WORLD HEALTH ORGANIZATION
Regional Office for the Western Pacific

STI

HIV

LABORATORY TESTS FOR THE DETECTION OF REPRODUCTIVE TRACT INFECTIONS

1999

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STI/HIV

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WHO Library Cataloguing in Publication Data

Laboratory Tests for the Detection of
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ISBN 92 9061 148 0

ABBREVIATIONS AND ACRONYMS

BV	bacterial vaginosis
DFA	direct immunofluorescence assay
DNA	deoxyribunucleic acid
dNTP	deoxynucleotide triphosphates
dUTP	deoxyuridine triphosphate
EDTA	ethylenediaminetetraacetic acid
EIA	enzyme immunoassay
ELISA	enzyme-linked immuno-sorbent assay
FTA-ABS	fluorescent treponemal antibody absorption test
GAC-EIA	IgG antibody capture EIA
HIV	human immunodeficiency virus
HPV	human papillomavirus
HSV	herpes simplex virus
IQS	internal quantification standard
LCR	ligase chain reaction
LE	leukocyte esterase assay
LIA	line immunoassay
LPS	lipopolysaccharide
MHA-TP	microhaemagglutination assay
NASBA	nucleic acid sequence-based amplification reaction
PCR	polymerase chain reaction
RNA	ribonucleic acid
RPR	rapid plasma reagin
RTI	reproductive tract infection
STI	sexually transmitted infection
STD	sexually transmitted disease
TMA	transcription mediated amplification
UNG	uracil-N-glycosylase
VDRL	venereal disease research laboratory
WB	western blot

ACKNOWLEDGEMENTS

The Western Pacific Regional Office of the World Health Organization would like to thank the principal contributor to this manual, Ms J Kuyper, and the reviewers, Dr P Rowe, Dr P Shrestha, Dr S Lazarri, Dr Yoke-Fong Chiew and Dr SM Garland.

1 INTRODUCTION

Valid laboratory assays are necessary to:

- confirm suspected reproductive tract infections (RTI) when laboratory facilities are available and accessible;
- detect infections in asymptomatic individuals;
- investigate cases of resistance to usual treatment;
- monitor the evolution of pathogen sensitivity to antimicrobials; and
- conduct research on STI prevalence and incidence.

An optimal laboratory test for diagnosis of infection should be simple to perform, sensitive, specific, reproducible, objective, rapid and inexpensive, and should require no special equipment.

In recent years, new methods to detect RTI have been developed, using sophisticated molecular biology techniques. Some of these new methods meet many of the criteria for optimal tests and have improved the diagnosis of RTI. In other cases, older, standard assays remain valid and should still be used.

Molecular detection techniques do not rely on the ability to culture or directly observe intact organisms but are designed to detect specific cellular antigens or nucleic acids. Therefore, the stringent transport requirements for clinical samples are no longer needed. Viable and nonviable microorganisms can be detected in samples. The development of molecular detection assays has led to the ability to use one sample for the detection of many organisms, to test for multiple organisms in one assay and to automate assays. Due to their extreme sensitivity, newly developed nucleic acid amplification systems allow the use of samples obtained by noninvasive methods, including patient self-sampling and urine collection. In addition, some of these assays are used to determine the number of nucleic acid copies in a sample. The notes on methods of molecular assay describe in detail the general techniques for the molecular assays used to detect RTI.

This publication discusses state-of-the-art methods to detect eleven RTI. The types of assay presented fall into several categories and include detection of

the organism by direct microscopy, detection of metabolic products, culture, and the detection of specific antibodies, antigens, DNA or RNA. Not all organisms can be detected using all types of assay, nor can all laboratories perform all types of assay. Thus, the methods that are most useful in detecting each organism are summarized, as well as their sampling procedures, sensitivity and specificity, the advantages and disadvantages of laboratory testing, the appropriate level of use, the training and equipment required, the ease of performance, and the indicative cost of reagents (at current rates in the United States of America).

Detailed instructions for carrying out each test can be found in the manufacturer's manual accompanying each test kit and should be strictly adhered to. The sensitivity and specificity of an assay will vary depending on the method used as the standard and the prevalence of the disease in the population tested. The assay sensitivity and specificity figures in this publication are based on a range of values taken from many different sources, including all types of patient population.

2 LABORATORY TESTS FOR SPECIFIC DISEASES

Candidiasis

Candida is a common commensal yeast found in the normal vaginal microbial flora. While most colonized women show no symptoms of infection, in some, colonization progresses to symptomatic disease. Laboratory confirmation is often necessary to diagnose candidiasis in cases where symptoms are nonspecific. Infection is usually, but not always, associated with higher numbers of yeast organisms and the presence of the mycelial form, together with an inflammatory response (polymorph). The normal microbial flora, Gram-positive rods of lactobacillus and diptheroid species, are still present. However, the relationship between the number of organisms and infection is not clear [1].

Methods of detection

Detection by microscopy

A swab of the vaginal secretions is placed in saline and the evaluation for yeast is carried out immediately by adding a drop to a slide and mixing it with a drop of potassium hydroxide solution. The potassium hydroxide lyses the patient's cells, making the yeast easier to see. Alternatively, the swab is rolled onto a slide, fixed and Gram stained. This slide does not have to be stained or evaluated immediately. Both slides are examined by a light microscope (400x) for the presence of the yeast or mycelial form and inflammatory response.

Detection by culture

A swab of the vaginal secretions or an aliquot of a vaginal wash is inoculated into Sabouraud's agar within a few hours of collection and incubated for up to two days at 37° C. Colonies are identified as yeast by performing a Gram stain. (*Candida* will also grow as dull white colonies on horse blood agar.) The quantity of yeast is determined, with more than 10^3 colony-forming units/ml of vaginal secretions usually being associated with disease.

Detection of *Candida* antigen

A swab of vaginal secretions or a drop of a vaginal wash is mixed on a slide with a commercially available solution of latex beads that are coated with an antibody to mannan, a soluble polysaccharide that is the major constituent of the *Candida* cell wall and is found in the vagina when *Candida* are present. The antigen-antibody complexes are observed as agglutination of the suspension. Positive and negative control should be used.

Detection of *Candida* DNA

A commercially available, 40-minute, nonisotopic, automated test [2], is a hybridization assay that uses DNA probes to directly detect *Candida*, *Trichomonas* and *Gardnerella* in vaginal swab samples (Note 1). Samples are collected with a swab, placed into the collection tubes supplied in the kit, stored at 4º C. and tested within 24 hours.

***Table 1:* Characteristics of *Candida* detection assays**

	Microscopy *wet mount*	**Culture** *>10^{3}cfu/ml*	**Antigen detection**	**DNA detection**
Sensitivity[1]	35-45%	67%	61-81%	80%
Specificity[1]	99%	66%	97%	98%
Advantages	rapid, inexpensive	sensitive	rapid, also detects Trichomonas	rapid, objective, also detects Trichomonas and Gardnerella
Disadvantages	subjective	requires 24 hours	expensive	expensive, requires special equipment and test read immediately after completion
Level of use	exam room, on-site lab	on-site lab, intermediate lab	exam room, on-site lab	intermediate lab, referral lab
Training	moderate	moderate	minimal	moderate
Equipment	light microscope	incubator, light microscope	none	heat block, special processor
Ease of performance	easy	moderate	easy	easy to moderate, automated
Cost	US$ 1.00	US$ 2.00	US$ 12.00 (includes detection of Trichomonas)	US$ 12.00 (includes detection of Trichomonas and Gardnerella)

[1]Sensitivity and specificity are for clinical signs and symptoms of vulvovaginal candidiasis.

Trichomoniasis

Trichomonas vaginalis is a parasitic protozoan that causes vaginal inflammation. Infection with *Trichomonas* can range from severe vaginitis with discharge to asymptomatic carriage. Since asymptomatic carriage can account for as many as 50% of cases, diagnosis cannot be made solely on the basis of clinical presentation [3]. Laboratory detection of the parasite is required to confirm diagnosis.

Methods of detection

Detection by microscopy

Direct microscopic observation of motile characteristic parasites is made from a swab of vaginal secretions taken from the posterior fornix and then placed into a physiological saline solution and analysed immediately under a light microscope (100x).

Detection by culture

A swab of secretions taken from the posterior vaginal fornix is used within six hours of sample collection to inoculate a tube of Diamond's modified medium. The culture is incubated at 35°C. for up to four days with daily examination by wet prep for motile trichomonas. A culture system with a two-chambered bag is now available that, after inoculation with the swab, permits both incubation of the culture and immediate wet prep by microscopic examination through the upper chamber of the bag in one self-contained system [4].

Detection of *Trichomonas vaginalis* antigens

T. vaginalis antigens are detected using the *Trichomonas* Direct Enzyme Immunoassay and Fluorescent Direct Immunoassay. A swab of vaginal secretions is rolled onto a glass slide and air-dried in the examination room. In this one hour procedure, cocktails of peroxidase or fluorochrome-labelled monoclonal antibodies to various *T. vaginalis* antigens are added to the slide, incubated, washed and detected using substrates that change colour or fluoresce. Slides are examined under a light microscope for the colourimetric assay and under a fluorescent microscope for the fluorescent assay.

Another method uses a swab of vaginal secretions or a drop of a vaginal wash which is mixed on a slide with a commercially-available solution of latex beads that are coated with antibodies to *T. vaginalis* antigens. The antigen-antibody complexes are observed as agglutination of the suspension.

Detection of *T. vaginalis* DNA

A commercially available, 40-minute, nonisotopic, automated [2] hybridization assay that uses DNA probes to directly detect *Candida, Trichomonas* and *Gardnerella* in vaginal swab samples is now available (Note 1). The sample is collected with a swab and placed into the collection tube supplied by the kit, stored at 4º C. and tested within 24 hours.

Amplification and detection of *T. vaginalis* DNA

This assay is not commercially available [5]. Sample collection can be performed by obtaining a self-administered swab of the vaginal introitus or by a technique in which the patient inserts, then immediately withdraws, a tampon (commercially available). The sample is placed into transport media and can be tested for up to one month following collection. The DNA is extracted and added to a polymerase chain reaction (PCR) mixture (Note 2) containing primers TVA5 and TVA6 which amplify a 102 base-pair fragment of *T. vaginalis* DNA. The specific *T. vaginalis* DNA fragment is labelled during amplification and detected using DNA hybridization with probe TVB, a semi-automated ELISA assay for detection of PCR-amplified products (Note 3).

***Table 2:* Characteristics of *Trichomonas* detection assays**

	Microscopy	Culture	Antigen detection	DNA detection	
				Hybridization assay	*PCR*
Sensitivity[1]	38-82%	98%	86%	88-91%	93%
Specificity[1]	100%	100%	99%	100%	96%
Advantages	rapid, inexpensive	sensitive, diagnosis in men	rapid	rapid, objective, also detects Gardnerella and Candida	very sensitive, allows patient self-sampling
Disadvantages	low sensitivity, must be performed immediately, subjective	takes 1-4 days	expensive	expensive, requires special equipment and test read immediately after completion	expensive, requires expertise
Level of use	exam room on-site lab	on-site lab, intermediate lab	exam room, on-site lab	intermediate, referral lab	referral lab
Training	moderate	moderate	moderate/minimal	moderate	extensive
Equipment	light microscope	incubator, light microscope	light or fluorescent microscope/none	heat block, special processor	thermal cycler, microwell plate reader
Ease of performance	easy	easy	moderate/ easy	easy to moderate, automated	complex, automated
Cost	US$ 1.00	US$ 3.00	US$ 6.00-12.00 (includes detection of Candida)	US$ 12.00 (includes detection of Candida and Gardnerella)	$11.00

[1] Sensitivity and specificity are for detection of *T. vaginalis* by combined wet prep and culture results.

Bacterial vaginosis

Bacterial vaginosis (BV) is a condition in which the natural balance of organisms found in the vagina is changed from a predominance of Lactobacillus to an overgrowth of other bacteria including *Gardnerella vaginalis, Mobiluncus* and other anaerobes [6].

Methods of detection

Detection by microscopy and/or metabolic products

The most widely accepted method for diagnosis of BV is the presence of three of the following four criteria:

- a homogeneous vaginal discharge;
- a vaginal pH of greater than 4.5;
- the presence of clue cells; and
- a fishy odour after addition of potassium hydroxide to the vaginal secretions (the amine test) [6].

A swab of vaginal fluid from the posterior fornix is placed in normal saline. This sample is used immediately for the following tests. In the amine test, a drop is placed on a slide and examined under a light microscope for the presence of clue cells, which are epithelial cells heavily coated with bacteria so that the peripheral borders are obscured. Samples are generally considered positive for BV if clue cells comprise more than 20% of all epithelial cells. A drop is placed on a slide with a drop of potassium hydroxide solution. The release of amines produces a fishy odour. The pH value is determined using a pH dipstick onto secretions on a speculum.

A simpler and more specific assay is a Gram stain of a vaginal smear. A swab of vaginal secretions is rolled onto a glass slide and air dried. The sample is stable for days at ambient temperature. The slide is Gram stained and a standardized, 0-10 point scoring method is used to evaluate the smears [7]. Points are given by estimating the number of three different bacterial morphotypes from 0 to 4+, including large Gram-negative rods, small Gram-negative/variable rods, and curved Gram-negative/variable rods.

The proline aminopeptidase test [8] is an indirect test for a chemical produced by the organisms associated with BV. Vaginal secretions are added to a substrate and incubated for four hours. A colour change denotes the test result.

The vaginal swab from the posterior fornix is placed into normal saline and can be held at 4°C. for up to four hours and then frozen until testing. Samples can be shipped on ice.

Detection of *G. vaginalis* DNA

A commercially available, 40-minute, nonisotopic, automated [9] hybridization assay that uses DNA probes to directly detect *Candida*, *Trichomonas* and high concentrations of *Gardnerella* in vaginal swab samples (Note 1). Samples are collected with a swab and placed into the collection tubes supplied with the kit, stored at 4ºC. and tested within 24 hours.

***Table 3:* Characteristics of bacterial vaginosis detection assays**

	Microscopy and metabolic product detection			DNA detection
	3 of 4 criteria	*Gram stain*	*Proline aminopeptidase*	*Hybridization assay*
Sensitivity[1]	81%	89%	93%	94%
Specificity[1]	94%	93%	93%	81%
Advantages	rapid, inexpensive	reproducible, standardized, inexpensive	objective	objective, can also detect Candida and Trichomonas
Disadvantages	subjective, some criteria nonspecific	requires expertise	takes longer than wet mount or stain	expensive, requires special equipment, test read immediately after completion
Level of use	exam room, on-site lab	on-site lab	on-site lab, intermediate lab	intermediate lab, referral lab
Training	moderate	moderate	minimal	moderate
Equipment	light microscope	light microscope	centrifuge, incubator	heat block, special processor
Ease of performance	easy	easy	easy	easy to moderate, automated
Cost	US$ 1.00	US$ 0.50	US$ 1.00	US$ 12.00 (includes detection of Candida and Trichomonas)

[1] Sensitivity and specificity are for diagnosis of BV by presence of 3 of 4 criteria and/or positive Gram stain.

Chlamydiosis

Chlamydia trachomatis is an important cause of urethritis and cervicitis. Laboratory detection of *C. trachomatis* is necessary because as many as 70% to 80% of women and up to 50% of men who are infected do not experience any symptoms. Untreated, infected individuals transmit chlamydial infections to sexual partners and are at risk for sequelae such as epididymitis in men and pelvic inflammatory disease and infertility in women [10].

Methods of detection

Detection by culture

The conventional method for the laboratory diagnosis of *C. trachomatis* has been inoculation of a cell culture with a genital specimen. This method is expensive, labour-intensive and time-consuming. It also requires considerable expertise to perform it correctly and meticulous handling of the specimen during transport to maintain viable organisms. For these reasons, culture tests are now used less frequently and antigen and nucleic acid detection techniques have become common methods for detection of *C. trachomatis* infection, allowing testing in laboratories that lack the facilities for tissue cell culture [10].

Detection of host response

The leukocyte esterase assay (LE) is a rapid, nonspecific urine dipstick test for the presence of an enzyme found in urine when leukocytes are present due to inflammation. The LE test can diagnose urethritis but cannot identify the specific cause of the infection. The sensitivity and specificity of LE for the detection of chlamydial and gonococcal infection are 54%-97% and 36%-95%, respectively. The LE test performs best as a screening test for chlamydial infection in asymptomatic adolescent and young men with positive tests confirmed by enzyme immunoassay [11].

Detection by microscopy

In the direct immunofluorescence assay (DFA), urethral or endocervical columnar epithelial cells collected on swabs are rolled onto glass slides, fixed and stained with fluorescein-labelled monoclonal antibodies specific for the major outer membrane protein of *C. trachomatis*. DFA allows for the visualization of the distinctive morphology and staining characteristics of chlamydial inclusions and elementary bodies. It also permits simultaneous assessment of the specimen adequacy.

For all chlamydial detection assays, samples must contain urethral or endocervical columnar cells and not exudate. The presence of ten or more elementary bodies is generally accepted for the test to be positive. DFA is useful as a confirmatory test for samples found positive by antigen and DNA detection assays.

Detection of *C. trachomatis* antigens

A common test for genital *C. trachomatis* infection is the detection of chlamydial genus-specific lipopolysaccharide (LPS) antigens in cervical and urethral swab specimens and in urine samples from men using any of several commercially-available enzyme immunoassay (EIA) kits. Because chlamydial LPS antibodies may cross-react with the LPS of other Gram-negative bacteria to give false-positive results, blocking assays are needed to confirm positive EIA results and thus improve specificity (Note 4). Confirmation can also be done by performing direct fluorescent antibody assay on the centrifuged specimen.

Rapid immunodot or latex agglutination assays are simple tests in which the swab sample is mixed with chlamydial LPS antibodies coated on a membrane or card. The antigen-antibody complexes are observed visually as agglutination.

Detection of *C. trachomatis* RNA

C. trachomatis ribosomal RNA is detected by hybridization with a chemoluminescent DNA probe (Note 5). A probe competition assay has been developed to confirm positive samples. A similar kit can be used to detect *N. gonorrhoeae* RNA in the same sample. Endocervical swabs, urethral swabs and urine samples from men can be tested. Specimen collection kits containing swabs and transport medium that lyses the organisms and releases the RNA are provided by the manufacturer. No stringent transportation conditions are required and the samples are stable at ambient temperature for one month.

Amplification and detection of *C. trachomatis* DNA or RNA

The polymerase chain reaction (PCR) amplification assay for *C. trachomatis* DNA is available in a semi-automated and a fully automated format. A kit is available that also amplifies *N. gonorrhoeae* DNA from the same sample and detects it in a separate hybridization well. To prevent false-negative results due to inhibitors of the amplification reaction in the patient samples, this kit includes an internal standard that is amplified in the same reaction tube and detected in a separate hybridization well (Note 6).

Another DNA amplification technique for *C. trachomatis* detection is ligase chain reaction (LCR) (Note 7). The amplicons are detected in an automated instrument that is designed to minimize false-positive samples due to contamination from carry-over. The enzymes used in LCR are not as sensitive as the enzymes in PCR to inhibitors in the sample.

LCR and PCR are excellent tests for the detection of *C. trachomatis* in endocervical, urethral and urine samples from both men and women (these tests do not have an internal control, such as ßglobin, to assess the adequacy of sample collection). In addition, other noninvasive specimen collection methods such as self-collected vaginal introitus swabs or tampons can be tested. Specimen collection kits are provided by the manufacturers. Swab and tampon samples can be kept at ambient temperature but urine samples should be refrigerated and are stable for up to four days. They will remain stable for up to 60 days when frozen.

A third amplification assay for *C. trachomatis* uses transcription-mediated amplification (TMA) of the 16S ribosomal RNA (Note 8). After initial heating to 95°C, the reaction occurs at one temperature. Amplification and product detection take place in one tube [12].

LCR, PCR and TMA are all highly sensitive assays. Unless specimens are carefully collected in the clinic, appropriately transported and handled carefully in the laboratory, they are susceptible to contamination and may give false-positive results. For kits, the manufacturer's instructions must be carefully followed and adhered to. For example, to ensure no contamination at the laboratory level, positive-displacement pipettes (or plugged pipettes) should be used. For "in-house" assays, there should be physical separation (in separate rooms) for the various steps of PCR, such as DNA extraction process, detection and preparation of master mixes.

***Table 4:* Characteristics of *Chlamydia* detection assays**

	Microscopy	**Antigen detection**		**RNA detection**	**Amplification & detection**	
	DFA	*EIA*	*Rapid*	*Chemoluminescent DNA probe*	*PCR*	*LCR*
Sensitivity[1]	74%-90%	71%-97%	52%-85%	75-%85%	90%	90%-97%
Specificity[1]	98%-99%	97%-99%	> 95%	98%-99%	99%-100%	99%-100%
Advantages	rapid, easy	can batch samples	rapid, easy	also detects N. gonorrhoeae, automated	can detect N. gonorrhoeae in same sample, allow noninvasive sampling	less affected by inhibitors
Disadvantages	labour intensive, subjective	requires confirmation	insensitive, requires confirmation	less sensitive than PCR, requires confirmation	false negatives	no test for sample inhibitors
Level of use	on-site lab, intermediate	intermediate, referral lab	exam room, on-site lab	intermediate, referral lab	intermediate, referral lab	intermediate, referral lab
Training	moderate to extensive	moderate	minimal	moderate	moderate to extensive	moderate
Equipment	fluorescent microscope	microwell plate reader	none	heat block, luminometer	thermal cycler, incubator, microwell plate reader	thermal cycler, LCx processor
Ease of performance	moderate	moderate	easy	moderate	moderate to difficult, automated	moderate, automated
Cost	US$ 6.00	US$ 6.00	US$ 13.00-16.00	US$ 8.00	US$ 11.00 (US$ 14.00 for N. gonorrhoeae detection also)	US$ 16.00

[1] Sensitivity and specificity are for detection of C. trachomatis by culture or by DNA amplification test.

Gonorrhoea

Infection of the genital tract with *Neisseria gonorrhoeae* can cause urethritis, cervicitis, proctitis, or Bartholinitis. Complications of untreated disease include epididymitis, prostatitis and infertility in men and pelvic inflammatory disease and infertility in women. Because most cases in females are asymptomatic, detection of infection using laboratory tests is needed to prevent sequelae and transmission to sexual partners and, for pregnant women, to neonates [13].

Methods of detection

Detection by microscopy

A direct Gram smear is performed as soon as the specimen is collected by rolling the swab onto a slide, staining, and viewing under an oil immersion lens (1000X). The presence of Gram-negative diplococci inside polymorphonuclear leukocytes in urethral smears from symptomatic men is diagnostic for presumptive gonorrhoea. However, the Gram stain is not as useful for endocervical smears (sensitivity of 50%-70%) because the presence of other Gram-negative diplococci make interpretation difficult.

Detection of host response

The leukocyte esterase assay (LE) is a rapid, nonspecific urine dipstick test for the presence of an enzyme found in urine when leukocytes are present due to inflammation. The LE test can diagnose urethritis but cannot identify the specific cause of the infection. The sensitivity and specificity of LE as a marker for chlamydial and gonococcal infection are 54%-97% and 36%-95%, respectively. The LE test performs best as a screening test for gonococcal infection in asymptomatic men with positive tests confirmed by DNA detection [11].

Detection by culture

The endocervical or urethral swab is used immediately after collection to inoculate a plate of selective media such as modified Thayer-Martin. The selective medium contains antimicrobial agents that allow the growth of *N. gonorrhoeae* and inhibit the growth of other bacteria. The agar plate is placed into a plastic zip-lock bag with a CO_2 generating tablet for transport to the laboratory, where the plate is incubated at 35°C for up to three days. Typical colonies are tested with Gram-stain, oxidase and catalase and/or superoxal tests for presumptive identification of *N. gonorrhoeae*. To confirm a presumptive culture, the isolated organism is tested for sugar fermentation by growth in standard carbohydrate fermentation tubes, or by using any of several rapid nongrowth methods for confirmation of *N. gonorrhoeae* isolates, including detection of preformed enzymes and agglutination with pools of mouse IgG monoclonal antibodies to the outer membrane protein. The monoclonal antibodies can be bound to staphylococcal Protein A or to easy-to-read coloured metal particles.

Detection of *N. gonorrhoeae* antigen

Gonococcal antigen is detected by an enzyme immunoassay that is similar to a Gram stain in sensitivity and specificity for presumptive diagnosis in men, but is less sensitive for endocervical swabs.

Detection of *N. gonorrhoeae* RNA

A two-hour DNA probe hybridization assay is available (Note 5) [14] and a probe competition assay has been developed to confirm positive samples. A similar kit can be used to detect *C. trachomatis* RNA in the same sample. Endocervical swabs and urethral swabs and urine samples from men can be tested. Specimen collection kits, containing swabs and transport medium that lyses the organisms and releases the RNA, are provided by the manufacturer. No stringent transportation conditions are required and the samples are stable at ambient temperature for one month.

Amplification and detection of *N. gonorrhoeae* DNA

The polymerase chain reaction (PCR) amplification assay (Note 2) is designed to also amplify *C. trachomatis* DNA from the same sample (Note 6). The reaction can be automated. To prevent false-negative results due to inhibitors of the amplification reaction in the patient samples, the kit includes an internal standard that is amplified in the same reaction tube and detected in a separate hybridization well.

Another DNA amplification technique for *N. gonorrhoeae* detection is ligase chain reaction (LCR) (Note 7). The amplicons are detected in an automated instrument that is designed to minimize false-positive samples due to contamination from carry-over. The enzymes used in LCR are not as sensitive as the enzymes in PCR to inhibitors in the sample [15].

LCR and PCR are excellent tests for the detection of *N. gonorrhoeae* in endocervical, urethral and urine samples from both men and women. In addition, other noninvasive specimen collection methods such as self-collected vaginal introitus swabs or tampons can be tested. Specimen collection kits are provided by the manufacturers. Swab samples and tampons can be kept at ambient temperature, but urine samples should be refrigerated and are stable for up to four days. They will remain stable for up to 60 days when frozen.

Table 5: Characteristics of *N. gonorrhoeae* detection assays

	Microscopy	Culture	DNA Detection	Amplification & Detection	
			Hybridization assay	*PCR*	*LCR*
Sensitivity[1]	90%-95%	81%-100%	86%-100%	89%-97%	95%-100%
Specificity[1]	98%-100%	100%	99%	94%-100%	98%-100%
Advantages	rapid, inexpensive	gold standard, isolates available for further testing	rapid, viable organisms not required	viable organisms not required, extremely sensitive, allow non invasive sampling can detect C. trachomatis in same sample	
Disadvantages	insensitive for females	stringent handling, requires up to 3 days	expensive	expensive, requires expertise no test for sample inhibitors	
Level of use	on-site lab	on-site lab, intermediate	intermediate, referral lab	intermediate, referral lab	intermediate, referral lab
Training	moderate	moderate	moderate	moderate to extensive	moderate
Equipment	light microscope	incubator, light microscope, candle jar	water bath, luminometer	microfuge, thermal cycler, incubator, microwell reader	heat block, thermal cycler, microfuge, Imx processor
Ease of performance	easy	moderate	moderate	moderate to difficult, automated	moderate, automated
Cost	US$ 0.50	US$ 1.00 (+1-3 to confirm positive isolates)	US$ 6.00	US$ 11.00 (US$ 14.00 for C. trachomatis detection also)	US$ 14.00

[1] Sensitivity and specificity are for detection of *N. gonorrhoeae* in urethral, endocervical and urine samples by culture except for microscopy, which is for detection in urethral samples from symptomatic men.

Syphilis

Syphilis, a chronic infection with clinical manifestations occurring in distinct stages, is caused by the spirochete *Treponema pallidum*. This bacterium cannot be cultured *in vitro* or stained using standard techniques. The rabbit infectivity test is the oldest and most sensitive method to identify infection with *T. pallidum*. While this test is not practical for routine laboratory use, it is the standard for measuring the sensitivity of other methods. Routine laboratory methods rely on demonstrating the presence of *T. pallidum* in a characteristic lesion or the presence of antibodies in serum. Not all methods can be used to diagnose all stages of syphilis. Antibodies are not present early in disease (1 to 4 weeks after a lesion has formed) and lesions are not present at all stages [16].

Methods of detection

Detection by microscopy

T. pallidum is detected in primary or secondary lesions by dark-field microscopy. A glass slide is touched to fluid expressed from a lesion or node aspirate, coverslipped, and examined immediately under a light microscope fitted with a dark-field condenser. *T. pallidum* is identified by its characteristic morphology and motility. Oral specimens are not suitable for this method because other spirochetes present in these specimens cannot be distinguished from *T. pallidum*.

Direct fluorescent antibody assay for *T. pallidum* detects and differentiates *T. pallidum* from nonpathogenic treponemes by an antigen-antibody reaction. The organism does not have to be motile for identification. The specimen is obtained in the same way as for dark-field microscopy but the smear is air-dried and fixed. A fluorescein-labelled polyclonal antibody to *T. pallidum* (IgG from humans or rabbits with syphilis that is absorbed with Reiter treponemes) is added to the smear. The slide is examined for fluorescing treponemes displaying typical morphology using a fluorescent microscope.

Detection of nontreponemal antibodies

Nontreponemal antibody tests for syphilis, which are used for screening patient serum, are based on detection of antibodies to a cardiolipid-cholesterol-lecithin antigen. Undiluted or serial two-fold dilutions of serum are added to the antigen on a slide or card. The reagents are then mixed and rocked and observed for flocculation. The rapid plasma reagin (RPR) test, in which the antigen is mixed with charcoal so the antigen-antibody complexes can be seen without a microscope, is the most common. The Venereal Disease Research Laboratory (VDRL) slide test, which is read microscopically, can also be used to detect antibodies in cerebral spinal fluid. For some immunoassays, the VDRL antigen is coated onto microwells to capture the antibodies in the patient serum which are then detected with anti-human immunoglobulins conjugated

to an enzyme [16] or coated onto erythrocytes [17]. A positive test provides a presumptive diagnosis for syphilis and must be confirmed using a specific treponemal antibody test. It is also advantageous to gauge response to appropriate treatment, as specific tests, once positive, usually remain so for life.

Detection of treponemal antibodies

The fluorescent treponemal antibody absorption test (FTA-ABS) and the microhaemagglutination assay for *T.* pallidum (MHA-TP) detect specific antibodies in serum to *T. pallidum*. For FTA-ABS, the Nichols strain of *T. pallidum* is fixed on a slide. Patient serum is absorbed with an extract of Reiter treponemes and added to the slide. Fluorescein-labelled anti-human immunoglobulin is added and the slide is examined for fluorescent spirochetes using a fluorescent microscope. In MHA-TP, erythrocytes are sensitized with *T. pallidum* antigen, mixed with patient serum and observed for agglutination.

Immunoassays have also been developed that use *T. pallidum* antigen-coated microwells to capture specific antibodies from the patient serum, which are detected with enzyme-labelled anti-human IgG monoclonal antibodies [18].

Detection of *T. pallidum* antigens

T. pallidum antigens are detected using an enzyme immunoassay [19]. Antigens are extracted within 72 hours of collection from swabs containing lesion exudate.

Amplification and detection of *T. pallidum* DNA and RNA

T. pallidum DNA is amplified in a PCR (Note 2). This assay has been described as a multiplex-PCR for the simultaneous amplification of herpes simplex virus and *H. ducreyi* DNA in the same sample [20].

A highly sensitive reverse transcriptase PCR assay has been described for the amplification and detection of *T. pallidum* 16S rRNA. This technique is especially useful for diagnosis of neurosyphilis where very low numbers of organisms exist [21].

Table 6: Characteristics of syphilis detection assays

	Microscopy	Antibody Detection		Antigen Detection	DNA Detection
	dark-field	*nontreponemal RPR*	*treponemal MHA-TP*		*multiplex PCR*
Sensitivity[1]	74%-86%	72%-100%	69%-90%	81%	91%
Specificity[1]	97%-100%	93%-98%	98%-100%	89%	99%
Advantages	positive early, rapid, specific, inexpensive	inexpensive, rapid, easy, antibody titer to follow treatment	specific, confirms non treponemal tests	detects T. Pallidum before antibodies are positive	sensitive, specific, allows self-collected sample
Disadvantages	insensitive, no oral sample, requires live treponemes	false positives, less sensitive for early disease	more difficult, more expensive	time consuming, expensive	inhibitors of PCR reaction cause false-negative results, complex, expensive
Level of use	exam room, on-site lab	on-site lab, intermediate lab	intermediate lab, referral lab	intermediate lab, referral lab	referral lab
Training	extensive	minimal	moderate	moderate	extensive
Equipment	light microscope with dark-field condenser	centrifuge, rotator	centrifuge	spectrophotometer	microfuge, thermal cycler, incubator, microwell plate reader
Ease of performance	easy	easy	moderate	moderate	complex
Cost	US$ 0.40	US$ 0.50	US$ 1.40	US$ 3.00	US$ 14.00 (includes detection of H. ducreyi and HSV)

[1] Sensitivity and specificity are for detection of primary syphilis. The sensitivity of both nontreponemal and treponemal antibody detection increases for detection of secondary syphilis. The sensitivity of nontreponemal antibody detection decreases for detection of latent and tertiary syphilis. The tests for *T. pallidum* are only relevant when lesions are present in primary and secondary syphilis. But these tests can detect latent untreated infection which can be important for patient outcomes, such as in pregnancy.

Genital Herpes

Herpes simplex virus (HSV) is one of the major causes of genital ulcer disease. Primary infection is followed by latency and variable periods of reactivation. Although clinical diagnosis may be accurate if based on the presence of typical vesicles, up to two-thirds of individuals acquire HSV asymptomatically and most infected persons shed virus during latent periods. Laboratory diagnosis is necessary to detect HSV in asymptomatically infected people to prevent transmission to sexual partners and to children born to infected mothers [22].

Methods of detection

Culture of HSV

Inoculation of cells in tissue culture with infected secretions or tissues is considered the standard method. Lesions are rubbed at their base with cotton or Dacron swabs (need to break intact vesticles), or fluid is extracted from vesicles, and immediately placed in transport medium. The sample can be stored at 4°C for up to five days before it is used to inoculate a fibroblast cell-line, although ideally this should be done as soon after collection as possible. Diagnosis is made by observation of a characteristic cytopathic effect on the cells after incubation for up to one week (although most positives occur within 48 hours of cell inoculation) and confirmation of the virus by staining the infected cells with monoclonal antibodies specific for HSV. A rapid culture and histochemical stain method uses a genetically engineered cell-line that is inoculated, incubated for 16 to 24 hours, and stained with a solution that causes HSV infected cells to turn blue [23]. Another rapid tissue 24-hour culture method is also available. Both of these rapid culture methods are as sensitive as conventional culture [24].

Detection of HSV antigen

In the direct immunofluorescence assay (DFA), cells collected from a lesion (with the base rubbed well with a swab) are concentrated and spotted onto a slide that is incubated with a fluorescein-conjugated monoclonal HSV type-specific antibody. The slide is observed under a fluorescent microscope for the presence of intracellular fluorescence (this approach is not as sensitive as a culture). In the immunoperoxidase assay, specimens are prepared as for DFA and incubated with a monoclonal HSV antibody that is detected with a second antibody conjugated to an enzyme and reacted with a colourimetric substrate.

Amplification and detection of HSV DNA

HSV DNA is amplified in a PCR. This assay has been described as a multiplex-PCR for the simultaneous amplification of *T. pallidum* and *H. ducreyi* in the same sample [20].

Table 7: Characteristics of genital herpes detection assays

	Culture	Antigen detection	DNA detection *multiplex PCR*
Sensitivity[1]	gold standard	70%-95%	more sensitive than culture
Specificity[1]	100%	90%-100%	98%-100%
Advantages	sensitive, specific	rapid, relatively inexpensive, more sensitive than culture for detection in late-stage lesions	very sensitive, specific, allows self-collected sample
Disadvantages	expensive, time-consuming, requires expertise	less sensitive	inhibitors of PCR cause false-negative results, complex, expensive
Level of use	referral lab	intermediate lab, referral lab	referral lab
Training	extensive	moderate	extensive
Equipment	CO_2 incubator, microscope, (centrifuge)	fluorescent microscope or light microscope or microwell plate reader	microfuge, thermal cylcer, incubator, microwell plate reader
Ease of performance	complex	moderate	complex
Cost	US$ 40.00	US$ 4.00-8.00	US$ 14.00 (includes detection of T. Pallidum and H. ducreyi)

[1] The sensitivity of culture varies depending on the type of medium used and can only be estimated since there is no gold standard on which to base the diagnosis of chancroid.

Chancroid

Chancroid is a genital ulcer disease caused by the bacterium *Haemophilus ducreyi*. As also seen in other genital ulcer diseases, inguinal lymphadenopathy is present in about 50% of cases, which sometimes progresses to an inguinal bubo. The accuracy of clinical diagnosis varies due to the atypical presentation of the ulcer.

Methods of detection

Culture of *H. ducreyi*

Before obtaining material for culture, the ulcer base should be exposed and free of pus. Culture material should be obtained from the base or margins of the ulcer with either a cotton or calcium alginate swab and immediately inoculated directly onto culture plates. *H. ducreyi* is a fastidious organism and requires special media for growth. An effective medium for *H. ducreyi* isolation contains Columbia agar base, foetal bovine serum, haemoglobin, IsoVitalex, activated charcoal and vancomycin. Plates are incubated for up to three days at 33-35°C in 5% CO_2 atmosphere. A Gram stain is performed on suspected colonies [26]. Gram-negative bacilli from colonies compatible with *H. ducreyi* can be identified based on their requirements for X but not V factor for growth, fluorescent monoclonal antibody (see below), or by DNA hybridization on spot blots [27].

Detection of *H. ducreyi* antigen

H. ducreyi can be detected in fixed smears of lesional material with a mouse monoclonal antibody specific for this organism by adding fluorescein-labelled antimouse immunoglobulin and examining the slide under a fluorescent microscope [26]. Rabbit polyclonal antibodies can detect the organism in genital lesion specimens using an enzyme immunoassay [28].

Amplification and detection of *H. ducreyi* DNA

H. ducreyi DNA is amplified in a PCR [20]. This assay has been described as a multiplex-PCR for the simultaneous amplification of *T. pallidum* and HSV in the same sample. Swab samples collected from the genital ulcer or aspirated from the bubo are placed in specimen transport medium. Samples are then frozen until ready to test.

Table 8: Characteristics of chancroid detection assays

	Culture	Antigen detection	DNA detection *PCR*
Sensitivity[1]	56%-90%[1]	not determined	77%-98%[2]
Specificity[1]	100%	not determined	98%-100%
Advantages	isolates available for further testing	faster	very sensitive
Disadvantages	insensitive, proper medium difficult to obtain	not commercially available	inhibitors of PCR cause false-negative results, complex, expensive
Level of use	on-site lab	referral lab	referral lab
Training	moderate	moderate	extensive
Equipment	incubator, light microscope, candle jar	fluorescent microscope or microwell plate reader	microfuge, thermal cycler, incubator, microwell plate reader
Ease of performance	difficult	moderate	complex
Cost	US$ 2.00 (without confirmation)	not available	US$ 14.00 (also detects T. Pallidum and HSV)

[1] The sensitivity of culture varies depending on the type of medium used and can only be estimated since there is no gold standard on which to base the diagnosis of chancroid.

[2] Resolved sensitivity of PCR vs *H. ducreyi* culture.

Donovanosis

Donovanosis, or granuloma inguinale, is one cause of genital ulcer disease. The disease is caused by *Calymmatobacterium granulomatis* which can be seen in infected tissue as intracellular bacterial inclusions known as Donovan bodies [29] but cannot be cultured on artificial media. Donovanosis is likely to be confused with other diseases affecting the genital region, making clinical diagnosis difficult. A limited number of laboratory techniques are available to aid in the diagnosis. Some (immunofluoresence and PCR) are not commercially available but are promising methods for future development.

Methods of detection

Detection by microscopy

Diagnosis of donovanosis is made by direct visualization under a light microscope of Donovan bodies in Giemsa or Warthin-Starry silver-stained thin sections made from biopsy specimens [30].

Alternatively, a piece of clean granulation tissue is removed from the leading edge of the genital ulcer with a scalpel and crushed and spread on a slide, or a smear is made by rolling a swab firmly over the ulcer surface and depositing the material collected by rolling the swab across a glass slide. The slide is air-dried and stained with Wright-Giemsa stain or a rapid staining technique called RapiDiff [31]. RapiDiff is a one-minute staining technique using eosin and thiazine dye solutions.

Detection of *C. granulomatis* antibodies

An indirect immunofluorescence technique has been developed [32]. Serological tests are not available for routine use but could be used as epidemiological tools.

Detection of *C. granulomatis* DNA

DNA extracted from biopsy specimens is added to PCR mixtures (Note 2) [33]. Detection of *C. granulomatis* by PCR is only available as a research tool.

Table 9: Characteristics of donovanosis detection assays

	Microscopy		Antibody detection	DNA detection
	tissue	*swab RapiDiff*		*PCR*
Sensitivity	60%-80%	60%-80%	not yet determined	not yet determined
Specificity	100%	100%	not yet determined	not yet determined
Advantages	very specific	very specific	sensitive	objective
Disadvantages	sample collection painful, lengthy	rapid	limited sensitivity for early detection	complex procedure, time-consuming, usefulness not yet determined
Level of use	intermediate lab/ referral lab	on-site lab, intermediate lab	referral lab	referral lab
Training	extensive	moderate	extensive	extensive
Equipment	microtome, light microscope	light microscope	microtome, fluorescent microscope	thermal cycler, PAGE apparatus, equipment for DNA sequencing
Ease of performance	difficult	easy	difficult	complex
Cost	US$ 7.00-8.00	US$ 0.50	not available	not available

Human Papillomavirus

Human papillomavirus (HPV) is a common genital infection and some genotypes are associated with the development of several anogenital cancers. The detection of HPV is complicated by the fact that it cannot be identified by conventional viral detection methods such as growth in tissue cell culture or serological assays. Cytological and histological methods have traditionally provided indirect evidence of HPV infection by demonstrating cellular dysplasia, the result of an HPV infection. The most accurate way to demonstrate the presence of an HPV infection is by detection of the viral DNA in the clinical sample. Although over 100 distinct types of HPV have now been detected, 12-15 types are found in the majority of cervical lesions. DNA detection assays have been shown to be as good or better than cytology for the detection of high-grade cervical dysplasia, the precursor lesion to cervical cancer [34].

Methods of detection

Detection by cellular morphology

Epithelial cells are collected from the endocervix and ectocervix using a plastic or wooden spatula, a plastic brush or a Dacron swab. Cells are either rolled onto a glass slide, which is fixed with alcohol and transported to the laboratory, or placed into a liquid cytology medium and sent to the laboratory for processing into a Thin-prep slide using a special instrument. Cervical cell samples are stained with the Papanicolaou stain and read by a cytotechnologist and/or pathologist. Particular abnormal cellular morphology is indicative of an HPV infection. The sample can be used for both cytological analysis and DNA detection.

Direct detection of HPV DNA

Hybrid Capture is a solution DNA-hybridization assay that detects DNA from 14 types of HPV using one probe mix. This mix contains probes to five low-risk HPV (not usually found in precancerous lesions) and another probe mix that contains probes to nine high-risk HPV (associated with precancerous and cancerous lesions) (Note 9). For sample collection, the cervical swab sample is placed into a tube of transport medium manufactured for the Hybrid Capture kit or into a liquid-based cytology medium [35]. Both samples are stable for days at room temperature.

In situ hybridization uses HPV DNA probes to hybridize HPV DNA present in formalin-fixed, paraffin-embedded tissue sections mounted on slides (Note 10). Individual biotin-labelled, high-risk type HPV DNA probes (HPV 16, 18, 31, and 33) are commercially available for *in situ* hybridization assays. A kit which provides probe mixes for high-risk HPV types 16 and 18, types 31, 33 and 51, and the *in situ* assay reagents is also available.

Polymerase chain reaction amplification and detection of HPV DNA

For PCR amplification, DNA is extracted from the cervical cell sample (Note 2). After amplification, the specific HPV DNA amplicons of five low-risk HPV types and nine high-risk HPV types are identified (Note 11) [36]. Because of the high analytical sensitivity of the PCR assay, a cervix/vaginal swab or tampon obtained by the patient can also be used, eliminating the need for a cervical swab obtained with a speculum in place [37].

***Table 10:* Characteristics of HPV detection assays**

	Cellular morphology	High-risk HPV DNA detection		
		Hybrid Capture	*In situ hybridization*	*PCR*
Sensitivity	62%-85%[1]	74%-93%	60%-80%[2]	80%-100%[3]
Specificity[3]	33%-96%	64%-68%	80%-90%	40%-61%
Advantages	detects HPV infection/disease	objective	confirm equivocal histological evaluation	very sensitive
Disadvantages	false-negative results, expertise required	no cellular morphology	labour-intensive, low sensitivity	low specificity for disease
Level of use	intermediate lab, referral lab	intermediate lab, referral lab	referral lab	referral lab
Training	extensive	moderate	extensive	extensive
Equipment	light microscope	water bath, shaker, luminometer	oven, slide incubator, light microscope	heat block, microcentrifuge, thermal cycler, microwell plate reader
Ease of performance	difficult	moderate	complex	complex
Cost	US$ 7.00-8.00	US$ 22.00	US$ 26.00	US$ 8.00

[1] Sensitivity is for detection of biopsy-confirmed high-grade dysplasia.

[2] Sensitivity is for detection of HPV in tissue confirmed by PCR amplification.

[3] Specificity is for detection of biopsy-confirmed high-grade dysplasia.

Human immunodeficiency virus

Most individuals can be diagnosed as infected with human immunodeficiency virus (HIV) with an assay based on the detection of HIV specific IgG antibodies. For detection of early infection before seroconversion occurs, or to detect HIV infection in neonates, assays that detect HIV p24 protein or HIV DNA or RNA are used. Quantitative HIV RNA assays are not necessary for diagnosis of infection but are useful for monitoring treatment.

Methods of detection

Detection of HIV antibodies (screening and confirmation)

HIV antibodies can be detected in serum samples using any of more than 100 commercially available tests from more than 40 companies. The problem of the "window phase" of early HIV infection, during which antibodies are not detectable, and the variability of the virus have resulted in continual improvements in HIV antibody detection tests since 1985, with some assays being marketed as third and fourth generation [38]. Assays for HIV antibody detection include enzyme immunoassay (EIA), simple immunodot assay, line immunoassay (LIA), particle agglutination assay, and western blot (WB). Most HIV antibody screening tests are antigen sandwich EIA which detect both HIV-1 and HIV-2 in one assay (Note 12) using mixtures of recombinant antigens and synthetic peptides common to all HIV types. New fourth generation screening EIAs, which permit the simultaneous detection of HIV antigen and antibodies, have been developed, reducing the diagnostic window for HIV infection [40].

HIV antibody assays provide sensitivities and specificities with oral specimens comparable to those achieved with serum samples. Oral fluid is collected by dribbling or by using one of several commercially available devices that use a chemically treated cotton fibre pad which is placed between the lower gum and cheek. Comparative studies have shown no significant differences in sensitivity between these two methods of oral fluid collection. A special EIA called IgG antibody capture EIA (GAC-EIA) has been developed to optimize antibody detection from oral fluid [41].

Whole blood can be collected and dried on filter paper and stored for up to six weeks before testing. The blood is eluted from the paper and analysed by an EIA [42].

Tests used to confirm the results of the screening assay and distinguish between HIV types 1 and 2 include WB, immunodot assay, LIA (Note 13), and a combination of monospecific EIA tests [39].

Detection of HIV antigen

The presence of the p24 antigen of HIV-1 in serum is detected using an enzyme-labelled antibody. The p24 antigen assay becomes positive approximately 16 days after the subject becomes infectious.

Detection of HIV DNA

HIV-1 DNA is detected in the peripheral blood mononuclear cells pelleted from whole blood collected in EDTA tubes using a PCR assay (Note 2). This assay is as sensitive as HIV culture for diagnosing perinatal HIV infection, and much faster. The assay can be performed on as little as 0.1 ml of blood and on dried blood spots. The primers used for amplification and the probes used for detection target a highly conserved region of the HIV genome. Two commercially available HIV-1 DNA detection assays use primers that amplify regions of the HIV-1 *gag* gene (Note 14) [43].

Detection of HIV RNA

Several commercial assays are available for the quantification of HIV-1 RNA in peripheral blood. These assays are based on different techniques including competitive reverse transcriptase-PCR, nucleic acid sequence-based amplification, branched-chain DNA assay (Note 15) and transcription-mediated amplification (Note 8). All the assays have similar reproducibility and equal reliability. All the assays give similar results for HIV-1 clade B. However, some do not recognize subtype A or E virus. Plasma is a better sample to use than serum, and acid citrate dextrose or EDTA is a better anticoagulant than heparin [43].

Table 11: **Characteristics of HIV detection assays**

	Antibody detection		Antigen detection	DNA detection	RNA detection
	EIA	*Dot*		*PCR*	*Quantitative*
Sensitivity	100%	100%	detect earlier than antibody tests	as sensitive as culture	earliest detection
Specificity	95.8%-100%	99%-100%	100%	100%	100%
Advantages	sensitive, inexpensive automated	sensitive, specific, differentiate HIV-1 and 2	early detection	perinatal diagnosis, sensitive	monitor HIV levels
Disadvantages	false-positive results, no serotyping	expensive	insensitive	expensive, time-consuming	expensive, time-consuming
Level of use	intermediate lab	on-site lab, intermediate lab	referral lab	intermediate lab, referral lab	referral lab
Training	moderate	minimal	moderate	moderate	extensive
Equipment	centrifuge, microwell plate reader	none	centrifuge, microwell plate reader	microfuge, thermal cycler, microwell plate reader	depends on method[1]
Ease of performance	moderate	easy	moderate	moderate	extensive
Cost	US$ 2.00-3.00	US$ 6.00-7.00	US$4.00-5.00	US$ 12.00	US$ 60.00

[1] For RT-PCR: microfuge, thermal cycler, incubator, microwell plate reader. For bDNA: ultracentrifuge, luminometer. For NASBA: microfuge, luminometer.

3 NOTES ON METHODS OF MOLECULAR ASSAY

1 Pathogen-specific, oligonucleotide DNA probes are coupled to nylon beads which are embedded in a dipstick to form a probe-analysis card. The sample is placed in a lysis solution and heated to release DNA into the solution. A hybridization buffer is added and the sample solution is hybridized to the probe which is attached to the beads on the card. The card is moved through wells containing a wash buffer, a second probe that is biotin-labelled and hybridizes to the target DNA captured on the beads, and reagents that react with the biotin to produce a colour change in positive samples.

2 A PCR mix contains salts, magnesium, *Taq* DNA polymerase, deoxynucleotide triphosphates (dNTP), and paired oligonucleotide DNA primers designed so that they hybridize to opposite strands of the target DNA, usually between 100 to 800 base-pairs apart. For some procedures, degenerate primers are used. These are mixtures of oligonucleotides whose sequences differ from each other by one or more bases. They are used to amplify consensus regions of DNA found among related strains or variants of microorganisms. The PCR takes place in a thermal cycler which incubates the reaction mix through 30 to 40 cycles of three temperature steps of denaturation, annealing and extension. DNA is synthesized in both directions to produce a double-stranded piece that has one primer sequence on each end. One of the primers or one of the dNTP's can be labelled with a protein to facilitate later detection of the synthesized fragment (amplicon). Each time a cycle is repeated, the number of amplicon copies is doubled. In this way, one copy of a target DNA can be amplified over a million-fold. The reaction can include a second primer pair to amplify a human DNA sequence (internal control, such as ßglobin) to determine if the sample has an adequate amount of DNA for analysis and if the sample contains inhibitors of PCR. Amplicons are usually detected using oligonucleotide probes that bind to sequences on the amplicon that are internal to the primers.

3 The DNA detection assay uses digoxigenin-labelled dUTP in the reaction mixture to label the amplicons during PCR amplification. The labelled amplicons are hybridized with a target-specific biotin-labelled probe, captured in microwells coated with streptavidin, and detected with an enzyme-labelled anti-digoxigenin antibody and a colourimetric substrate to the enzyme. The absorbance values of the wells are read in a microwell plate reader. The detection can be automated using the ES 300 analyser.

4 In direct EIA, LPS extracted from elementary bodies in the specimen binds to microwells. The bound antigen is detected with enzyme-labelled antibodies that recognize all species of chlamydia. In indirect EIA, a primary murine anti-

LPS antibody is used to bind the LPS extracted from the sample and then a second enzyme-labelled anti-murine IgG antibody is added to the wells. Detection of the enzyme-labelled antibodies is performed using a substrate that changes colour and by measuring the absorbance in a spectrophotometer. To confirm a positive test, the EIA is repeated in the presence of non-labelled monoclonal antibodies to chlamydial LPS, which block LPS binding to the enzyme-labelled antibody. A reduced signal with the addition of the blocking antibody is interpreted as confirmation of the positive EIA result.

5 Cells in the swab or urine samples are lysed to release 16S rRNA which is hybridized to an acridine ester-labelled single-stranded DNA probe. The RNA:DNA hybrids are captured on magnetic beads coated with anti-RNA:DNA hybrid antibodies and an unhybridized probe is hydrolyzed in a hybridization protection assay. The beads are separated from the hybridization solution and washed. The chemoluminescence of the hybridized sample is read in a luminometer.

6 The PCR mixture contains two biotinylated primer pairs, one each for amplification of *C. trachomatis* and *N. gonorrhoeae* DNA. The internal control DNA, which is a synthetic DNA fragment containing identical primer binding sequences and an internal sequence distinct from the *C. trachomatis* amplicon, is added to each reaction. The amplicons are detected in three separate microwells, each containing an immobilized oligonucleotide probe that captures a specific amplicon. The detection kits are purchased separately. The biotinylated captured amplicons are detected using enzyme-labelled avidin and a colourimetric substrate. A positive signal in the well containing the internal control DNA probe indicates that the sample does not contain inhibitors of the polymerase enzyme. To minimize carry-over contamination of the samples with previously amplified DNA, the kit also contains uracil-N-glycosylase (UNG). During amplification, uracil is incorporated into the amplicons instead of thymidine. During the first step of the amplification reaction, UNG cleaves the uracil-containing DNA so that it can't be used as a template for DNA synthesis. If the sample is contaminated with DNA from a previous reaction, it will be destroyed and not available for amplification. Both the chlamydia and the CT/NG kits are available in the microwell format and the fully automated format that performs amplification, detection and results reporting.

7 In the LCR test, two labelled oligonucleotide probes bind to adjacent sites on one strand of the target DNA and two other labelled probes bind to adjacent sites on the opposite strand. One probe of each pair is labelled with fluorescein and the other is labelled with biotin. The gap of 1 to 2 nucleotides between the adjacent probes is filled by DNA polymerase and the two probes are joined by ligase. The two ligated probe pairs anneal to each other and form the templates for successive reactions. The reaction is done in a thermal cylcer for 30-40 cycles of denaturation, annealing and ligation to produce a logarithmic amplification of the target sequences. The products are detected in the automated IMx instrument. Anti-fluorescein antibody-coated microparticles capture the amplicons that are labelled at one end with fluorescein. Anti-biotin antibody conjugated to an enzyme binds to the opposite, biotin-labelled end of

the captured amplicons. A fluorescent substrate is added and the samples are read in the automated processor.

8 Transcription-mediated amplification (TMA) is very similar to NASBA (Note 15). The reaction contains RNA polymerase and reverse transcriptase and, after initial heating to melt the nucleic acids in the sample, is isothermal. The RNA amplicons are detected in the same tube as the amplification reaction using a hybridization protection assay in which a single-stranded chemoluminescent DNA probe is added and the RNA:DNA hybrids are captured with an anti-hybrid antibody (Note 5).

9 Hybrid Capture uses full-length genomic HPV RNA probes to hybridize to denatured target HPV DNA sequences in solution. Two probe mixes are supplied. Probe A consists of RNA transcripts of HPV types 6, 11, 42, 43 and 44. Probe B consists of RNA transcripts of HPV types 16, 18, 31, 33, 35, 45, 51, 52 and 56. The RNA:DNA hybrids are captured by antibodies immobilized in tubes. The captured hybrids are detected using antibodies conjugated to alkaline phosphatase and the enzyme is detected using a chemoluminescent substrate. The results are read as light units in a luminometer.

10 Formalin-fixed, paraffin-embedded tissue is dewaxed and rehydrated, then permeabilized with proteases and the probe, which is labelled with biotin, is added and allowed to hybridize. The biotin is detected using avidin conjugated to horseradish peroxidase which is detected using a colorimetric substrate. The rest of the tissue is counterstained. HPV-DNA-positive cells are visualized using a light microscope.

11 The biotin-labelled PCR amplicons are denatured and added to a tube containing hybridization buffer and either of the two probe mixes. Probe A consists of full-length genomic HPV RNA transcripts of HPV types 6, 11, 42, 43 and 44. Probe B consists of full-length genomic HPV RNA transcripts of HPV types 16, 18, 31, 33, 35, 45, 51, 52 and 56. After hybridization, each sample is transferred to a microwell coated with streptavidin. The streptavidin captures biotin-labelled amplicons and those that are hybridized to an RNA probe are detected using an anti-RNA:DNA antibody that is conjugated to alkaline phosphatase. The enzyme is reacted with a colourimetric substrate and the absorbance values in the wells are read on the microwell plate reader.

12 Several variations of the sandwich-type capture EIA have been developed for HIV-antibody-detection assays. Second generation assays are usually indirect assays in which the microwells are coated with anti-human IgG antibodies that capture all IgG antibodies from the sample. The HIV-specific antibodies are detected using HIV antigens that are conjugated to an enzyme. The enzyme is detected using a colourimetric substrate and the absorbance is read in a microwell plate reader. Third generation tests are usually direct sandwich EIA in which the microwells are coated with HIV recombinant antigens and synthetic peptides from the core, env and p24 proteins. The immobilized antigens capture HIV-specific antibodies from the sample, which are then detected using enzyme-labelled antigens or anti-human IgG antibodies. In a

double-antigen sandwich EIA, HIV antibodies bind to biotin-labelled and digoxigenin-labelled antigens in solution. The immunocomplexes then bind to streptavidin-coated microwells and are detected with an anti-digoxigenin antibody conjugated to an enzyme.

13 Rapid simple dot, strip or line immunoassays use membranes or nylon strips or disks that are impregnated with HIV-1 and HIV-2 recombinant antigens and synthetic peptides. The antigens can be combined into a screening assay for the simultaneous detection of antibodies against HIV-1 and HIV-2 or coated separately onto the membrane to distinguish between the types. The HIV antibodies in the sample bind to the antigens and are detected in the same way as for a western blot using enzyme-labelled anti-human IgG.

14 One test kit uses biotinylated primers that amplify a 124 base pair region of the HIV-1 *gag* gene. Detection of amplicons is carried out in microwell plates coated with a probe that hybridizes to the HIV-1 *gag* sequences, which are detected using an enzyme-linked colourimetric absorbance assay. Another test detects PCR-amplified HIV-1 *gag* sequences using a hybridization protection assay in which a chemoluminescent acridine ester-labelled RNA probe binds to the amplicons. After hybridization, a base is added which hydrolyzes any probe that is not hybridized to target DNA, destroying its ability to luminesce. The intensity of the chemoluminescent signal, measured in a luminometer, is proportional to the number of probe:target hybrids.

15 The RT-PCR assay uses r*Tth* polymerase to reverse transcribe HIV RNA into cDNA and PCR-amplify a 124 base pair fragment of the HIV-1 *gag* gene in the same reaction tube. Quantification is accomplished using a competitive assay in which a known amount of an internal control RNA (IQS) is added to the sample prior to RNA extraction. The HIV-1 primers amplify a similar size fragment from the IQS cDNA but the internal sequences are different. Detection of amplicons is done in separate microwells containing probes for the HIV-1 fragment and for the IQS fragment. The absorbance values of the HIV and IQS wells for each sample are compared and the amount of HIV RNA in the sample can be calculated.

For the branched-chain DNA assay, the virions in the sample are formed into pellets by ultracentrifugation. They are then lysed and the HIV-1 RNA is captured using a probe that hybridizes to the HIV-1 *pol* gene sequences. The signal is amplified by hybridizing branched DNA-amplifier molecules to the immobilized hybrids, then hybridizing multiple enzyme-labelled probes to each branched DNA molecule. Detection is based on light emission of a chemoluminescent substrate.

The nucleic acid sequence-based amplification reaction (NASBA) is able to directly amplify specific sequences of single-stranded RNA. The NASBA reaction mixture contains T7 RNA polymerase, RNase H, reverse transcriptase, nucleoside triphosphates, and two specific primers. One end of the first primer is complementary to the target sequence while the other end contains a promoter for the T7 RNA polymerase. In the reaction, the target RNA is tran-

scribed into cDNA, from which the RNA hybrid is hydrolyzed. The cDNA is synthesized to double-stranded DNA and as many as 100 copies of RNA are transcribed from the T7 promoter. The reactions take place at one temperature and become cyclic when each new RNA molecule is available to begin the cycle again. The reaction is made quantitative by adding known amounts of internal standard RNA to the sample. Amplified RNA is detected using labelled probes in a chemoluminescent reaction. The amount of HIV-1 RNA in the sample is calculated from the ratio of the target signal to the signal of the standards.

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ISBN 92 9061 148 0