program designated as “HLAB56.”
12. Answer “Yes” sequentially to all built-in options (e.g., distilled water, sieve check, etc.).
13. Wait for ~20 min until the system reaches the desired SSO conditions.
14. Load SSO probe strips (1 and 2) to the 30-well hybridization tray.
15. Make sure that all SSO strips have the colored top (probe side) facing up.
16. Add 10 µL of denaturing buffer to the top of each trough.
17. Load 10 µL of PCR product to the spot of denaturing buffer.
18. Insert tray into the Auto LiPA 30 and press any key on display panel to continue.
19. Select start position 1 and stop position 30 using the < > keys.
20. Select “No” when prompted for “Last aspiration.”
21. Select “Yes” when prompted for “Procedure Inc 1.”
22. When assay is done, press the “Pause” button then the “Continue” to allow aspiration of washing solution from the troughs.
23. Take out the used 30-well tray and load another empty tray.
24. Replace all the buffer containers with beakers filled with distilled water.
25. Repeat the auto cleaning procedures (*see* step 4 in this section).
26. Press “No” to disallow decanting water from tubes.
27. Exit program and switch off the power.

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## Protocol for the Clonal Analysis of NK Cell Effector Functions by Multi-parameter Flow Cytometry

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### Abstract

Natural killer (NK) cells provide a first line of defense against viral infections and prepare the ground for subsequent action of virus-specific T cells in a concerted way. Human NK cells use a sophisticated system of inhibitory and stimulatory receptors of the killer cell immunoglobulin-like receptor (KIR) gene family, which are expressed in a clonally distributed manner. Several studies suggest that KIR play a critical role in NK cell-mediated protection against HCV and HIV infection. As each NK cell expresses an individual set of KIR receptors that enables them to sense differences in HLA class I expression, classical measurement of NK cell function by analysis of target cell killing does not enable one to define and isolate the clinically relevant NK cell effector subsets. Here, we have developed a flow cytometry-based protocol to measure cytolytic activity together with KIR expression at a clonal level. Combined analysis of KIR expression in conjunction with cell surface mobilization of CD107 enables precise enumeration of cytolytic NK cells with defined specificity for HLA class I. Moreover, via inclusion of intracellular perforin or alternatively granzyme B, NK cells with deficient loading of cytotoxic granula can be identified. The present protocol enables identification and isolation of cytotoxic NK cells on a clonal level and enables reliable measurement in healthy as well as in pathological settings such as virus infection and hematological disease.

**Key words:** NK cells, NK cell function, Cytotoxicity, CD107, Granzyme B, Perforin, Flow cytometry

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### 1. Introduction

Natural Killer (NK) cells are lymphocytes that play a vital role in the early immune defense against tumor cells and viral infections. In this regard, it has long been known that patients with NK-cell deficiencies suffer from recurrent viral infections such as herpes viruses (1). Moreover, genetic and functional evidence is now accumulating that the course of HIV infection is significantly influenced by NK cells with respect to resistance to infection, protection from maternal-infant transmission, and the delay in disease

progression (2–4). Similarly, a protective role of NK cells was suggested in hepatitis C by a study showing linkage between NK cell receptor genes and resolution of infection (5).

In contrast to lymphocytes of the B- and T cell lineage, NK cells do not express an antigen-specific and somatically rearranged receptor. Instead, NK cells express a variety of MHC class I-specific inhibitory and stimulatory receptors, either encoded by the killer cell immunoglobulin-like receptor (KIR) family in primates and humans or the Ly-49 family in rodents (6). In humans, 12 functional KIR genes are known that can be expressed in a clonally distributed fashion, i.e., a single NK cell can express all possible combinations of KIR starting from one receptor up to all receptors encoded in the genome of that individual. The system is complemented by the more conserved CD94:NKG2A heterodimer that is specific for HLA-E and is expressed in a clonally distributed manner as well (7). Restrictions to the repertoire of expressed receptors on each NK cell are set by the HLA class I ligands, which introduce a bias towards expression of cognate inhibitory receptors for self class I molecules (8, 9). Nonetheless, NK cells lacking receptors for self and NK cells not expressing any HLA class I-specific NK receptors are commonly found in the repertoire of most individuals (10). As these NK cells are potentially autoreactive, mechanisms should be in place to inhibit their inappropriate activation. Indeed, in recent years it was shown that only NK cells expressing an inhibitory receptor for self are fully activated by MHC class I-negative target cells whereas NK cells that lack cognate NK cell receptors are hyporesponsive with regard to cytokine production and target cell killing (10, 11).

The classical method to determine cytotoxicity of NK cells is the chromium release assay (12). In this assay, a bulk population of NK cells is cocultivated with  $^{51}\text{Cr}$ -labeled target cells. When target cells are killed, chromium is released to the supernatant, which can be analyzed for radioactivity in a gamma counter. In recent years, nonradioactive cytotoxicity assays are becoming more popular. In particular, carboxyfluorescein succinimidyl ester (CFSE)-labeling of target cells in combination with propidium iodide (PI) staining of dead cells is now a reliable alternative to measuring target cell killing by flow cytometry (13).

Although the lysis of target cells provides a good estimation of the cytotoxicity of lymphocytes, it does not enable direct identification and enumeration of cytotoxic NK cells. To this end, the recently developed CD107 mobilization assay fills exactly this gap. Originally developed for the identification of cytotoxic T cells (14), it was quickly adapted for measuring cytotoxic NK cells (15). Recent studies have integrated the CD107 assay in multicolor flow cytometric protocols thereby enabling identification and isolation of cytolytic NK cells that express defined NK cell receptors (9).

The CD107a and b molecules, also known as lysosome-associated membrane proteins (LAMP1 and 2) line the inner membrane of lytic granules. In the course of the degranulation process the granules fuse with the outer cell membrane and the CD107 molecules become accessible to the cell surface. Consequently, whereas resting NK cells are negative for CD107 cell surface expression, activated cytolytic NK cells mobilize CD107 to the cell surface and expression can be measured by flow cytometry. Importantly, CD107 is not a direct marker for killing but a suitable marker for degranulation. Thus, only if the granules are filled with the necessary lytic components, does the CD107 assay constitute a suitable correlate of cytotoxic function. Lytic granules contain granzyme B, a serinprotease, and perforin, a membrane-disrupting protein (16). Both proteins cooperatively induce cell death by apoptosis in the target cell (17). Importantly, NK cells do not always express the same amount of lytic substances: under certain circumstances, for example when analyzing immature NK cells or in certain pathological settings, lytic granules might not be properly loaded with perforin or granzyme B and in these cases the lytic exocytosis will not be effective (data not shown).

Here, a flow cytometry-based protocol is presented, in which cell surface mobilization of CD107 is analyzed in combination with intracellular perforin and granzyme B staining. These functional parameters are measured together with three different inhibitory KIR receptors, which together enables a high-resolution analysis of NK cell receptor repertoires on a clonal level. Moreover, clonal analysis of NK cell function is then compared with flow cytometric analysis of target cell killing using a CFSE/PI assay.

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## 2. Materials

### 2.1. Cell Culture

1. K562 cells (HLA-class I-deficient erythroleukemic cell line).
2. 10 mL peripheral blood from patients and healthy controls.
3. Rosette Sep NK cell enrichment cocktail (Stemcell Technologies).
4. PBS/EDTA: PBS, 2 mM EDTA, pH 8.0.
5. Ficoll separating solution.
6. K562 medium: DMEM, 4.5 g/L glucose, 2 mM<sub>L</sub>-glutamine, 10 % (v/v) fetal calf serum (FCS), 100 U/mL penicillin, 0.1 mg/mL streptomycin.
7. NK medium: RPMI 1640, 25 mM HEPES, 2 mM<sub>L</sub>-glutamine, 10 % (v/v) FCS, 5 % (v/v) human AB-serum, 100 U/mL penicillin, 0.1 mg/mL streptomycin, 1,000 U/mL rh interleukin-2 (IL-2).

**Table 1**  
**Antibody staining panel for CD107 assay**

Antibody <sup>a</sup>	Clone	Conjugate <sup>b</sup>	Company	Excitation/ emission (nm)
CD107	H4A3	FITC	BD Biosciences	488/518
NKG2A or perforin or granzyme B	Z199 δG9 GB11	PE	Beckman Coulter BD Biosciences BD Biosciences	488/575
KIR3DL1	Z27.3.7	ECD	Beckman Coulter (custom conjugate)	488/620
CD56	N901	PE-Cy5	BD Biosciences	488/670
KIR2DL1/S1	EB6	APC	Beckman Coulter	633/660
KIR2DL2/3/S2	GL183	APC-Cy7	Beckman Coulter (custom conjugate)	633/775
CD3	UCHT1	Pacific Blue	Beckmann Coulter	405/455

<sup>a</sup>Instead of surface staining for NKG2A, intracellular staining for either granzyme B or perforin can be performed

<sup>b</sup>FITC fluorescein isothiocyanate, PE R-Phycoerythrin, ECD PE-Texas Red, APC allophycocyanin, Cy5 cyanine dye 5, Cy7 cyanine dye 7

## 2.2. Degranulation Assay

1. Phorbol 12-myristate 13-acetate (PMA)—Prepare a dilution of 50 µg/mL in distilled water.
2. Ionomycin—Prepare a dilution of 10 µg/mL in distilled water.
3. 96-well plate (u bottom).
4. NK Medium: RPMI 1640, 25 mM HEPES, 2 mM L-glutamine, 10 % (v/v) FCS, 5 % (v/v) human AB-serum, 100 U/mL penicillin, 0.1 mg/mL streptomycin, 1,000 U/mL rh IL-2.
5. Golgistop containing monensin (BD Biosciences).
6. Monoclonal antibodies against CD107, CD56, CD3, KIR, NKG2A, granzyme B, and perforin (see Table 1 for details of specificity, labeling, and clones).
7. Cytofix/Cytoperm Fixation/ Permeabilization Solution Kit (BD Biosciences).
8. PBS/EDTA: PBS, 2 mM EDTA.
9. FACSCanto II with FACS Diva Software 6.0.
10. FlowJo Software v8.7.

## 2.3. CFSE Killing Assay

1. Vybrant® CFDA SE Tracer Kit (Invitrogen)—Prepare a dilution of 0.5 mM CFDA-SE in DMSO.
2. PBS.

3. PBS/FCS: PBS, 20 % (v/v) FCS.
4. PBS/EDTA: PBS, 2 mM EDTA.
5. 96-well plate (u-bottom).
6. Propidium iodide staining solution (PI).
7. FACSCanto II with FACS Diva Software 6.0.
8. FlowJo Software v8.7.

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## 3. Methods

### 3.1. Cell Isolation and Culture Conditions

1. K562 cells were cultured in K562 medium at 37 °C and 5 % CO<sub>2</sub>.
2. To isolate NK cells from peripheral blood, use the NK cell enrichment cocktail (see Notes 1 and 2).
3. Add 50 µL of the NK cell enrichment cocktail per mL blood (see Note 3) and incubate for 20 min at room temperature (RT).
4. Dilute the blood 1:1 with PBS and add it slowly to a 50 mL tube, prefilled with 15 mL Ficoll. Centrifuge the sample for 35 min at 440×g and RT without brake. Carefully harvest the interphase (see Note 4).
5. Wash the cells with PBS/EDTA (7 min, 440×g, RT).
6. To isolate mononuclear cells (MNCs) from peripheral blood, add 15 mL Ficoll to a 50 mL tube and slowly add 30 mL of blood, diluted 1:1 with PBS. Centrifuge the sample for 35 min at 440×g and RT without brake. Carefully harvest the interphase and wash the cells with PBS/EDTA (7 min, 440×g, RT).
7. Primary NK cells or MNCs are cultured overnight in NK medium at a concentration of 0.5–1×10<sup>6</sup>/mL at 37 °C and 5 % CO<sub>2</sub>.

### 3.2. CD107 Degranulation Assay

1. Count and harvest the required number of NK cells or MNCs. The cells are pre-incubated with IL-2 for at least 16 h (see Note 5). For the optimal 1:1 ratio, you need at least 1×10<sup>5</sup> NK cells or 1×10<sup>6</sup> MNCs. For controls, 0.5–1×10<sup>5</sup> NK cells or MNCs are sufficient (Table 2).
2. Count and harvest the appropriate number of K562 cells to reach a 1:1 ratio with NK cells during cocultivation (see Notes 6 and 7). Although a 1:1 ratio is usually best, it is recommended to perform titration of effector/target ratios when setting up the method for the first time (Table 2).
3. Centrifuge the cells (7 min, 440×g, RT).
4. Prepare a 96-well plate with 5 µL of CD107 antibody in each well including negative and positive controls.

**Table 2**  
**CD107 degranulation assay: exemplified sample setup**

Sample	NK cells <sup>a</sup>	K562	PMA/ionomycin	CD107-antibody
Unstained control	0.5–1 × 10 <sup>5</sup>	–	–	
Negative control	0.5–1 × 10 <sup>5</sup>	–	–	+
Positive control	0.5–1 × 10 <sup>5</sup>	–	+	+
10:1	1 × 10 <sup>6</sup>	1 × 10 <sup>5</sup>	–	+
5:1	5 × 10 <sup>5</sup>	1 × 10 <sup>5</sup>	–	+
1:1	1 × 10 <sup>5</sup>	1 × 10 <sup>5</sup>	–	+
1:5	1 × 10 <sup>5</sup>	5 × 10 <sup>5</sup>	–	+
1:10	1 × 10 <sup>5</sup>	1 × 10 <sup>6</sup>	–	+

<sup>a</sup>Calculate a 10× higher cell number when using MNCs instead of NK cells

5. Prepare the positive control: 10 μL of PMA (final concentration: 2.5 μg/mL) and 10 μL of ionomycin (final concentration: 0.5 μg/mL).
6. Resuspend cell pellets in NK medium at a concentration of 1 × 10<sup>7</sup> cells/mL.
  - (a) Add 10 μL of K562 (1 × 10<sup>5</sup>) and 10 μL of NK (1 × 10<sup>5</sup>) cells to the sample well (see Note 8).
  - (b) Add 10–50 μL of NK cells (0.5–1 × 10<sup>5</sup>) to the negative, positive, and unstained control wells.
  - (c) Fill up the wells to 200 μL with NK Medium (see Note 9).
7. Incubate for a total of 6 h at 37 °C and 5 % CO<sub>2</sub> (see Note 10).
8. After 1 h, add 2 μL monensin to the wells (see Note 11).
9. Harvest the cells in 4 mL round bottom tubes (“FACS tubes”).
10. Wash the cells with PBS/EDTA (7 min, 440×g, RT) and resuspend the pellet in 100 μL PBS/EDTA.
11. Stain the cells with the following monoclonal antibodies (different protocols for staining with NKG2A (step 11a) or perforin and granzyme B (step 11b)):
  - (a) CD56, CD3, KIR, and NKG2A-specific mAbs according to Table 1. Put 5 μL of each antibody (in case of CD56 and CD3, signals are usually strong and 2.5 μL might also be sufficient) to the cells and mix briefly (see Note 12).
  - (b) CD56, CD3, and KIR-specific mAbs. Put 5 μL of each antibody (in case of CD56 and CD3, see step 11a) to the cells and mix briefly.
12. Incubate for 20 min at 4 °C.

13. Wash the cells with PBS/EDTA (7 min,  $440\times g$ , RT). For NKG2A-stained samples, continue directly with flow cytometrical analysis (step 21). For additional analysis of granzyme B and/or perforin, continue with step 14.
14. Start with the intracellular staining protocol using the Cytofix/Cytoperm kit. Resuspend the cell pellet in 250  $\mu\text{L}$  Permwash Solution and incubate for 20 min at 4 °C.
15. Prepare Permwash Buffer by diluting 5 $\times$  Buffer 5:1 with distilled water.
16. Wash 2 $\times$  with Permwash Buffer and centrifuge the cells (7 min,  $440\times g$ , RT). Resuspend in 100  $\mu\text{L}$  Permwash Buffer.
17. Add 20  $\mu\text{L}$  of antibody against granzyme B or perforin and mix well.
18. Incubate for 30 min at 4 °C.
19. Wash with Permwash Buffer and centrifuge 2 $\times$  (7 min,  $440\times g$ , RT).
20. Wash the cells with PBS/EDTA (7 min,  $440\times g$ , RT), resuspend the pellet in 300  $\mu\text{L}$  PBS.
21. To start flow cytometrical analysis, set a gate on the lymphocyte population in an FSC versus SSC dot plot (Fig. 1) (see Notes 13 and 14).
22. Create a dot plot of CD56-PE-Cy5 versus CD3-Pacific blue and determine the percentage of NK cells by gating on CD56<sup>+</sup>CD3<sup>-</sup> lymphocytes (see Note 15).
23. Analyze these NK cells in a CD107 histogram and gate on CD107-positive cells in comparison to the negative control (Fig. 1).
24. Do the same for perforin and/or granzyme B to determine the frequency of NK cells stained for these intracellular markers. Calculate the frequency of the respective marker as percentage of NK cells (see Notes 16 and 17).

### 3.3. CFSE Killing Assay

1. Harvest the required number of K562 cells and wash with PBS (7 min,  $440\times g$ , RT). For every well, at least  $1\times 10^5$  K562 are required. For the calculation of the required number of cells, see Table 3.
2. Incubate the required number of NK cells (see Table 3) overnight in NK medium (at least for 16 h, Note 5).
3. Centrifuge the NK cells (7 min,  $440\times g$ , RT).
4. Resuspend K562 cells in PBS at a concentration of  $1\times 10^7$  cells/mL and put 2  $\mu\text{L}$  of CFDA-SE (0.5 mM) per mL into the tube.
5. Incubate for 10 min at 37 °C, invert 2 $\times$  during the incubation.
6. Wash K562 cells 2 $\times$  with PBS/FCS (7 min,  $440\times g$ , RT).

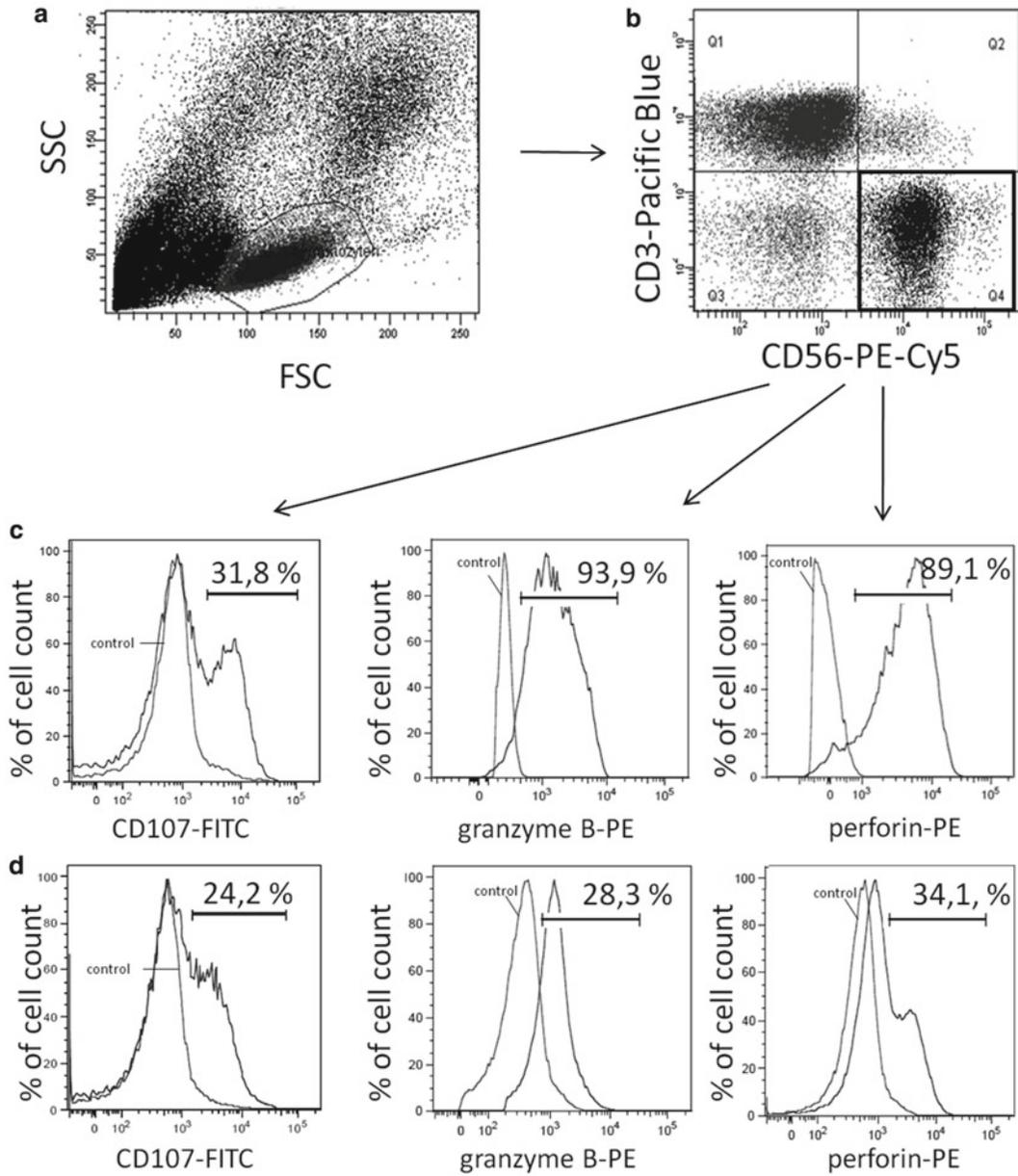


Fig. 1. Analysis of CD107 mobilization and intracellular expression of perforin and granzyme B in neonatal and adult NK cells. Mononuclear cells (MNCs) from umbilical cord blood and adult peripheral blood were each co-cultured with K562 target cells for 6 h and subsequently surface-stained with mAbs against CD56, CD3, KIR, and CD107. Surface staining was followed by intracellular staining for either granzyme B or perforin. **(a)** For flow cytometrical analyses, lymphocytes are displayed in an FSC versus SSC dot plot, as shown here for an MNC sample. **(b)** NK cells were defined as CD56<sup>+</sup>CD3<sup>-</sup> fraction of lymphocytes and analysis is restricted to NK cells as shown here for an MNC sample. The results of the CD107, granzyme B, and perforin analysis are presented as histogram overlays (*dark grey*) together with the respective negative control samples (*control, light grey*) from a healthy adult **(c)** and a neonatal sample **(d)**. Maximum cell counts are set to 100 %. The frequency of NK cells expressing CD107 after degranulation is comparable in both samples (*left panel*). Notably, the percentage of cells expressing granzyme B (*middle panel*) and perforin (*right panel*) among NK cell differs substantially. In the adult sample (*upper panel*), most NK cells contain granzyme B and perforin, whereas in the neonatal sample (*lower panel*) only a minority of cells contain both effector molecules.

**Table 3**  
**CFSE assay: exemplified sample setup**

Sample	NK cells	K562	PI
Unlabeled control	–	$0.5\text{--}1 \times 10^5$	–
CFSE-labeled control	–	$0.5\text{--}1 \times 10^5$	–
CFSE-labeled negative control	–	$0.5\text{--}1 \times 10^5$	+
10:1	$1 \times 10^6$	$1 \times 10^5$	+
5:1	$5 \times 10^5$	$1 \times 10^5$	+
1:1	$1 \times 10^5$	$1 \times 10^5$	+
1:5	$1 \times 10^5$	$5 \times 10^5$	+
1:10	$1 \times 10^5$	$1 \times 10^6$	+

7. Resuspend both NK cells and K562 pellets to a final concentration of  $1 \times 10^7$  cells/mL in NK medium.
8. The following controls have to be prepared (Table 3):
  - (a) 10–50  $\mu\text{L}$  unlabelled K562 ( $0.1\text{--}0.5 \times 10^6$ ).
  - (b) 10–50  $\mu\text{L}$  CFSE-labeled K562 cells as control for the CFSE staining ( $0.1\text{--}0.5 \times 10^6$ ).
  - (c) 10–50  $\mu\text{L}$  CFSE-labeled K562 as a negative control ( $0.1\text{--}0.5 \times 10^6$ ) (see Note 18).
9. Add 100  $\mu\text{L}$  NK cells ( $1 \times 10^6$ ) and 10  $\mu\text{L}$  K562 ( $1 \times 10^5$ ) in one well for an optimal E:T ratio of 10:1 (see Note 19).
10. Add NK medium to all wells to a final volume of 200  $\mu\text{L}$ .
11. Incubate the 96-well plate for 6 h at 37 °C and 5 %  $\text{CO}_2$ .
12. Harvest the wells in 4 mL round bottom tubes (“FACS tubes”) for flow cytometry.
13. Wash the cells with PBS/EDTA (7 min,  $440 \times g$ , RT) and resuspend the pellet in 300  $\mu\text{L}$  PBS.
14. Stain the cells with PI shortly before analysis (except unstained and CFSE controls).
15. Start flow cytometrical analysis and create FSC versus SSC dot plot (see Notes 20 and 21).
16. Determine the CFSE-positive cells in a histogram by setting a gate (see Note 22).
17. Show the CFSE-positive cells in a new dot plot.
18. Determine the frequency of CFSE<sup>+</sup>PI<sup>+</sup> cells as percentage of CFSE-positive K562 in a dot plot or histogram.
19. Calculate the number of killed target cells (Fig. 2) (see Note 23).

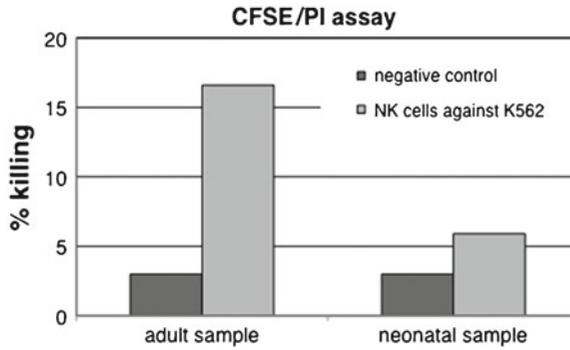


Fig. 2. Analysis of NK cell function with the CFSE/PI assay. Mononuclear cells from the same donors shown in Fig. 1 were co-cultured with CFSE-labeled K562 for 6 h in a 10:1 ratio and after co-culture stained with PI. Double-positive cells (CFSE<sup>+</sup>PI<sup>+</sup>, *light grey bars*) were compared to a negative control (CFSE-labeled K562 without effector cells, *dark grey bars*). Consistent with the results shown in Fig. 1 for granzyme B and perforin, the adult sample showed a substantially higher killing frequency compared to the neonatal sample.

#### 4. Notes

1. Flow cytometrical analysis of enriched NK cells is in some cases superior to direct analysis of MNCs due to higher and more discriminative signals for CD107, granzyme B, and perforin. Nonetheless, analysis can also be done directly from MNCs.
2. Depending on the blood source and frequency of lymphocytes one usually obtains  $5\text{--}10 \times 10^6$  NK cells from 10 mL blood.
3. It is possible to use less kit, i.e., up to 1/3 of the recommended volume. In this case, the purity of NK cells will go down to about 70 % but the recovery rate will increase considerably.
4. Sometimes it is difficult to locate the cells in the interphase. It is then advisable to remove some of the serum or ficoll along with the enriched cells in order to ensure their complete recovery.
5. Stimulation with IL-2 is recommended for the CD107 assay as well as the CFSE killing assay to increase the frequency of responding NK cells in order to ensure reliable statistical analysis.
6. We have observed that K562 is very well suited for CD107 analysis, whereas 721.221, an HLA-class I-deficient B cell lymphoblastoid cell line, gave the best results for stimulation of IFN- $\gamma$  production.
7. Typically, an effector/target ratio of 1:1 is optimal for K562, but may have to be adjusted for other target cells.
8. Whenever possible, increase the number of analyzed cells. A higher number of recorded NK cells leads to a better identification of small populations.

9. Optimal volume in each well is 200  $\mu$ L medium but smaller medium volumes might be used if the number of cells is limited.
10. Shorter incubation periods (4 h) lead to weaker CD107 signal, longer incubation periods (12–16 h) are possible but do not necessarily increase CD107 signal or frequency.
11. Addition of monensin is recommended to avoid internalization of CD107. Of note, in pilot experiments without monensin CD107 signals were somewhat weaker but could still be evaluated.
12. Inclusion of KIR antibodies into the flow cytometry panel enables functional analysis of NK cells at the clonal level. Calculate percentage of desired KIR-positive NK cells that have mobilized CD107.
13. Lymphocytes are small in comparison to K562. Additionally, intracellular staining leads to even smaller cell volumes. Carefully adjust FSC and SSC channels to enable exact enumeration of lymphocytes.
14. Depending on the number of killed K562, many dead cells will be present in FSC and SSC dotplots. Be sure to exclude the dead cells when gating on lymphocytes.
15. Compensation between the strong signals of PE-Cy5 and ECD is critical. Generally, do not use CD56 and CD3 in fluorochromes that are analyzed in neighboring channels.
16. To calculate the frequency of degranulated NK cells, subtract the percentage of CD107<sup>+</sup> NK cells present in the negative control from the percentage of CD107<sup>+</sup> NK cells present in coculture with K562.
17. Not only the frequency but also the mean fluorescence intensity (MFI), i.e., the expression levels per cell, of perforin, granzyme B, and CD107 can vary between samples and should be analyzed when evaluating effector function of NK cells.
18. A CFSE-labeled negative control is necessary to determine the number of K562, which lyse spontaneously.
19. A typical suitable effector/target ratio for CFSE with K562 is 10:1, but has to be adjusted for every given cell line separately. For every new set up, prepare a killing curve with different E:T ratios (i.e., 10:1, 5:1, 1:1, 1:5, and 1:10) and determine the optimal ratio with the best killing efficiency and discriminative power in flow cytometry.
20. In a CFSE/PI assay the dead target cells must also be measured. Take care to keep the FSC threshold low; otherwise, dead cells might be excluded from analysis.
21. PI is measured best in ECD or PE-Cy5 channel. Care has to be taken to enable good compensation between the strong signals of CFSE and PI.

22. The CFSE signal can be very strong. In this case, adjust the voltage of the CFSE channel accordingly.
23. To calculate the frequency of lysed target cells, subtract the percentage of spontaneous lysis of K562 from the percentage of K562 in coculture with NK cells.

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## Protocol for a Mammalian Cell-Based Assay for Monitoring the HIV-1 Protease Activity

Chitra Rajakuberan, Brett J. Hilton, and Roland Wolkowicz

### Abstract

Proteases are essential at different stages of the viral life cycle and for the establishment of a successful infection. Monitoring the catalytic activity of proteases in an easy and straightforward manner can thus drastically facilitate the discovery of novel antivirals, as well as help elucidate the activity and mechanism of action of the viral protease under study. In our laboratory, we have developed an assay in T-cells with a robust read-out to monitor the proteolytic activity of HIV-1 Protease (PR). The assay utilizes the prototypic transcription factor Gal4, which consists of the N-terminal DNA-binding domain and the C-terminal trans-activation domain. The assay is based upon (1) introduction of PR in between the two Gal4 domains to obtain a PR/Gal4 fusion protein and (2) utilization of the enhanced Green Fluorescent Protein as reporter of PR activity.

In order to overcome the possible cellular cytotoxicity of PR, the fusion protein in our assay is under the control of a tetracycline-inducible promoter. This ensures that it will be expressed only when needed, upon the addition of tetracycline or doxycycline. When active, PR has autocatalytic activity and cleaves itself from the Gal4 domains, resulting in the inability to induce eGFP expression. However, if PR activity is blocked or it is inactive, the two domains remain intact, resulting in eGFP expression. The assay can therefore be utilized to analyze the inhibitory effects of factors, peptides or compounds, designed on a rational- or nonrational-based approach, in the natural milieu of infection, where eGFP serves as a biosensor for PR activity.

**Key words:** HIV-1 protease, T-cell, Cell-based assay, Gal4 transcription factor, DNA-binding domain, Trans-activation domain, Green fluorescent protein (GFP), Drug screening, Drug discovery, Protease inhibitor, Autocatalytic activity

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### 1. Introduction

Proteases are crucial at different stages of the viral life cycle and thus absolutely required in the establishment of a successful infection. HIV-1 encodes for an 11 kDa aspartic protease (PR) as part of a precursor poly-protein (1, 2). PR must cleave itself from the poly-protein before it can cleave the gag-pol precursor and nef

(except for Envelope (3, 4), which is cleaved by host enzymes (5, 6). Hindering the proteolytic activity of HIV-1 PR, results in immature virions and aberrant infection. Hence, it is imperative to develop innovative assays to monitor HIV-1 PR activity, as well as to screen for new inhibitory compounds in a high-throughput manner. This assay can thus facilitate the development of novel antiretroviral drugs.

**1.1. The assay**

In our laboratory, we have developed a novel and straightforward assay to monitor HIV-1 PR activity (illustrated in Fig. 1) (7). While PR is active at the membrane or within the virions within the viral life cycle, in our assay PR is active in the cytoplasm and is totally membrane independent. To maximize the relevance of hits that will be found using this assay, it has been designed in T-cells, a cell line readily infected by HIV-1, in order to mimic the natural milieu of HIV infection. The assay utilizes Gal4 (8), which consists of an N-terminal DNA-binding domain (DBD) and a C-terminal trans-activation domain (TAD). The DBD recognizes and binds to the Gal4 responsive upstream activation sequences (UAS), which allows the TAD to activate transcription of the downstream reporter gene, the enhanced Green Fluorescent Protein (eGFP). The DBD and TAD must act in conjunction, and neither domain can act independently as a transcription factor (9).

The assay can be used to monitor the catalytic activity of other proteases: viral as well as host (10). The constructs described in

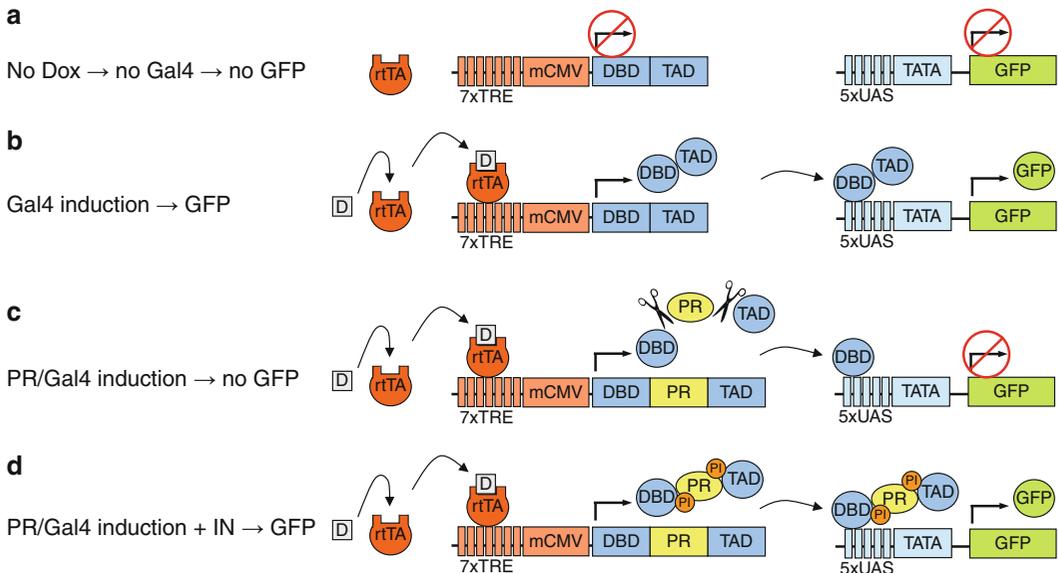


Fig. 1. Outline of the assay. *D* Doxycycline, *rtTA* Reverse tetracycline trans-activator, *DBD* DNA-binding domain, *TAD* Trans-activation domain, *TRE* Tet-responsive element, *UAS* Upstream activation sequence, *PR* Protease, *PI* Protease inhibitor, *GFP* Green fluorescent protein, *mCMV* Minimal Cytomegalovirus promoter, (adapted from Hilton and Wolkowicz (7)).

this paper can be used for this purpose as well, since the HIV-1 PR, including the cleavage sites, can be easily exchanged with any other protease of interest, and as such can serve as a platform to monitor proteases, provided their recognition/cleavage sites are included.

In our assay, wild-type PR, along with the PR cleavage sites, is introduced in between the DBD and TAD of Gal4 (PR/Gal4) (7, 11). When PR is active, it cleaves itself from the fusion protein, effectively separating the domains, resulting in no eGFP expression. However, if PR activity is inhibited or it is inactive, the domains remain intact and eGFP expression is induced. Thus, in our assay, eGFP acts as a biosensor of HIV-1 PR activity. A schematic of the assay is outlined in Fig. 1.

### **1.2. Tetracycline Inducible System**

In order to circumvent the possible cytotoxicity of HIV-1 PR in mammalian cells (12), we adapted the tetracycline (tet) inducible system in order to express the PR fusion protein in an inducible manner. The tet-inducible (off/on) system consists of two elements: (1) Tet-responsive element (TRE), which has seven copies (7×) of the tet operator sequences (TetO) and a minimal CMV promoter, and (2) reverse tet-controlled trans-activator (rtTA). When doxycycline (dox) is added to the system (dox is a stable form of tet), it binds to rtTA, resulting in a conformational change, which allows the complex to bind to TetO, resulting in the transcription of downstream genes. In short, PR expression is tightly regulated by the tet system, and induced only upon the addition of dox.

### **1.3. Expression and Clonal Selection**

All the assay elements are expressed via retro/lentiviral vectors to enable stable expression in mammalian cells. Two to three days after transduction with retro/lentiviral particles, T-cells are induced with dox. Cells expressing high levels of the reporter eGFP gene, with minimal background (highest dynamic range) are selected. A clonal population is generated to rule out genotypic differences and ensure that the population is homogenous.

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## **2. Materials**

### **2.1. Plasmids**

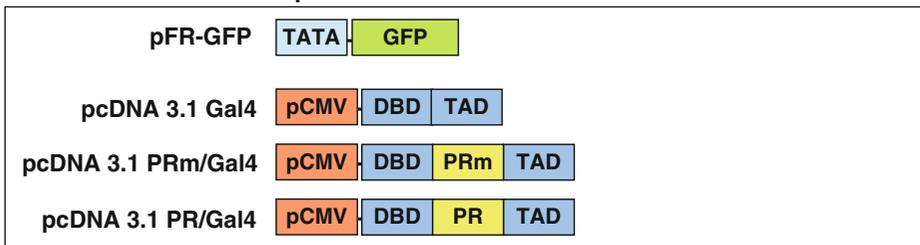
The plasmids used to construct the clones for the assay are depicted in Fig. 2 (Plasmids are available upon request).

1. Transient expression: pFR-GFP (reporter construct), pcDNA 3.1 Gal4, pcDNA 3.1 PRm/Gal4, and pcDNA 3.1 PR/Gal4 (Zeocin), for transient expression studies (Fig. 2a) (see Note 1).
2. Stable expression: pH-5× UAS-GFP (reporter construct), pH-7× TRE-PR/Gal4, for stable but inducible expression of the PR/Gal4 fusion protein. pH-TRE is a self-inactivating lentiviral vector (with most of the 3'LTR U3 region removed) for

safety purposes as well as to ensure that the LTR promoter does not interfere with the UAS promoter of the reporter construct (Fig. 2b upper panel) (see Notes 2 and 3).

3. rtTA plasmid: pBMN-rtTA-i-mCherry is a Murine Leukemia Virus (MLV)-based plasmid for the stable expression of rtTA. The plasmid also expresses mCherry under internal ribosome entry site (IRES), enabling sorting for rtTA positive cells based on mCherry expression (Fig. 2b upper panel) (see Note 4).
4. Plasmids for viral production: pCMVΔ8.2 (packaging plasmid, expresses all the HIV-1 proteins, except Envelope), pCI-VSVg (expresses the Vesicular Stomatitis Envelope glycoprotein), pRSV-Vpr (expresses HIV-1 Viral Protein R, shown to enhance viral titers) (Fig. 2b lower panel).

**a Vectors for transient expression**



**b Vectors for stable expression**

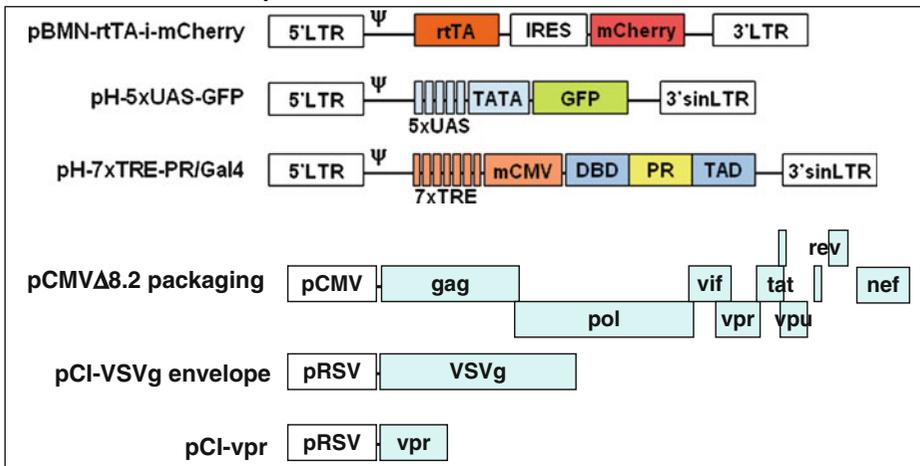


Fig. 2. Schematic representation of the plasmids. (a) Vectors for transient expression include the reporter pFR-GFP and Gal4, PRm/Gal4 and PR/Gal4 expression plasmids. (b) Vectors for stable expression include, in the upper panel transfer vectors: retroviral vector expressing rtTA (pBMN-rtTA-i-mCherry), lentiviral reporter vector (pH-5x UAS-GFP), and lentiviral vector expressing inducible PR/Gal4 fusion protein (pH-7x TRE-PR/Gal4) (see Note 4); and in the lower panel vectors for the production of HIV-based lentiviral particles: pCMVΔ8.2 packaging, pCI-VSVg envelope, and pCI-vpr regulatory plasmids. LTR Long terminal repeat, sinLTR Self-inactivating-LTR, Ψ Packaging signal, rtTA Reverse tetracycline trans-activator, IRES Internal ribosome entry site, DBD DNA-binding domain, TAD Trans-activation domain, TRE Tet-responsive element, UAS Upstream activation sequence, mCMV Minimal Cytomegalovirus promoter, PR Protease, GFP Green fluorescent protein, VSVg Vesicular Stomatitis Virus glycoprotein, pRSV Rous Sarcoma Virus promoter.

## **2.2. Validation of the Assay with Transient Expression**

1. Cells:
  - (a) HEK293T (ATCC CRL-11268) are adherent cells used for transient expression studies.
2. Plasmids:
  - (a) pcDNA Gal4, PR/Gal4, PRm/Gal4, and pFR-GFP (Fig. 2a).
3. Media:
  - (a) HEK293T are grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with penicillin–streptomycin and 2mM L-Glutamine and 10 % (v/v) fetal calf serum (FCS).
  - (b) Cells are resuspended in 1× Phosphate Buffer Saline (PBS) for flow cytometric analysis (see Note 5).
4. Reagents:
  - (a) Polyethylenimine linear 25 kD (PEI) is dissolved in nano pure water to obtain a stock concentration of 2 mg/mL. Aliquots are stored at –80 °C.
  - (b) Stocks of FDA approved PR Inhibitors (PI), i.e., Darunavir are dissolved in DMSO and stored at –20 °C at a concentration of 10 mM. Working stocks (at various concentrations as needed) are stored at 4 °C. The reagents were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH.
5. Flow cytometry:
  - (a) Flow cytometer with a 488 nm laser for the detection of GFP.

## **2.3. Production of Stable and Inducible Clones for Screening**

1. Cells:
  - (a) HEK293T (ATCC CRL-11268) and Phoenix GP cells are adherent cells used for the production of lentiviral and retroviral particles, respectively.
  - (b) SupT1 (ATCC CRL-1942), a T-cell line readily infected by HIV-1 (see Note 6).
2. Plasmids:
  - (a) pCI-VSVg, pRSV-Vpr and pCMVΔ8.2 for the production of retroviral particles and pH-7× TRE-PR/Gal4, pBMN-rtTA-i-mCherry and pH-5× UAS-GFP for the assay elements (Fig. 2b) (see Note 7).
3. Media:
  - (a) HEK293T and Phoenix GP cells are grown in DMEM, supplemented with penicillin–streptomycin and 2mM L-Glutamine and 10 % (v/v) FCS.
  - (b) SupT1 cells are grown in RPMI, supplemented with penicillin–streptomycin and 2mM L-Glutamine and 10 % (v/v) FCS at 37 °C and 5 % CO<sub>2</sub>.

(c) Cells are sorted in 1× PBS with 1 % (v/v) FCS. (see Note 5)

4. Reagents:

(a) Polyethylenimine linear 25 kD (PEI) is dissolved in nano pure water to obtain a stock concentration of 2 mg/mL. Aliquots are stored at  $-80^{\circ}\text{C}$ .

(b) Polybrene (Hexadimethrene Bromide) is dissolved in nano pure water at 5  $\mu\text{g}/\text{ml}$  and stored at  $-20^{\circ}\text{C}$ .

(c) Stocks of FDA approved PR Inhibitors (PI), i.e., Darunavir are dissolved in DMSO and stored at  $-20^{\circ}\text{C}$  at a concentration of 10 mM. Working stocks (at various concentrations as needed) are stored at  $4^{\circ}\text{C}$ . The reagents were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH.

(d) Stock of doxycycline is dissolved in nano pure water at a concentration of 1mg/mL and stored at  $-20^{\circ}\text{C}$ .

5. Flow cytometry:

(a) Fluorescence Activated Cell Sorter (FACS) with Automatic Cell Deposition Unit, (ACDU) (see Note 8).

**2.4. Verification of Assay Responsiveness to FDA Approved Inhibitors**

1. Cells:

(a) Selected SupT1 clones from Subheading 3.3.

2. Media:

(a) RPMI, supplemented with penicillin–streptomycin and 2 mM L-Glutamine and 10 % (v/v) FCS at  $37^{\circ}\text{C}$  and 5 %  $\text{CO}_2$ .

(b) Cells are resuspended in 1x PBS for flow cytometric analysis. (see Note 5)

3. Reagents:

(a) Stocks of FDA approved PR Inhibitors (PI), i.e., Darunavir are dissolved in DMSO and stored at  $-20^{\circ}\text{C}$  at a concentration of 10 mM.

(b) Stock of doxycycline is dissolved in nano pure water at a concentration of 1 mg/mL and stored at  $-20^{\circ}\text{C}$ .

4. Flow cytometry:

(a) Flow cytometer with a 488 nm laser for the detection of GFP.

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## 3. Methods

### 3.1. Plasmids

Prepare stock solutions of all necessary plasmids listed in the materials section to ensure pure plasmid samples (Maxiprep).

### **3.2. Validation of the Assay with Transient Expression**

In order to validate the expected behavior of the proteins in the assay, transfect cells in the presence and absence of PI (see Note 9).

1. Grow HEK293T cells in a 6-well plate to about 50–60 % confluency (around 300,000 cells per well, 24 h prior to transfection) (see Note 10).
2. Incubate the cells in a humidified incubator in atmosphere enriched with 5 % CO<sub>2</sub> at 37 °C.
3. For transfections in the presence of PI, add PI (Darunavir) to the cells to a final concentration of 1 μM, at least 10 min *prior* to transfection (see Note 11).
4. In a 0.6 mL Eppendorf tube, add 125 μL DMEM only (without FCS, PenStrep or L-Glutamine), 15 μL of polyethylenimine linear 25 kD and 3 μg of plasmids (pcDNA Gal4, PR/Gal4 and PRm/Gal4, along with pFR- GFP) drop-wise and mix thoroughly.
5. Leave this mixture at room temperature for 20 min.
6. Add this mixture to all cells drop-wise.
7. Put the cells back in the humidified incubator with 5 % CO<sub>2</sub> at 37 °C.
8. 24 h post-transfection, replace the media of transfected cells.
9. 48 h post-transfection analyze the cells by flow cytometry and/or fluorescence microscopy.

In the presence of the reporter, PR/Gal4 and 1 μM Darunavir there should be maximal activation of eGFP. On the other hand, reporter alone or reporter with PR/Gal4 in the absence of PI should have minimal eGFP expression.

### **3.3. Production of Stable and Inducible Clones for Screening**

Development of a stable cell line enables preselection of an optimal clonal cell line, which exhibits the most robust and reliable readout for PR inhibition. Due to the potential cytotoxicity of PR in cells, its expression is induced after the cells have been grown and prepared for screening.

Generation of viral particles for transduction of the target SupT1 cells (see Note 12).

Production of Retroviral Particles (pBMN-rtTA-i-mCherry)

1. Plate Phoenix GP cells at about 50–60 % confluency in 10 mL of fresh medium in a 10 cm plate (Phoenix GP cells express gag-pol of MLV and require only a transfer vector and an envelope-expressing vector, for the production of MLV-like particles).
2. After 24 h, transfect (same procedure as in Subheading 3.2) with 3 μg of pBMN-rtTA-i-mCherry and 3 μg of pCI-VSVg.
3. Incubate the cells in a humidified 5 % CO<sub>2</sub> incubator.

4. Replace the media 24 h post-transfection.
5. Collect the viral supernatant 48 h post-transfection, (see Note 13) filter out cellular debris using 45  $\mu\text{m}$  filters (Pall Corporation) and store the viral stocks at  $-80\text{ }^{\circ}\text{C}$  in aliquots (see Note 14 and 15).

#### Production of Lentiviral Particles (pH Vectors)

1. Grow HEK293T cells at about 50–60 % confluency in a 10 cm plate for each transfer vector used (pH vectors).
2. Transfect as described in Subheading 3.2 with 3  $\mu\text{g}$  of the transfer vector (pH-7 $\times$  TRE-PR/Gal4), 3  $\mu\text{g}$  pCI-VSVg, 1.5  $\mu\text{g}$  of pRSV-Vpr (shown to enhance viral titers) (13), and 2  $\mu\text{g}$  of the packaging vector, pCMV $\Delta$ 8.2, (kindly provided by Didier Trono, EPFL, Switzerland) (14).
3. Replace the media 24 h post-transfection.
4. Incubate the cells at 37  $^{\circ}\text{C}$  in a humidified incubator with 5 %  $\text{CO}_2$ .
5. Collect the viral supernatant 48 h post-transfection, filter out cellular debris using 45  $\mu\text{m}$  filters (Pall Corporation), aliquot the viral stocks and store them at  $-80\text{ }^{\circ}\text{C}$ .

#### Establishment of rtTA Cell Line (See Note 16)

1. Thaw the frozen viral stock of rtTA-i-mCherry.
2. Seed cells at a density of about 250,000 cells/well in 3 mL of fresh medium for each well of a 6-well plate.
3. Add 12  $\mu\text{L}$  of polybrene (stock at 5  $\mu\text{g}/\text{mL}$ ) to the cells.
4. Add 1 mL of the viral stock to the polybrene-treated cells, followed by fresh media up to 3 mL and mix well (see Note 17).
5. Centrifuge the plate at 1,500 $\times g$  for 80 min. at 32  $^{\circ}\text{C}$  in a hanging bucket rotor (Spin infection).
6. Resuspend the cells in the plate at the end of the cycle.
7. Incubate the cells in a humidified incubator with 5 %  $\text{CO}_2$ .
8. Resuspend the cells in fresh media 24 h post-infection.
9. 72 h post infection, analyze the cells by flow cytometry and sort a minimum of 50,000 mCherry positive cells (see Notes 5 and 18).
10. Expand the sorted cells, and reanalyze to verify the purity of rtTA-mCherry population.
11. If necessary, sort again to further purify the population.

#### Co-infection of the rtTA Cell Line with the Reporter and the Inducible PR/Gal4 Viral Particles

1. Thaw the 5 $\times$  UAS GFP reporter and, PR/Gal4 fusion viral stocks.

2. Count the rtTA mCherry expressing cells and seed about 250,000 cells/well of a 6-well plate in 3 mL of fresh medium.
3. Add 12  $\mu$ L of polybrene to the cells.
4. Add 1 mL of the viral stocks of pH-5 $\times$  UAS-GFP and 1 mL of the pH-7 $\times$  TRE-PR/Gal4 to the cells (see Note 17).
5. Add fresh medium to bring the final volume to 3 mL.
6. Centrifuge the plate at 1,500 $\times g$  for 80 min at 32 °C in a hanging bucket rotor (Spin infection).
7. Resuspend the cells at the end of the cycle in fresh medium.
8. Incubate the cells in a humidified incubator with 5 % CO<sub>2</sub>.
9. Resuspend the cells in fresh media 24 h post-infection.

#### Establishment of an Efficient Reporter Cell Line

1. Expand the transfected HEK293T cell lines generated above for about 1 week.
2. Place 300,000 cells/well in 3 mL of fresh medium in each well of a 6-well plate.
3. Add Darunavir and dox to a final concentration of 1  $\mu$ M and 1  $\mu$ g/mL, respectively (see Notes 11 and 19).
4. After about 48 h, verify the presence of eGFP, under a fluorescent microscope.
5. Analyze the cells and sort for the cells expressing high levels of eGFP in an FACS.
6. Allow the sorted eGFP population to expand until fluorescence diminishes.
7. Add dox again to the cells and sort out the negative population to eliminate background activation.
8. Repeat purification once more (see Note 20).

#### Generation of Clonal Populations

1. Activate the cells with Darunavir and dox, as above.
2. Sort cells into at least two 96 well plates at a single cell per well.
3. Visualize the wells under a microscope to confirm that the wells truly contain single cells. Ignore and omit wells containing no cells or multiple cells.
4. Allow the cells to expand (this may require a few weeks).
5. Activate individual expanded clones with 1  $\mu$ g/mL dox alone, and with both 1  $\mu$ g/mL dox and 1  $\mu$ M Darunavir.

Analyze cells by FACS to identify the ideal clone to be used for screening purposes as well as to monitor the catalytic activity of PR. The optimal clonal cell line will be one which has the lowest

eGFP ratio between cells with dox only compared to cells with dox and PI (i.e., cells with dox only should have little to no eGFP expression, but when PI is also added, these cells should have close to 100 % eGFP expression and the highest mean fluorescence intensity of eGFP as well).

### **3.4. Verification of Assay Responsiveness to FDA Approved Inhibitors**

*Expand the selected clonal cell line and test other FDA approved PI to verify that the responsiveness of the clone is not limited to a specific inhibitor.*

1. In a 96-well plate, seed 50,000 cells/well in 3 mL fresh medium from the selected clonal population.
2. Pre-incubate these cells with the specific PI at varying concentrations for at least 10 min.
3. Activate with 1 µg/mL dox.
4. Analyze responsiveness to the PI using flow cytometry as described above.

#### **Titration of Doxycycline**

*In order to avoid over-activating the PR/Gal4 fusion protein, titrate the concentration of dox required to maximally induce eGFP expression in the presence of a PI.*

1. Perform the titration experiment in a 96-well plate with 50,000 cells/well.
2. Incubate cells in each well with 1 µM Darunavir and add varying concentrations of dox.
3. Analyze the cells by flow cytometry and determine the saturating point of dox.

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## **4. Notes**

1. pFR-GFP and pcDNA 3.1 PR/Gal4 constructs harbor the same elements as pH-5× UAS-GFP and pH-7× TRE-PR/Gal4 vectors, respectively, and are described in Fig. 2 top panel; the only difference being that they are not retro/lentiviral in nature.
2. When amplifying retroviral/lentiviral plasmids, grow bacteria harboring plasmids at 30 °C or 32 °C rather than 37 °C, since at higher temperatures there is a greater chance of recombination.
3. For the production of stable cell lines with inducible elements, the vectors used in this assay are highly recommended. Nevertheless, other vectors displaying similar characteristics can also be used.

4. rtTA is also available in a plasmid carrying a zeocin antibiotic resistance marker pBMN-rtTA-i-Zeocin, and therefore can be selected for with zeocin, rather than fluorescence (mCherry)-based sorting.
5. RPMI contains residual compounds that are considered to interfere with the fluorescent properties of the samples, and therefore it is typically recommended to fully remove the media before analyzing the samples by flow cytometry. However, we have not noticed any interference with the signals, by analyzing samples directly in their growth medium. However, for sorting, cells MUST be resuspended in the proper sorting buffer.
6. The cell line for the assay chosen here is SupT1. Other relevant cell types for HIV-1 such as other T-cell lines, macrophages, and so on can be used as well. Naturally, if adherent cells are used instead, tissue culture practices should be adapted to adherent versus non-adherent cells.
7. pH-7 $\times$  TRE-PR/Gal4 is described here. A control vector for Gal4 activity (pH-7 $\times$  TRE-Gal4) and another one for a mutant catalytically inactive version of PR (pH-7 $\times$  TRE-PRm/Gal4) were utilized in the original assay to corroborate the expected behavior of the assay. While obviously Gal4 will serve as a control for maximum eGFP induction irrespective of the presence of inhibitors, the PRm/Gal4 mutant fusion will serve as a control for insertion of PR within the Gal4 domains. As this PR is inactive, eGFP expression is expected even in the absence of inhibitor.
8. Clones were sorted here using a flow cytometer with an automatic cell deposition unit. However, without this technology, the cells can be counted, diluted to one cell/well and incubated. This method of clonal expansion is far less reliable and requires careful observation of plated wells to confirm that only a single cell was deposited in each well. However, if a cell-sorter is not available, it is still a viable option.
9. A transient experiment to validate the expected behavior of the constructs *prior* to the more elaborate and time-consuming task of obtaining the final cell lines is highly recommended, but not compulsory.
10. All the transfections were done with cells at a confluency of 50–60 %; however, lower or higher cell concentrations can be used. In this case, transfection efficiency could be lower, or cell death higher, and therefore should be tested.
11. Any of the FDA approved PIs can be used to activate the expression of eGFP in the validation and selection steps outlined here. However, Darunavir has been observed in this assay to have the lowest IC<sub>50</sub> and minimal cell death at higher

concentrations and is therefore recommended as a PI control. Indinavir was used as control in the original experiments (7).

12. While replicative virus is not used in the experiments outlined here, because of the production of MLV, and HIV particles (infectious for one round, but non-replicative), a Biosafety Level-2 environment is required for the development of the assay.
13. It is recommended that the viral supernatant be collected at 48 h. However, virus can still be collected at later time points, up to about 72 h. Harvesting outside of the 48–72 h window, however will probably significantly reduce the viral titer.
14. Filtering the viral supernatant with 45  $\mu\text{m}$  filters is not mandatory, but it improves the infection efficiency. The cellular debris from the supernatant can also be removed by centrifugation at  $525 \times g$  for 5 min.
15. Upon freeze thawing the viral stock, it is typically assumed that about 50 % of the infectivity is lost. However, we have not noticed such drastic loss in infectivity. The virus can be stored at 4 °C for about 3 days. However, there is about 50 % loss in infectivity with each day at 4 °C. In other words, freeze thawing cycles should be avoided.
16. Development of an rtTA cell line *prior* to co-infection can be bypassed if infection efficiencies are high enough to infect the cells with all three viral particles (carrying each of the assay elements) simultaneously.
17. When transducing cells, it is a good idea to infect at various Multiplicities of Infection (MOI) to ensure efficient infection. High expression of reporter from one infectious event could prevent the need for an MOI larger than one.
18. While 50,000 cells is recommended in our protocol, sorting more or less cells is acceptable; however, growth and recovery times will then vary.
19. For the tetracycline inducible system, we use dox to induce expression, since it is more stable at room temperature. However, tetracycline would be expected to be a comparable alternative.
20. The method used here for enrichment of a responsive cell line to select the most inducible clone with the clearest eGFP read-out was two rounds of sorting, first in the presence of PI and dox, and then only with dox. Other selection methods can also be used, such as using resistant markers (resistance to blasticidin, zeocin, and so on) and then selecting with the proper antibiotics. However, these methods should still incorporate FACS to enrich for the most responsive population and to eliminate background.

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## **Self-Collection of Specimens for Nucleic Acid-Based Diagnosis of Pharyngeal, Cervicovaginal, Urethral, and Rectal *Neisseria gonorrhoeae* and *Chlamydia trachomatis* Infections**

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### **Abstract**

Commercially available nucleic acid amplification tests (NAATs) for *Neisseria gonorrhoeae* and *Chlamydia trachomatis* detection allow for self-collection including home-based collection from multiple anatomic sites such as the urethra, cervicovagina, rectum, and pharynx. Verification studies need to be done prior to processing pharyngeal and rectal specimens. We review specimen collection and test characteristics of NAATs at different anatomical sites.

**Key words:** Self-collection, Gonorrhea, *Chlamydia*, Nucleic acid amplification test, NAAT

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### **1. Introduction**

Nucleic acid amplification tests (NAATs) for *Neisseria gonorrhoeae* (NG) and *Chlamydia trachomatis* (CT) detection have revolutionized screening for sexually transmitted infections. NAATs have greatly improved sensitivity over culture and allow testing from noninvasive specimens (e.g., urine), and multiple anatomic sites such as the urethra, cervicovagina, rectum, and pharynx. Testing from any of these anatomic sites allows for self-collection of specimens making non-clinic-based specimen collection, including home-based specimen collection, feasible. The use of other sample types obtained through other noninvasive means (oral wash (1), glans swab (2)) has been evaluated. Those specimens, however, are not yet recognized as reliable specimens for testing. Self-collected vaginal swab specimens are equivalent in sensitivity and reliability compared to traditional clinician-collected endocervical swab

specimens and more sensitive and reliable than first-catch urine samples for the detection of CT and NG (3). The test characteristics (sensitivity, specificity, and accuracy) of NAATs performed with self-collected rectal swab specimens were similar or slightly better than those of NAATs performed with clinician-collected rectal swabs (2).

Currently in the USA, there are three widely used and studied commercially available NAATs which use proprietary methods of target capture, amplification, and signal identification: Amplicor CT/NG using polymerase chain reaction (PCR) (Roche Diagnostics Corp., Branchburg, NJ), Probe Tec using strand displacement amplification (SDA) (Becton Dickinson Co., Sparks, MD), and Aptima Combo 2 using transcription mediated amplification (Gen-Probe Inc., San Diego, California) (4–8). NAATs detect and amplify bacterial DNA or RNA sequences specific for the targeted organism with each NAAT using a slightly different target and method of amplification (Table 1). False positive results for NG can occur with the current Roche and Becton-Dickinson assays as their targets may cross-react with other *Neisseria* species such as *N. cinerea* and *N. subflava*. The Aptima assay uses a ribosomal RNA target with minimal cross-reactivity with genes from nongonococcal *Neisseria* species. Other benefits of the Aptima assay include a target capture step which eliminates false negatives due to amplification inhibitors, and the presence of thousands of copies of the ribosomal RNA target in each bacterium in contrast to far fewer copies of the DNA target in the other assays. Due to potential false positive results and the lower specificity of certain NAATs, the Centers for Disease Control and Prevention (CDC) recommends confirmatory testing of specimens positive for CT and NG by NAATs when the positive predictive value is <90 % (9). Large-scale studies, however, have found that confirmatory testing using NAATs is not needed for genital specimens (10–12).

The CDC recommends annual CT screening of all sexually active women aged  $\leq 25$  years (13). Focus groups have voiced the need to “normalize” CT testing, favoring home-based specimen collection (14, 15). Recent efforts have successfully linked Internet-based educational sexual health information and self-collection test kits for STDs with partner notification. The Internet educational Website [www.iwantthekit.org](http://www.iwantthekit.org) has tested over 1,200 women in Maryland, Washington, DC, and West Virginia using free test kits for self-collected vaginal samples with mailing to a laboratory for CT and NG testing. Women using this site described high acceptability of the service and had a high overall CT prevalence of 9 % (16). A study comparing results of cervico-vaginal swabs tested with Gen-Probe PACE 2 assay for CT sent to a state laboratory by courier in a relatively controlled environment to swabs transported by the US mail in hot summer months in a southern state found 99 % agreement between courier and mailed specimen results (17).

**Table 1**  
**Commercially available nucleic acid amplification test characteristics and performance in rectal and pharyngeal specimens compared with culture for the diagnosis of *Neisseria gonorrhoeae* and *Chlamydia trachomatis* infections<sup>a</sup>**

	PCR (COBAS Roche AmpliCor)	SDA (BD ProbeTec)	TMA (Gen-Probe Aptima)	Culture
Nucleic acid amplified	For <i>Chlamydia trachomatis</i> (CT), multicopy cryptic CT plasmid and major outer membrane protein For <i>Neisseria gonorrhoeae</i> (NG), chromosomal primer based and 16S rRNA	For CT, multicopy plasmid region For NG, a region within the multicopy pilin gene inverting protein homologue	For CT, specific region of the 23S rRNA For NG, a specific region of the 16S rRNA via DNA intermediates	
Amplification method	Primer binds to and amplifies DNA gene sequence. Oligonucleotide probe binds to the DNA copies (amplicons) which are detected by spectrophotometer	Primer binds to DNA and displaces one strand prior to amplification with amplified gene sequences detected by fluorescent probe	Primer-bound rRNA target binds to a magnet prior to amplification allowing substrate inhibitors to be cleansed from the sample. Amplified target detected using two different light-producing labels	
Sensitivity and specificity (mean), NG rectum	Sensitivity 54 % Specificity 99 %	Sensitivity 78 % Specificity 100 %	Sensitivity 100 % Specificity 100 %	Sensitivity 53 % Specificity 100 %
Sensitivity and specificity (mean), NG pharynx	Sensitivity 66 % Specificity 89 %	Sensitivity 75 % Specificity 100 %	Sensitivity 95 % Specificity 100 %	Sensitivity 42 % Specificity 100 %
Sensitivity and specificity (mean), CT rectum	Sensitivity 91 % Specificity 96 %	Sensitivity 77 % Specificity 100 %	Sensitivity 100 % Specificity 100 %	Sensitivity 76 % Specificity 100 %

<sup>a</sup>PCR polymerase chain reaction, SDA strand displacement amplification, TMA transcription-mediated amplification. Unable to determine sensitivity of NAATs for pharyngeal CT due to low prevalence of infection in available studies (26, 27)

Based on the significant prevalence of CT and NG infections among men who have sex with men (MSM) and the often asymptomatic clinical presentation of those infections, the Centers for Disease Control and Prevention (CDC) guidelines also recommend screening sexually active MSM using NAATs from genital and extra-genital sites for NG and CT at annual or more frequent intervals as influenced by risk (13). Among over 6,000 MSM in San Francisco, 85 % of rectal infections were asymptomatic. In this same study, 53 % of CT infections and 64 % of NG infections were at non-urethral sites. These data support the need for routine screening of genital and extra-genital sites (the pharynx and rectum) in MSM (18). Increasing evidence suggests that the pharynx might be an important sanctuary site for the development of cephalosporin-resistant *N. gonorrhoeae*, emphasizing the importance of routine pharyngeal screening and treatment particularly in MSM (19). For MSM diagnosed with gonorrhea at any site, 10–25 % have only pharyngeal infection (20).

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## 2. Materials and Methods

1. NAATs used for the diagnosis of NG and CT infections can be performed on noninvasively collected specimens: urine, self-collected vaginal swab, and self-collected rectal swab and pharyngeal swab (see Note 1). Self-collected pharyngeal and rectal swabs are reliable for CT and NG detection (21). The collection kit sent home with the patient will depend on the NAAT assay being used. Each NAAT will have specifications for storage time and conditions as well as acceptable transport media (Table 2). Due to the ability to store these specimens without freezing for days to months, and the fact that the Roche Amplicor and the GenProbe Aptima Combo2 can be automated, the specimens can be accepted around the clock and the assay performed on specific days. This saves both time and expense as the microbiologist is using one set of internal and external controls to test numerous specimens.

Patients should be instructed on how to perform the swab collection. Vaginal swabs should be inserted 3 to 5 cm and once inserted rotated for 30 s. The swab is then placed into a tube and capped. Rectal swabs should be inserted 3 to 5 cm into the anus and rotated for 5–10 s. If needed prior to insertion, the swab can be moistened with water or saline solution. Self-collected pharyngeal swabs should make contact with five key anatomic landmarks: bilateral tonsils, bilateral posterior walls, and the uvula. Users should wash their hands between specimen collection if collecting a specimen from more than one anatomic site. Patient instructions for self-collection of

**Table 2**  
**Specifications for collection and transport of specimens for nucleic acid amplification testing**

	<b>PCR (COBAS Roche Amplicor)</b>	<b>SDA (BD ProbeTec)</b>	<b>TMA (Gen-Probe Aptima)</b>
Sample collection	Dacron-, rayon-, or calcium alginate-tipped swabs with plastic or non-aluminum	<i>Chlamydia trachomatis</i> / <i>Neisseria gonorrhoeae</i> amplified DNA assay endocervical specimen collection kit	Unisex swab specimen collection kit
Recommended maximum storage time prior to testing, room temperature <sup>a</sup>	1 h	4–6 days	60 days
Recommended maximum storage time prior to testing, 2–8 °C	7 days	30 days	60 days
Recommended maximum storage time prior to testing, <–20 °C	30 days	N/A	90 days
Transport media	2SP culture transport media, Bartels Chlamtrans, SPG, and M4 culture transport media	Included in kit	Included in kit

<sup>a</sup>Specimen must be processed for assay or frozen at conclusion of transport/storage time

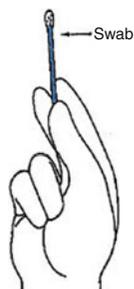
pharyngeal, rectal, and vaginal swabs using the Gen-Probe Aptima kit are presented in Figs. 1–3 (22).

2. Verification studies need to be done prior to processing pharyngeal and rectal specimens. The currently available commercial NAATs are not currently FDA cleared for marketing for use on extra-genital specimens. To adhere to the US Clinical Laboratory Improvement Amendments (CLIA) regulations, clinical laboratories need to perform a verification study prior to reporting NG and CT test results from pharyngeal and rectal specimens. Verification is a one-time procedure, completed before extra-genital NAAT testing is offered and conducted on clinical specimens. The goal of a verification study is to evaluate a test system to determine whether the claims outlined in the manufacturer's package insert as they relate to product, process, results, or interpretation can be reproduced. A document published by the American Society of Microbiology (ASM) ([www.asm.org](http://www.asm.org)), Cumitech 31- Verification and

## Pharyngeal Swab Collection Instructions



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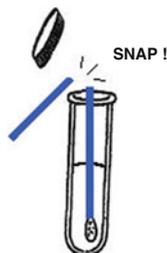
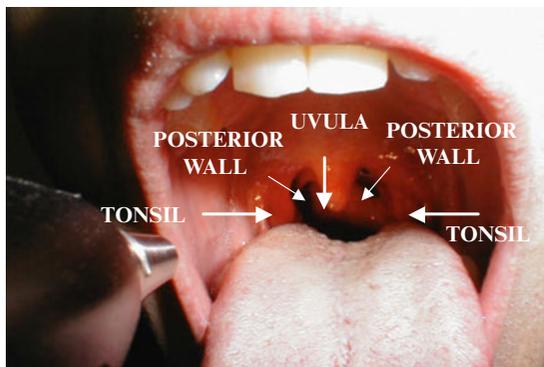


### Step 1.

Open kit and remove tube and package with green writing. Remove the swab with the **BLUE** shaft. **USE BLUE SHAFT SWAB ONLY.**

### Step 2.

Instruct patient to open mouth widely. Be sure to make good contact with 5 key areas of the throat (See below).



### Step 3.

**Remove cap** from test tube. Place swab in test tube. Do not puncture the foil cap.

Break swab shaft at the score mark.



### Step 4.

Put cap back tightly on test tube to prevent any leaking. Try not to splash the liquid out the tube.

### Step 5.

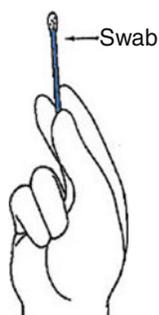
Discard wrapper and unused swab. **Wash your hands.**

Fig. 1. Pharyngeal swab collection instructions using the GEN-PROBE APTIMA kit for gonorrhea and chlamydia testing.

## Rectal Self-Swab Collection Instructions

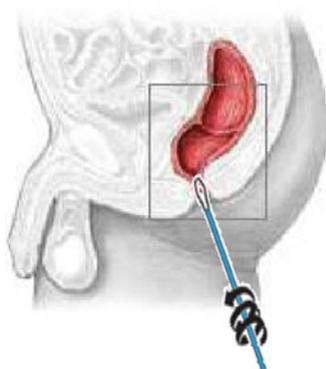


A landmark in prevention



### Step 1.

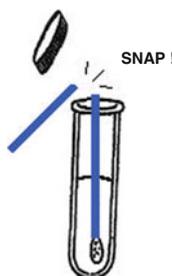
Open kit and remove tube and package with green writing. Remove the swab with the **BLUE** shaft. **USE BLUE SHAFT SWAB ONLY.**



### Step 2.

Insert swab 1 inch into the anus and turn for 5 – 10 seconds.

If needed, before inserting swab, wet swab with water or saline solution.



### Step 3.

**Remove cap** from test tube. Place swab in test tube. Do not puncture the foil cap.

Break swab shaft at the score mark.



### Step 4.

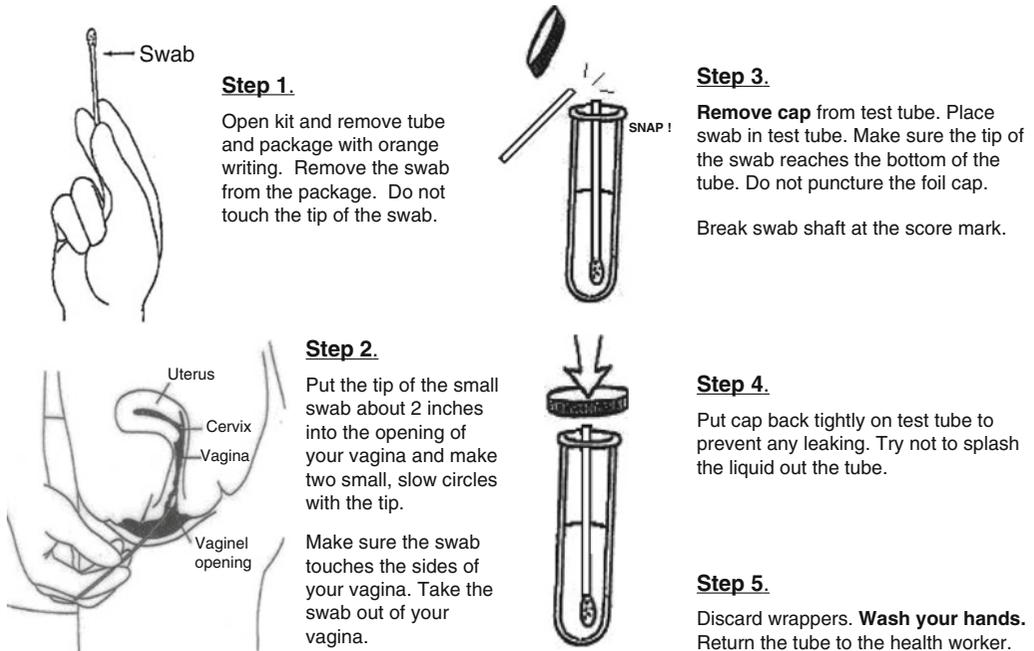
Put cap back tightly on test tube to prevent any leaking. Try not to splash the liquid out the tube.

### Step 5.

Discard wrapper and unused swab. **Wash your hands.** Return the tube to the health worker.

Fig. 2. Rectal self-swab collection instructions using the GEN-PROBE APTIMA kit for gonorrhea and chlamydia testing.

### Vaginal Self-Swab Collection Instructions



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Fig. 3. Vaginal self-swab collection instructions using the GEN-PROBE APTIMA kit for gonorrhea and chlamydia testing.

Validation of Procedures in the Clinical Microbiology Laboratory, provides general guidance. Verification studies to use NAATs for CT and NG diagnosis in rectal and pharyngeal specimens have employed a reference standard that includes a positive culture result, a second confirmatory commercially available NAAT, or a research NAAT using a different primer target (23). An alternative approach is to work with a reference laboratory that has previously verified an NAAT test for extra-genital samples. The reference laboratory can (1) verify an adequate number of test samples and (2) provide a panel of samples previously tested and characterized by the reference lab to be tested at the lab undergoing verification. The minimum number of samples recommended for a verification of an FDA-cleared indication by the ASM is 20 positive and 50 negative samples and by the National Committee for Clinical Laboratory Standards is 50 positive and 100 negative (24). Given that the confidence interval of the sensitivity estimate will be broader with fewer positive specimens tested, use of fewer than ten specimens is not recommended. A typical verification protocol uses a reference panel of at least 50 positive and 50 negative specimens, obtained from a laboratory that has successfully completed verification. The positive, negative, and control samples are run on a CLIA-approved NAAT

system in the verifying laboratory. Specimens are tested by at least two microbiologists and by a single microbiologist on different days to show consistent results among different operators and from day to day. The goal is to obtain sensitivity and specificity estimates similar to that demonstrated in the reference laboratory that provided the samples (see Note 2).

3. Amplification inhibitors in NAATs using PCR and SDA can produce false negative results. Urine specimens are more commonly associated with inhibitors compared to male urethral or female endocervical/vaginal specimens. Assay inhibitors in urine include hemoglobin, glucose, nitrites, beta human chorionic gonadotropin, and crystals. Inhibitors can be detected in NAATs using PCR and strand displacement by means of an internal control (IC). The Roche Amplicor CT/NG Amplification kit includes an IC that permits the identification of processed specimens containing substances that may interfere with PCR amplification. The IC is a noninfectious recombinant plasmid containing primer regions identical to those of the CT target sequence, a randomized internal sequence of similar composition as the CT and NG target sequences, and a unique probe binding region that differentiates it from the target amplicon. The IC is introduced into each individual amplification reaction to be co-amplified with the target DNA from the clinical specimen.

There are several options for detecting inhibition using the BD ProbeTec CT/NG amplified DNA assay. The positive controls supplied in the ProbeTec ET CT/NG amplified DNA assay can be used as ICs or a separate amplification control is available which can be used to detect inhibition. The users may develop their own ICs as described by CLSI C24-A3. These controls would be added to a separate aliquot of the suspect specimen and run in tandem.

The Aptima Combo2 assay has a novel target capture step where the primer-bound nucleic acid target binds to a magnet prior to amplification, allowing inhibitors to be washed from the sample. This minimizes the effect of inhibitors on the specimen. In spiking studies conducted by Chong and colleagues, the false negative rate for specimens run using the Aptima assay was 0.48 % compared to 13 % for specimen tests run using ligase chain reaction. Repeat testing after overnight storage reduced the false negative rate to 0 % for Aptima and 5.4 % for ligase chain reaction tests. (24) It further helps to decrease the number of false negatives due to amplification inhibitors if the specimen is free of mucus and the patient has not recently used lubricant as some lubricants have been shown to inhibit PCR. Dilution, heat treatment, or freeze thawing of samples have been found to reduce amplification inhibition (25).

4. Care must be taken to avoid sample contamination as this can lead to false positive results. If it is necessary to pipette individual specimens, the microbiologist must be cautious not to cross contaminate samples through inadvertently touching more than one specimen with the same pipette tip or to allow any specimen to drip from the pipette tip once processing is finished. The microbiologist needs to be aware of the state of the specimen container. Improper seals and a buildup of pressure inside the specimen container can lead to aerosolizing of the specimen and potential contamination as well as a biohazard for the microbiologist.

Due to the molecular nature of the NAATs, it is very important to maintain a unidirectional work flow. The optimal arrangement is to maintain dedicated rooms for the pre-amplification and post-amplification stages. However, in an established laboratory this may not be possible and in this situation at least separate areas should be established for the pre-amplification and post-amplification procedures that are physically separated but with the ability to maintain a unidirectional flow. The processing should be designed to start with reagent preparation and move through product amplification without physically crossing back into a section of the lab used in an earlier part of the process. At each step, the work area must be thoroughly cleaned with at the very least a 1:1 bleach and water mix to avoid contamination from the previous batch of specimens. Maintaining work flow discipline is crucial as extremely large numbers of amplicons are created from a relatively small amount of nucleic acid. A very small number of amplified target accidentally crossing back into the pre-amplification area can cause false positives through several batch runs. Gloves should be changed after cleaning the area, pipetting specimens, mixing reagents, preparing the controls, and between the pre-amplification steps and post-amplification steps.

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### 3. Notes

1. The preferred anatomic specimen for NG/CT testing for urogenital infection is urine in males and self-collected vaginal swab in women. NAATs are FDA cleared for use with male urethral, female endocervical, and urine specimens. The APTIMA assay is FDA cleared for use with self-collected vaginal swab specimens.
2. An indispensable part of any quality program is the use of controls. Each of the NAAT systems mentioned in this chapter is FDA approved and includes controls for continuous quality

maintenance. However, it is important to remember that the manufacturer's performance claims for these assays need to be verified in each laboratory environment before any system is used to test patient samples. Verification requires conformation of the test performance characteristics including sensitivity and specificity. Sensitivity is a measure of the test's ability to accurately detect patients with a specific disease. Mathematically, this is expressed as the number of true positive results divided by the number of true positive results plus false negative results multiplied by 100. Specificity is the measure of a test's ability to accurately identify all noninfected patients. Mathematically, this is expressed as the number of true negative results divided by the number of true negative results plus false positive results multiplied by 100. The clinical laboratory director is responsible for verification studies. The exact procedures for each verification study will vary depending on the lab. Consultation with local CLIA authorities is imperative. The general principle is to test a number of known specimens to assure that the results, that is the specificity and sensitivity, at least match the results published in the manufacturer's package insert. This can be accomplished using specimens spiked with a specific amount of organism procured from an institution such as the American Type Culture Collection (ATCC) and specimens that are not spiked. Once this basic level of accuracy is accomplished, then a larger number of known patient specimens—that is positive specimens that have not been manipulated and known negative specimens—need to be tested. It is from these results that the legitimate performance verification can be ascertained by the laboratory director or the decision made to do further testing with a larger number of samples. Documentation of the verification study must be maintained by the clinical laboratory director; it may be requested by CLIA inspectors.

In general, testing should be targeted to those at the highest risk. This general recommendation prevents excessive false positive results, which, regardless of the test, are always more likely in low-prevalence populations. The positive predictive value (PPV) is defined as the proportion of subjects with a positive test result who are correctly diagnosed.

$$\text{PPV} = \frac{\text{number of true positives}}{\text{number of true positives} + \text{number of false positives}}$$

For example, the Aptima Combo 2 assay package insert states that the test for *N. gonorrhoeae* has a sensitivity of 99.2 % and a specificity of 98.7 % when cervical swab specimens are used and a sensitivity of 91.3 % and a specificity of 99.3 % when urine specimens obtained from women are used. This would translate to a

PPV for the test in a female population with a 1 % prevalence of *N. gonorrhoeae* of 43 % and 57 % for cervical and urine specimens, respectively. The PPV increases to 87 % for NG in a hypothetical population with an NG prevalence of 5 %.

Despite the concern of a lower PPV using NAATs in low-prevalence populations, a study which tested 59,664 unduplicated cervical or urine specimens from women of which 280 (0.47 %) tested positive for NG directly estimated PPV for the APTIMA assay in this very-low-prevalence population. This group performed confirmatory tests on all specimens that yielded positive results and found a PPV of 97 %, far superior to that reported in APTIMA's package insert (12).

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# Chapter 29

## Sexually Transmitted Diseases: Reflections on Metaphors and Ethics

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### Abstract

Sexually transmitted diseases (STDs) are probably the most tabooed diseases we know. The taboos and the related stigmata shape patients reality and influence significantly health care policies, medical research, and actual problems in medical ethics. To better understand these complex influences of ancient but still powerful taboos, related metaphors associated with illness and disease are analyzed according to their historical development and actual impact on society. It becomes obvious that research and health care policies cannot be satisfyingly successful in helping people affected by STDs as long as they do not take the mechanisms of taboos and associated metaphors into account.

**Key words:** Sexually transmitted diseases, Health care policies, Medical ethics, Medical history, Metaphors

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### 1. Introduction

Talking about the pathological means talking about the normal. The understanding of the discourse about disease and health as a discourse about normality, norms, and values is one of the central and often contested theses in the influential work of Georges Canguilhem “Le normal et le pathologique” (see Note 1) (1). Venereal diseases are entangled in multiple discourses about the normal and the abnormal, proximity and distance, and “us” and “them.” The immense political potential of the debate lies in the tempting possibility to mix and exchange—more or less veiled—the different underlying concepts of normality, be it with regard to sexual behavior, disease, or other human conditions. Canguilhem’s work can serve as a heuristic guideline to analyze the historical and

actual problems with and the corresponding debates about sexually transmitted diseases (STDs). The important role of metaphors in the communication of underlying norms and values in the context of STDs has been lucidly outlined by the American writer Susan Sontag in her historically rich essay “Aids and its metaphors” (2). In contrast to a purely body-centered or scientific approach, she states that our attitudes towards STDs are highly dependent on the myths and metaphors shaping our past and current understanding and “image” of venereal diseases. Therefore, people aware of having an STD are suffering from a tabooed, morally and metaphorically overloaded disease that affects their private life in a multidimensional, highly context-sensitive, and complex manner. This short review reflects upon the major metaphors and taboos dominant in the debates on STDs, and their influence on actual ethical and social problems in the treatment of persons with STDs. The analysis is guided by Canguilhem’s concept of normality and pathology to further elucidate the complex genesis of stigma and its consequences for people affected by STDs, health care policies, and research. It might shed some light on the question why it is so difficult to design and perform effective strategies to reduce the suffering of those affected or menaced by a venereal disease.

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## 2. Major Persisting Metaphors

### 2.1. *The War*

One of the most dominant group of metaphors attached to illness in general are military metaphors. We are well accustomed to “fight against” a disease, to talk about defenses, and to stop the “invasion” of the human body or the whole society by dangerous “intruders.” We use military metaphors in many other contexts, too (e.g., war on drugs, another discourse associated with STDs), with the effect that a state of emergency is confirmed—or created—where, even in modern times, excessive expenditure of resources as well as a dramatic change in priorities are justified without further debate about profitability or desirability of specific measurements (like in a war against terror). However, the rhetoric on war against diseases is not fully understood when it is merely seen, as a means to acquire more research and health care funding. As Sontag emphasizes: “The metaphor implements the way particularly dreaded diseases are envisaged as an alien “other” (see Note 2), as enemies are in modern war; and the move from the demonization of the illness to the attribution of fault to the patient is an inevitable one, no matter if patients are thought of as victims” (2, p. 97). This general note is even more important for our context of infectious diseases, because the use of military metaphors contributes to the stigma not only of the disease but also of the affected

patient—the carrier of the dangerous, society-threatening agent. For example in the case of HIV: being infected by the virus means—up to now—being a menace for others for the rest of one’s life. AIDS, or HIV carriers, as well as social groups, prisons, cities, or whole nations have been repeatedly described as “ticking time bombs” (see Note 3) (3). Syphilis, firstly named this way after its mass distribution in the army of the French king Charles VIII and its further spread from Italy to the rest of Europe from the late fifteenth century onwards, received—among many other names—those of the (hostile) countries where it was supposed to have originated: *morbis gallicus*, *m. neapolitanus*, *m. italicus*, and others (4).

This perception of infectious diseases had in the cases of Syphilis and HIV political implications that were directed at excluding infected and possibly infected from society. In addition to framing “otherness” the military discourse also serves self-affirmation in terms of normality (the healthy, the friend, the compatriot) and offers a possibility to promote strategies to hold “aliens” outside, even when the political situation might not favor such attempts. This is especially obvious in the case of HIV and AIDS. Associated groups are those already marginalized because of other reasons: homosexuals, immigrants (see Note 4), black communities, or illegal drug consumers. Adding the notion of belonging to a “high-risk,” i.e., dangerous community fuels already existing attempts to exclude “them” from the “normal” society. During the first period of panic associated with the emergence of AIDS, e.g., the former US President Ronald Reagan advocated the mandatory testing of “aliens” applying for permanent residence status (5). First ideas to confront the new nationwide threat included the installation of detention camps and quarantine measurements (meaning a life-long exclusion of the ill) as well as ideas to tattoo persons being tested positive to warn potential sex partners—a modern version of the leper’s bell (5). Thus, governments have been inclined to potentially use their power to take drastic measurements to control the spread of an epidemic disease: segregation, lifelong quarantine, and even killing of the dangerously ill are measures that have been taken throughout history (4, 5). That such measurements are not possible in today’s democratic societies should not mask the still existing perception of persons carrying a lethal and highly infectious disease, able to generate an epidemic, as a public menace. The first reflex to keep “those” out is still familiar to us. Patients being exposed to a highly medialized political and public “war” against what they are suffering from are likely to develop a sort of identification with this public enemy—in the case of HIV even a global enemy, an enemy to mankind. This factor understandably drives many to hide their illness rather than to show it openly. Secrecy about STDs is one of the major problems in their treatment today, especially with regard to young persons (6).

## **2.2. Pollution and the Sacred**

Alongside with the notion of the ill as being “the other” (or the diseases as “foreign”) comes the metaphor of pollution—something attributed to the “wrong,” the “other” that does not have a place in society: a “matter out of place” (7, 8). According to the theory put forward by the anthropologist Mary Douglas, in traditional societies disease is considered to be a pollutant, often the result of moral contamination—the sacred state of the person or object is violated, and the affected may in turn contaminate others. One thesis is that when faced with (life-threatening) uncertainty and the menace of chaos, people fall back on these earlier beliefs about the “sacred and the profane” (9). Being “contaminated” (with a virus, bacteria, or a mysterious entity—like the HI virus at the beginning of the perceived AIDS onset in the 1980s) leads other people not to touch, speak of, or even look at the concerned individual. This is—at least according to secularization theories and scientific beliefs—especially the case as long as a scientific explanation of the transmission pathways is not available, uncertain, or obscured by other reasons. At a first glimpse, these pre-modern models might not seem relevant for today’s situation. However, recent studies in different social groups reveal that even after more than 20 years of immense educational efforts in most countries worldwide, knowledge about the transmission pathways of AIDS or Syphilis—and the corresponding ways to protect oneself and others from infection—is still not equally distributed and ancient beliefs grounded on ideas of the profane and the sacred persist (like divine healing or touching the “pure” virgin). And this is not only due to well-known factors like socioeconomic differences, ethnic contexts, or still absent sexual education for young homosexual men, e.g., at school (10–12), but also because myths or misconceptions persist beyond the scientific rationality (13). People perceive themselves as being contaminated and outside the “normal” community, they (often realistically!) fear exclusion and tend to hide their infection—or even prefer not to know about it and thereby remain a part of their society. It is quite obvious that in a situation like this, purely scientific driven efforts for prevention and containment of STDs cannot be 100 % effective. One has to admit that scientism is not appealing to everyone and maybe one must accept seemingly “irrational” reactions of society as a fact that shows us the limits of scientific public education. Other approaches and further research to find ways to overcome this gap are needed.

## **2.3. Sexuality**

The mentioned general factors contributing to the stigmatization and discrimination of illness and the ill are dramatically enforced and rendered more complex when combined with other ancient taboos and highly normative issues. For example, sexual behavior was and still is subject to social rules of high rigidity. STDs always strengthened the position of those holding abstinence and monogamy

for the “normal” and therefore desired sexual comportment. The “normality” of sexual abstinence before the (monogamous) marriage is still more or less openly addressed. Apparently, the reluctance of many officials to promote education about “safe sex” was and is often grounded in anxiety not to be regarded as promoting promiscuity or sexual intercourse before or outside marriage (another “sacred stage”). This was the case when the former US president Reagan refused public funding for a brochure written by the US surgeon general in the mid 1980s, explaining what was known about AIDS and the prevention of its transmission, because the governmental line was the emphasis on sexual abstinence (5). Since nonmonogamous life forms are now—politically—less easily condemned on moral and religious grounds than, e.g., 30 years ago, the shift from sexual norms to a medical, consequence-oriented argumentation against multiple sex partners became mandatory for those wishing to stay in the mainstream political debate. This shift from the moral to the seemingly neutral medical discourse to promote a monogamous lifestyle is well illustrated in an editorial remark on a study about genital chlamydial infections in a 1993 edition of the *Australian and New Zealand Journal of Obstetrics and Gynecology*. This editorial stated, “Regardless of moral considerations, a life-long unshared partnership looks a better proposition than ever before (14).” It provoked a heated discussion (15). Many authors’ anger about this seemingly moderate statement demonstrates how much scientists “fighting” for an open “rational” treatment of STDs see their work hampered every day by a society not willing to accept other moral norms of sexual behavior than those anchored in their belief system. However, as is to be expected, there is no homogeneous attitude among scientists either. In a study about sexual activities among African Americans—considered as being especially affected by HIV and AIDS—Doherty and colleagues found social networks of discordant sexual behavior that place black women in a relatively high risk for heterosexual infection with HIV. And although listing some of the many factors contributing to the vulnerability of black Americans, they come to a surprisingly simple goal of a desired governmental action: “HIV prevention strategies in the US should extend beyond individual-level interventions to include policies concerning income support, education, and sentencing inequity and other criminal justice issues to foster long-term monogamous sexual relationships, which are the bulwark against transmission of HIV” (16). For today’s public health care, the reality that monogamy is not the desired life form for a large part of the society (i.e., these people are, therefore, not excludable as a distinctive group from the rest of society) has to be taken into account and strategies to reach those having multiple sex partners by public health promotions actively try to present promiscuity as a “normal” behavior, but add the necessity of self-protection (the use of condoms, e.g.) as the imperative for rational

and responsible actions (see Note 5). Thereby, the concept of the poorly known sex partner as a potential menace (the “alien”) towards whom every reasonable person approaches only with adequate protection in place evokes xenophobic attitudes within the realm of an intimate relationship, which were discussed above for the societal level.

### 2.3.1. *Sexual Education*

The hesitation to promote sexual activity (or appearing to do so) is still an active factor especially when programs for adolescents are concerned and it is seen as a major obstacle for an open discussion and effective treatment of STDs, e.g., in the debate about (compulsory) HPV vaccination programs in school (17). Apart from—but often hidden behind—the arguments contesting efficacy, adequacy, safety, etc. of the available vaccines, there is a high reluctance among politicians now labeled as “conservative” to accept the picture of young people having sexual intercourse. Likewise, parents often do not want STD-preventive measures or even education—especially for girls—because they do not want their children exposed or introduced to a life form allowing sexual activity as a “normal” practice among young people (18). Of course, monogamy and sexual self-control is an active and effective dispositive for adults, too. But it seems to be more easily publicly addressed when adolescents are concerned.

### 2.3.2. *Sexual Behavior and Privacy*

However, even without a political interest in the promotion of certain life models, questioning people about their sexual relationships is always a delicate matter. For example one of the first obstacles to the morally highly contested strategy of “contact tracing” in cases of STDs is, of course, the undeniable disclosure of another sexual relationship, which puts the “stable” partnership at risk, sometimes with far-reaching consequences, e.g., in the not uncommon cases where the HIV-positive partner is an immigrant and relies strongly on the support of the partner. Underlying the strategy of contact tracing is the assumption that the HIV-positive patient would not tell his/her partner(s) about the infection, which means putting a whole group of people under the general suspicion of behaving in a morally and legally problematic manner. Furthermore even to confess certain sexual practices to medical staff is, for most people, associated with a sense of shame and fear of being marked as “not normal.” In this case, confessing to opt for the risk of acquiring a sexually transmitted infection as a probable consequence of a desired life form—that includes unsafe sexual practices based on a general trust in others—means confessing not to act according to the rules of a self-controlled society, which tends to prioritize health issues as the rationale for decisions about life forms. This means by default that one’s actions are irrational (see Note 6).

### 2.3.3. Homosexuality

A special subject within the sexuality discourse is homosexuality—especially male homosexuality. That a disease afflicts homosexual men in a disproportionate ratio still provides new powerful arguments to those opposed to homosexuality because of other normative reasons than medical rationality. “That anal intercourse is the major mode of [HIV] transmission in gay men places it further beyond the boundaries of “normality”, as does a multiplicity of partners which has been identified as a risk factor” (9). Here, a disease is stigmatized by its association with a “morally threatening group” (see Note 7) (9) and in turn stigmatizes all those attainted of the disease. Deviance and delinquency are readily associated with homosexuality—and AIDS. The applied moral judgments are not only related to religious beliefs or concepts of a natural order that rules out homosexual behavior as “not normal.” There has also been a strong political use of the concept of homosexuality during the onset of AIDS as a recognized epidemic in the 1980s, e.g., in the (at the same time collapsing) former Soviet Union and associated nations. “[...] the “myth of universal heterosexuality and patriotic sexual restraint” in the late Soviet period suggested that to be “queer” meant that one was not a patriot” (19). As in other countries, hetero- and homosexual intercourse was no longer the affair of the individual but a public matter. In a study about the political development and its influence on the perception of homosexual persons in Poland (and in part the Soviet Union), the author points out that “themes of violence, abuse, and general decay dominated socialist accounts of homosexuality” (19). During the infamous Hyacinth actions of the mid 1980s in Poland, men suspected of being gay were arrested in schools, universities, and enterprises, every detail about their sexual life, fingerprints, and photographs were registered, and they were forced to cooperate with the secret police and thereby visibly put “out of” society. This in turn designated gay men only as risk factors not as being at risk (and in need of help) themselves. HIV and AIDS were used as a pretext to persecution of gay men in that country dominated by a traditional catholic society (19).

When dealing with education programs, e.g., campaigns for routine testing among the so-called high-risk groups and other public policies like contact tracing, we have to bear in mind that homosexuality has a long, dark, multifaceted, and still fluctuating history. It was and still is (at least in some countries) considered as a crime or sin, as a disease with different “treatment options,” at least from the nineteenth century until officially 1992, when the WHO eliminated homosexuality from the ICD list (see Note 8) and, at least officially, acknowledged it as accepted private behavior. To assume a homogenous social attitude towards homosexual behavior is a dangerous fault when education, prevention, and treatment strategies are conceptualized on this assumption. The moral perception of homosexuality varies over time and

space—even on a small scale. The obvious changes in the political landscape since the early years of the twenty-first century, when in the course of the gay pride movements, more and more prominent politicians had their “coming out” as homosexuals, thereby demonstrating their support for an integration of homosexual people into “normal” society, should not conceal the fact that all the other concepts—evolved during the historical movements of medicalization and de-medicalization of a generally tabooed human behavior—are still applied to it and HIV/AIDS/Syphilis, respectively, contributing to a burden of stigma that prevents people from disclosing their medical status to anyone, including medical staff. Besides general measurements with regard to efficient data protection in medical and research contexts (see below), this problem lead, e.g., to the development of HIV home kits for self-testing that are seen to provide fairly good results whilst ensuring complete privacy (20). Of course, other problems like lack of adequate counseling with regard to the interpretation of results, medical options in case of positive results, etc. do occur.

Again, the moral reservations against homosexuality that are not openly addressed by those wishing to create a liberal, modern, democratic image of themselves find their expressions in a discourse that focuses on “medical consequences” of homosexual behavior, thereby allowing to discriminate the “abnormal” behavior by other means than yesterday’s moral convictions—like in the promiscuity discourse. In a situation where, from all the diverse reasons mentioned, homosexuality is not easily addressed, public education is a delicate matter. The lack of homosexual education for adolescents, e.g., is considered as one reason why so many young men get infected with the HI virus. They simply do not know much about gay sexual practices and are, therefore, not able to link AIDS- or Syphilis-related advices to their often vague expectations (12).

#### **2.4. Death**

What has contributed and still contributes to AIDS as a taboo is its association with inevitable death. Moreover, as Susan Sontag finely formulates, cancer and AIDS are often perceived as synonymous with death (2). Thus with death being a powerful taboo in most societies, another ring of silence surrounds this disease. AIDS as a metaphor for death was created during the age in which no treatment was available and the pathways and mechanisms leading from an HIV infection to the development of the Acquired Immune Deficiency Syndrome were more obscure than they are today. But the principal availability of highly active antiretroviral therapy (HAART) should not mislead one to believe that the horror of AIDS has been overcome. The diagnosis of AIDS is still perceived as a “death sentence,” dependent on the country where one lives in (for example AIDS-related mortality in the USA is higher than that in Central and Western Europe, and highest in some African countries), the socioeconomic status of the affected person, and

the moral, legal, and political contexts that people are exposed to. A social death is not necessarily perceived as being less threatening than a physical death. However, even if a person is in a position to receive and permanently adhere to HAART, he or she is still a life-long carrier of the virus, living with a hopeless prognosis, and remaining menace of a possibly lethal manifestation of AIDS. Additionally, all the mentioned stigmatizing factors will be active and may lead to isolation from friends, family, and colleagues and a reshaping of one's social life from the roots.

This is not only a private problem. Being a potentially lethal danger to intimate partners and, e.g., in the cases of HIV-positive blood transfusions, to unknown others, has the potential to render actions where the infection of others is involved under the jurisdiction of criminal law. Being knowingly infected with HIV and having unprotected sex have been brought to court several times (21). Most cases have been related to forced exposure to body fluids (rape, bites...) and were judged according to existing laws (21). In 1987 an informed HIV-positive man in the USA was charged with attempted murder following a sexual attack on a woman. However, there have been other cases, in the USA or Europe, where men and women (see Note 9) have been charged with criminal conduct for having sex after being tested positive under existing laws (see Note 10) (5, 21). Under these conditions, the "right not to know" about one's medical condition becomes even more delicate.

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### 3. Actual Issues in Medical Ethics

#### 3.1. *The Right Not to Know*

In general medical practice, the right not to know about one's medical status is considered as equal to the right to know about it. When it comes to a dangerous, potentially lethal infectious disease, this right is especially challenged. In view of the highly stigmatizing effects of an STD diagnosis, and of course the fundamental rights of an individual for respect of his or her bodily sovereignty and integrity, the above-mentioned ideas of forced testing of all suspected individuals have not been realized in most democratic countries. Public health interventions to influence sexual risk behavior focused, at least until the beginning of the twenty-first century, almost entirely on the general (presumably uninfected) population in order to avoid further stigmatization and stress for the patients (22). For a couple of years now, strategies have changed towards an active inclusion of HIV-positive individuals due to several developments—the most important probably being the availability of treatments like HAART and their manifold consequences on people's sexual behavior (22). It has been shown that people who know about their infectious status generally change their

sexual risk behavior and that most new infections are attributed to people that were unaware of their infection (22). Routine testing for HIV is, therefore, again on the agenda of several states. In 2006, the Center for Disease Control (CDC, USA) published its revised screening recommendations where pretest counseling and a specific affirmative agreement from patients prior to testing were withdrawn (opt-out solution) in order to increase the number of persons tested (23). These measurements have received some criticism because they violate, in the view of some, autonomy rights of patients and the principle of the “least infringement.” According to this principle, “public health policies” may infringe upon “general moral considerations” in order to achieve a public health goal, but if two policies provide the same public health benefit, then policymakers should choose the one that infringes least upon “general moral considerations” (23). Of course, testing without detailed prior counseling will, in many cases of positive testing, provoke immense personal problems. The authors argue that data are not sufficient to provide evidence that the measures really increase public health benefits in a way that justifies the violation of the principle of autonomy. However, even if they had proved to be effective, the question of the moral justification of such a violation of an individual’s right would still be left open.

The right not to know is equally challenged in the context of contact tracing. In addition to the other problems of contact tracing mentioned above (personal relationships, stigmatizing of homosexuals), contacting someone because of the HIV status of his/her sex partner involves several steps that need intimate private information and that, therefore, has the potential to violate the fundamental right to privacy. Accordingly, laws regulating the procedures of contact tracing are necessary to ensure patients’ and partners’ rights. They are not established everywhere and even within a country differences in the legal coverage of contact tracing persist (24).

In the context of several STDs, the right not to know is even more important when we consider the immense burden imposed by a diagnosis of the infection at a point where the person is still healthy. Being infected does not automatically tell you if, how, and when the symptoms will appear. Nevertheless, the fears are immense and decisions regarding treatment options, eventual changes to social and intimate life, etc. have to be taken immediately. The suffering provoked by the diagnosis of a severe future disease has been largely discussed with regard to genetic testing (25). Wehling and Viehöver (26) have called this the de-temporalization (“Entzeitlichung”) of a disease as a form of medicalization: a person who is not feeling ill is confronted with the diagnosis of a possible future illness (comparable with the diagnosis of late-onset genetic diseases, like Huntington’s Chorea) and becomes a patient through the very act of knowing about a “disposition,” a carrier of a disease.

The right not to know has been abandoned to some extent for pregnant women, where routine HIV testing has been introduced in many countries. The justification for this routine screening of a highly stigmatizing condition has strengthened since the risk of the transmission of HIV can be minimized to about 1–2 % (27) when adequate treatment (HAART treatment of mother and baby, elective cesarean section, and abdication of breastfeeding) occurs. Before the availability of treatment, testing a pregnant woman for HIV had, in case of a positive result, only the alternative of abortion—with all the moral problems well known from other debates about prenatal diagnosis (5). Apart from the fact that existing treatment options are, of course, not available in all countries (27), the solution of routine testing of pregnant women is still not self-evident. Before the availability of combination antiretroviral therapy, mother-to-child infections ranged between 12 and 45 %, depending on various factors (28). Since the antiretroviral therapy is not without side effects, some people might not regard the treatment option as an adequate alternative in light of a risk factor that appears lower than mere chance. Here, again, the question is not “is it medically reasonable to test and to take all available measurements”—that seems to be proven—but rather “is it justified to put the burden of knowing about their medical status on all, including those not wishing to go through the offered treatment options because they might have other priorities in their moral and personal belief system.” Like in other routine testing contexts it is crucial that adequate free and informed consent is sought. Simple opt-out procedures with no requirements of specific and comprehensive counseling and informed consent can be problematic (see Note 11).

To conclude, one has to state, of course, that the right not to know collides with other person’s right to know, such as potentially infected partners, as well as the right to treatment. The right not to know is surrounded by a variety of complicating factors (like gender issues, the above-described stigma potential, and others) especially, but not exclusively, in the so-called low-income countries, as shown above (29). Routine testing is still seen as one of the best options in preventing the spreading of STDs by many. Rising awareness about the complex environments in which testing takes place should be seen as a prerequisite to an ongoing sensible evaluation of the many more or less hidden effects of routine testing—with a special emphasis of maintaining individual rights as far as possible (30, 31).

### **3.2. Data Protection**

Closely connected to questions of screening and the right not to know are issues of data protection. Data protection is one of the most delicate topics in the context of STDs. With regard to the highly stigmatizing potential of STDs, personal medical data are more delicate than for the majority of other diseases (with some exceptions,

e.g., mental diseases), again especially for incurable diseases like AIDS. As analyzed above, the mere existence of HAART, apart from it not being accessible to all patients, does not change some of the essential stigmatizing aspects of the disease complex.

Major concerns surround the employment and insurance sectors, but research, especially when using large databases, is chronically subject to data-protection problems, too. Some aspects that are directly linked to the stigma attached to STDs should be shortly outlined: Already in the beginning of the AIDS epidemic in the 1980s, employers were anxious about liability issues: “Employers wonder what type of liability they will be exposed to if an employee infects someone else in the workplace” (5). The fact that employers want to know the health status of their employees in order to judge if they are capable of fulfilling their future tasks is largely recognized. Of course, employers also like to know if the candidate is trustworthy, confident, etc., i.e., if his or her moral character is flawless. STDs, as has been shown above, are associated with morally inadequate behavior—like promiscuity, homosexuality, and “abnormal” sexual practices (such as visiting bathhouses). Medical data about the STD status of a person always convey an image of his or her sexual life and thereby his or her moral character (see Note 12). In the context of STDs, behind or besides the fear of liability or the argument of acting in favor of the candidate when ensuring his or her physical capability to perform a certain job, the desire to have “morally correct” staff members is also prevalent. Legal problems related to the permissibility of demanding health tests for STDs persist today and are solved according to national standards. For example in Germany, the German National Ethics Council points out in its 2005 “Opinion” for employments in the public sector: “At any rate, questions about whether the candidate belongs to one of the major groups affected by HIV and about sexual orientation have certainly been considered permissible with a view to deciding whether an HIV antibody test should be carried out” (see Note 13) (32). The private sector has other means of avoiding the financial and social burden of STD-affected employees—like outsourcing production parts, reduction in employees’ benefits, or—wherever legally possible—preemployment screening, e.g., in South Africa (33).

Issues of data protection of course play a role in the context of health insurances, mainly concerning private insurances. The selection of clients or the level of demanded fees in cases of “high risks” can be a crucial barrier to the access to basic health care, at least in states where no general insurance system (e.g., compulsory insurance or universal coverage) exists. In the United Kingdom providers were allowed to ask for STD risk-related private information prior to the insurance contract. The so-called gay question was highly contested and led to a change in insurers’ questioning policy—without altering the right to ask about sexual behavior (34).

In research, data protection is a prominent issue that frequently provokes discussion and scandals (see Note 14). Ensuring the anonymity of patients taking part in clinical trials or of patients whose medical data are used for research purposes is not a simple issue and subject to ongoing adjustments and refinements. Furthermore, research on STDs always means exploring people's deepest intimacy, an endeavor that is faced by concrete practical problems—like men not admitting that they had sex with other men (or even outside their stable relationship) leading to a distorted picture about heterosexual transmission rates. There is also a debate about study designs leading to the creation of “groups” that are regarded as not only artificial but also creating new social realities, e.g., MSM (35). This seemingly neutral scientific term, now widely used in medical, social, and other research about STDs, clusters a group that “in real life” is highly inhomogeneous. But “inventing” a group for scientific reasons will in turn—via public health care policy, media, etc.—have affects on the (self) perception of people finding themselves associated with others they probably never even thought of before. One single, seemingly “neutral,” scientifically reduced character—having sex with other men—places concerned men in the context of different highly stigmatized groups. Furthermore, research on persons affected by STDs is in most cases research on vulnerable subjects (discriminated, drug addicts, the poor). Special emphasis has to be put on the protection and treatment of persons who can be subject to discrimination, family or partnership problems, etc. merely by participating in a research study.

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## 4. Conclusion

Major ethical issues of our understanding and treatment of STDs are centered on the manifold sources of stigma, historically and ethically, associated with these diseases: Infectious or individuals suspected of being infected as well as various other groups are excluded from society by diverse mechanisms of “othering.” They can be understood as the results of applying certain ideas of normality and abnormality with regard to sexual behavior, danger, or contamination of the assigned subjects. The respective metaphors applied are associated with war, impurity, death, and others. Their frequent usage creates a climate where people often prefer not to know about their medical status or not to confess it to others, leading them not to seek help and health care. The mechanisms of exclusion serve at the same time to reassure those worried and disoriented by the menace of a severe infectious disease that they still belong to their society.

Furthermore, the medical discourse about STDs is used by various groups to influence moral and social norms with regard to not only medical but also sexual, political, or other behavior. The right not to know and the need for data protection are especially challenged, when law and public health policies interfere with the most private issues of the individual's life. The need to protect people from discrimination, i.e., exclusion from society, has to be sensibly balanced against their right to information and treatment.

In addition to scientifically driven efforts to help people suffering from STDs it is important to search for additional ways to access the complex patient realities built up by norms and values other than scientific ones: diverse social, religious, or moral considerations, even if they might seem irrational at first (scientific), are often deeply rooted in communities and should be taken into account when designing public health policies. The highly normative discourses underlying the public perception of STDs, especially with regard to the rigid norms about "normal" sexual behavior, have to be kept in the public awareness, as they contribute to an immense potential for discrimination in various fields—such as in employment, insurance, and research issues.

Research on STDs is challenged by all these factors. Furthermore, research itself constitutes to the public discourse, not only by finding new treatment options but also by creating new social realities that are not adequately addressed by focusing on the scientific aspects alone.

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## 5. Notes

1. Written in 1943 as a doctoral thesis and later reedited and translated.
2. We still—though not uncontested—basically describe our immune system within the metaphors of "defense," the self versus nonself (or "other").
3. <http://www.thezimbabwean.co.uk/news/35203/prisons-ticking-time-bomb-for-hiv-positive-inmates.html>, <http://edinburghnews.scotsman.com/hivandaids/City39s-ticking-timebomb-of-100.6558410.jp>, <http://www.zimintransition.com/news/gender-issues/post-election-violence-zimbabwe-sitting-hiv-and-aids-ticking-time-bomb>; last access September 2011.
4. See, e.g., the Daily Mail article from November 2008 titled "HIV illegal immigrant may have infected more than 400 women" that comprised a detailed description of a Jamaican(!) immigrant who presumably infected "British" women, as well

as patients in a mental hospital (the connection of mental illness and STDs is manifold, see below).

5. See, e.g., the long-lasting German promotion strategy “Gib AIDS keine Chance” (Don’t give AIDS a chance) invoking scenes of liberal, romantic (protected) love as a self-evident matter of choice: of course when asked to choose between, e.g., “discover feelings” (Gefühle entdecken) and “risk AIDS” the message is simple: liberal sexual behavior yes but only under the rationale of self-protection (“use condoms”) <http://www.machsmitt.de/>.
6. As a side remark that cannot be analyzed in detail, here, the linkage between rationality, normality, “abnormal” sexual appetite, and mental disorders should at least be mentioned at this point. Apart from the medical linkage between some STDs and mental disorders, the four topics are highly merged in the “normality discourse” about the pathological and the healthy. It should just be mentioned that the image of the different STDs (mainly AIDS and Syphilis) is not the same with regard to associated mental disorder. Syphilis, in spite of being associated with a high “sexual appetite” and apart from all negative aspects of course dominating its image, had, at least in the late-nineteenth- and early-twentieth-century Europe, a flair of causing a heightened creative mental activity due to the several well-known writers and artists like Schubert, Van Gogh, Baudelaire, or Maupassant who were afflicted with Syphilis (2). By contrast, at present, AIDS evokes no positive association at all.
7. In this context it is important to realize that research plays a role in creating stigmatized groups, e.g., with the invention of the “MSM” factor (men who have sex with men, see below).
8. However, homosexuality is still subject to medical endeavor, e.g., in Germany where the listing of “therapy options” to “cure” homosexuality from the League of Catholic Physicians (<http://www.bkae.org/index.php?id=439&L=0>) provoked some discussion (36). And the possibility that homosexuality will be re-medicalized is already under discussion (37).
9. Like the German singer Nadja Benassai in 2010—see, e.g., CNN news from August 26 [http://articles.cnn.com/2010-08-26/world/germany.hiv.case\\_1\\_hiv-positive-sexual-partners-youth-court?\\_s=PM:WORLD](http://articles.cnn.com/2010-08-26/world/germany.hiv.case_1_hiv-positive-sexual-partners-youth-court?_s=PM:WORLD).
10. Like in the homosexuality discourse, men are remarkably more in the core attention of risk perception than women. It seems that associations in all discussed metaphorical contexts (military, normal sexual practices, etc.) are more readily linked to males as the aggressor, the “wrong.” This gender bias should be kept in mind when discussing the gender topic with regard

to women, e.g., as victims of violence and as a disadvantaged group concerning access to medical care, social pressure, and especially the problems of power inequality between men and women disabling women to ask for condom use, medical help, etc. (see, e.g., ref. 38) or the homepage of Women, HIV and AIDS <http://www.avert.org/women-hiv-aids.htm> (last access September 19 2011). Gender biases are active in both ways.

11. The aspect of the child's welfare in this context is comparable to problems arising when people reject established treatment options for their child in other medical conditions (e.g., Jehovah's Witnesses refuse blood transfusion, if medically necessary in the course of operations). There, as well as for STDs, the parents' right to reject a treatment for their child is usually accepted.
12. Of course there are cases of "innocent" infections, but apart from the problem of having to explain the conditions of STD acquirement, even then suspicions are likely to arise.
13. In Germany, state employees can still have lifetime positions. There are sharp differences concerning the right to demand tests between the public and the private sector.
14. The German Competence Network for HIV/AIDS (<http://www.kompetenznetz-hiv.de/>) has been under discussion for having used data in international research cooperation without prior permission by the patients (<http://www.ondamaris.de/?p=142>).

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