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Detection of HIV-1 and HCV amplification products

Blood banks will be able to take advantage of automated assays to screen the blood supply with speed, specificity, and cost-effectiveness.

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Nucleic acid amplification technology is now the method of choice for identification of a large and still growing number of microorganisms such as *Mycobacterium tuberculosis*, human immunodeficiency virus (HIV), and hepatitis C virus (HCV).[1,2] Nucleic acid amplification techniques include the polymerase chain reaction (PCR), ligase chain reaction (LCR), nucleic acid sequence-based amplification (NASBA), strand-displacement amplification (SDA), and transcription-mediated amplification (TMA).[3–7] Several FDA-approved diagnostic products incorporate these molecular diagnostic methods (see Table I). Nucleic acid amplification technology tests involve not only amplification, but detection methodologies as well. The promise of molecular diagnostics lies in the improvement of its specimen-processing, amplification, and target-detection steps, and in the integration of these steps into an automated format.

Many methods for the detection of amplification products are commercially available. While some are standard fare in molecular biology laboratories, many are tedious, technically difficult, or inappropriate for diagnostic laboratories. IVD manufacturers have just begun to answer customers' needs by bundling specimen processing, amplification, and detection technologies in laboratory diagnostic kits. So, the current generation of tests offers much room for improvement in terms of ease of use, cost, speed, sensitivity, and specificity. Clearly, changes in the nucleic acid amplification technology marketplace lie ahead.

FDA- Approved Assays	Amplification Product:	Nucleic Acid Amplification	Commercial Source

	Detection Method	Method	
*C. trachomatis,N. gonorrhoeae, M. tuberculosis,*HIV-1	Heterogeneous: Colorimetric	PCR	Roche Diagnostics
C. trachomatis, N. gonorrhoeae	Heterogeneous: Chemiluminescence	LCR	Abbott Laboratories
C. trachomatis, N. gonorrhoeae	Homogeneous: Fluorescence	SDA	Becton Dickinson
C. trachomatis, M. tuberculosis	Homogeneous: Chemiluminescence (HPA)	TMA	Gen-Probe

Table I. FDA-approved nucleic acid amplification tests and detection methodologies for infectious diseases.

The sensitivity and specificity of current serologic screening assays for HIV-1 and HCV have significantly reduced transmission of these viruses through transfusion, but it has recently become apparent that nucleic acid amplification technology narrows the seroconversion windows.[8] While FDA-approved PCR-based assays for the quantitation of HIV-1 viral load exist, there are currently no FDA-approved nucleic acid amplification tests for HIV-1 or HCV for blood bank use. The blood bank industry's goal of a zero-risk blood supply has driven the demand for molecular diagnostic tests for HIV-1 and HCV to be used in blood screening. This article first discusses several issues associated with the detection of amplification products in general, and then specifically focuses on detection of HIV-1 and HCV amplification products in the blood bank environment.

Detection Methodologies

Amplified-product detection schemes are of two basic types: heterogeneous and homogeneous. Heterogeneous detection is characterized by a distinct step, such as washing, designed to remove unhybridized probes from hybridized probes, whereas in homogeneous detection there is no physical separation step to remove free probe from bound probe. Multiple heterogeneous and homogeneous detection methods exist.[9]

Heterogeneous Detection. Southern blotting is a heterogeneous detection technique. In Southern blotting, electrophoresis is used to separate amplification products by size and charge. The size-fractionated products are transferred to a membrane or filter by diffusion, vacuuming, or electroblotting. Labeled detection probes are then hybridized to the membrane-bound targets in solution, the filters are washed to remove any unhybridized probe, and the hybridized probe on the membrane is detected by any of a variety of methods.

Other types of heterogeneous detection are based on specific capture of the amplification products by means of enzyme-linked immunosorbent assays (ELISAs).[9] One method used with PCR involves labeling one primer with a hapten or a ligand, such as biotin, and, after amplification, capturing it with an antibody- or streptavidin-coated microplate. The other primer is labeled with a reporter such as fluorescein, and detection is achieved by adding an antifluorescein antibody, horseradish peroxidase

(HRP) conjugate.[10] This type of method is not as specific as using detection probes that hybridize to defined amplification products of interest.

The LCx probe system (Abbott Laboratories; Abbott Park, IL) and the Amplicor HIV-1 test (Roche Molecular Systems Inc.; Pleasanton, CA) are systems that use heterogeneous detection methods. In the LCx system, hapten-labeled oligonucleotide probes thermocycle in the ligase chain reaction. Either a capture hapten or a detection hapten is covalently attached to each of the four primer oligonucleotides. Upon amplification, each amplified product (amplicon) has one capture hapten and one detection hapten. When amplification is complete, the LCx system instrument transfers the reaction to a new well where antibody-coated microparticles bind the capture haptens. Each microparticle is then irreversibly bound to a glass-fiber matrix. A wash step removes from the microparticle any probe that contains only the detection hapten. The LCx instrument adds an alkaline phosphatase (AP)–antibody conjugate that binds to the detection hapten. A fluorigenic substrate for AP is 4-methylumbelliferyl. Dephosphorylation of 4-methylumbelliferyl by AP converts it to 4-methylumbelliferone, which is fluorescent.[11]

The Amplicor HIV-1 test uses an ELISA format. After amplification by PCR, the amplicon is chemically denatured. Amplicon-specific oligonucleotide probes capture the denatured strands onto a coated microplate. The operator washes away any unincorporated primers and unhybridized material in a wash step and then adds an avidin-HRP conjugate to each well. The conjugate binds to the biotin-labeled amplicon captured on the plate. The operator then adds 3,3',5,5'-tetramethylbenzidine (TMB), a chromogenic HRP substrate. When hydrogen peroxide is present, HRP oxidizes TMB. The signal is determined colorimetrically.[12]

Each heterogeneous detection method has advantages and disadvantages. For example, Southern blotting of size-fractionated amplification products offers greater sensitivity than ethidium staining of agarose gels. ELISA and LCx feature sensitivity and specificity approaching that of Southern blots and provide much higher throughput than electrophoresis-based systems.

A significant disadvantage of heterogeneous detection methods is that they require laborious and time-consuming manipulations such as wash steps. In some systems, the wash steps and the transfer of amplification products from one reaction vessel to another increase the potential for contamination.

Homogeneous Detection. Because hybridized and nonhybridized detection probes are not physically separated in homogeneous detection systems, these methods require fewer steps than heterogeneous methods and thus are less prone to contamination. Among the commercially available kits that use homogeneous detection of fluorescent and chemiluminescent labels are the TaqMan system (Applied Biosystems; Foster City, CA), BDProbeTecET system (Becton Dickinson; Franklin Lakes, NJ), QPCR System 5000 (Perkin-Elmer Corp.; Norwalk, CT), and Hybridization Protection Assay (Gen-Probe Inc.; San Diego).

The TaqMan system detects amplicon in real time. The detection probe, which hybridizes to a region inside the amplicon, contains a donor fluorophore such as fluoroscein at its 5' end and a quencher moiety, for

example, rhodamine, at its 3' end. When both quencher and fluorophore are on the same oligonucleotide, donor fluorescence is inhibited. During amplification the probe is bound to the target. Taq polymerase displaces and cleaves the detection probe as it synthesizes the replacement strand. Cleavage of the detection probe results in separation of the fluorophore from the quencher, leading to an increase in the donor fluorescence signal. During each cycle of amplification the process is repeated. The amount of fluorescent signal increases as the amount of amplicon increases.[13]

Molecular beacons use quenchers and fluorophores also. Beacons are probes that are complementary to the target amplicon, but contain short stretches (approximately 5 nucleotides) of complementary oligonucleotides at each end. The 5' and 3' ends of the beacons are labeled with a fluorophore and a quencher, respectively. A hairpin structure is formed when the beacon is not hybridized to a target, bringing into contact the fluorophore and the quencher and resulting in fluorescent quenching. The loop region contains the region complementary to the amplicon. Upon hybridization to a target, the hairpin structure opens and the quencher and fluorophore separate, allowing development of a fluorescent signal.[14] A fluorometer measures the signal in real time.

The BDProbeTecET system uses a real-time detection method that combines aspects of TaqMan and molecular beacons. The probe has a hairpin loop structure and contains fluorescein and rhodamine labels. In this system, however, the region complementary to the target molecule is not within the loop but rather in the region 3' to the rhodamine label. Instead of containing the sequence complementary to the target, the single-stranded loop contains a restriction site for the restriction enzyme BsoBI. The single-stranded sequence is not a substrate for the enzyme. The fluorescein and rhodamine labels are near each other before amplification, which quenches the fluorescein fluorescence. Strand-displacement amplification converts the probe into a double-stranded molecule. The BsoBI restriction enzyme can then cleave the molecule, resulting in separation of the labels and an increase in the fluorescent signal.[15]

The QPCR System 5000 employs electrochemiluminescence with ruthenium labels. A biotinylated primer is used. After amplification, the biotin products are captured on streptavidin-coated paramagnetic beads. The beads are transferred into an electrochemical flow cell by aspiration and magnetically held to the surface of the electrode. Upon electrical stimulation, the ruthenium-labeled probe emits light.[16]

The Hybridization Protection Assay (HPA; see Figure 1) is used in Gen-Probe's nonamplified PACE assays as well as in amplified *Mycobacterium tuberculosis* and *Chlamydia trachomatis* assays. The detection oligonucleotide probes in HPA are labeled with chemiluminescent acridinium ester (AE) by means of a linker arm. Hybridization takes place for 15 minutes at 60°C in the same tube in which the amplification occurred. The selection reagent, a mildly basic buffered solution added after hybridization, hydrolyzes the AE on any unhybridized probe, rendering it nonchemiluminescent. The AE on hybridized probes folds inside the minor groove of the double helix, thereby protecting itself from hydrolysis by the selection reagent.

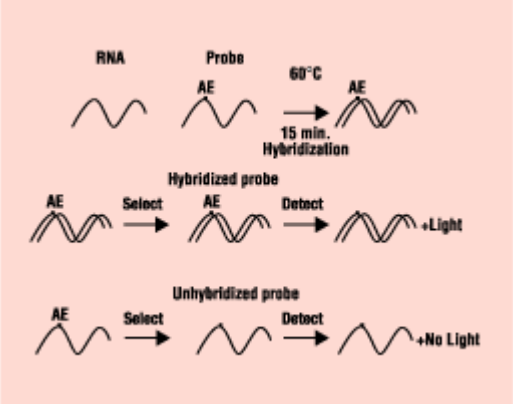

Figure 1. Gen-Probe Hybridization Protection Assay.

The AE emits a chemiluminescent signal upon hydrolysis by hydrogen peroxide followed by sodium hydroxide. A luminometer records the chemiluminescent signal for 2 seconds (a period termed a light-off) and reports the photons emitted in terms of relative light units (RLU). The period from addition of the probe to data collection can be as short as 40 minutes, depending on the number of samples.[17]

Critical Issues in Detection

Detection-probe design is critical in all methodologies that use probes to detect amplification products. Good detection probes hybridize only to specified amplification product and do not hybridize to nonspecific products. Other key issues in optimizing detection methodologies involve the labeling of probes and the maximization of sample throughput.

Probe Design. To design probes that differentiate between closely related species has always been a challenge. Bacterial or fungal assays using detection probes that target species-specific regions of ribosomal RNA (rRNA) can confront closely related bacterial species differing by only one or two bases in the rRNA target. Depending on the probe sequence, its length, and the position of the mismatch, a single mismatch can cause as much as a 5° to 10°C drop in melting temperature.

Assay developers can sometimes take advantage of these properties to design probes and hybridization conditions—hybridization temperature, salt concentration, and hybridization time—that can distinguish single base-pair differences among targets.[18] On the other hand, some assays require probes that tolerate mismatches. This is particularly important in assays for RNA viruses like HIV-1 and HCV that exhibit high sequence diversity. Even though highly conserved regions not under selective pressure are usually targeted, it can be difficult to design probes that hybridize to all strains.

High sequence conservation is only one element involved in the choice of a target region. Other factors affecting performance of a probe sequence include GC content and the presence of repetitive sequences or palindromes. High GC content increases nonspecific hybridization.[18] Repetitive sequences, such as long runs of one nucleotide (e.g., GGGGGG), can introduce unusual structures that inhibit specific binding.[19] Palindromic sequences can cause poor hybridization characteristics.[18]

One strategy for dealing with such problems is to amplify several discrete conserved regions of the target virus and use multiple detection probes.

Another is to use longer probes or chemically modified probes that tolerate mismatches. Combinations of probes that are homologous to all known mutations might also be employed. These strategies all have pitfalls. The use of longer probes or probe combinations increases the likelihood of nonspecific hybridization and of background problems.[18]

Labeling Methods and Reporter Molecules. Detection probes can be labeled several different ways. Enzymatic incorporation of ^{32}P or ^{35}S into the probes is the most common method for isotopic labeling. Following hybridization and washing, the signal is detected on autoradiographic film.

To perform nonradioactive detection, probes can be enzymatically labeled with a variety of molecules. Biotin can be incorporated enzymatically and then detected with streptavidin-conjugated alkaline phosphatase, using AP substrates like 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitroblue tetrazolium (NBT). Chemiluminescent substrates such as Lumi-Phos 530 or Lumi-Phos Plus (Lumigen, Southfield, MI) can also be used with AP. In addition, digoxigenin-11-dUTP can be incorporated enzymatically into DNA or RNA, and antidigoxigenin AP conjugates can be used with colorimetric or chemiluminescent detection.[9]

There are numerous other types of reporter molecules, including chemiluminescent moieties such as acridinium esters.[17] Many fluorescent moieties are available as well.[20] Electrochemiluminescent compounds such as tris (2,2'-bipyridine) ruthenium (II) can be used also.[21]

Throughput. Assay throughput is a major concern for high-volume diagnostic labs and large blood banks. In the blood bank environment, whole blood and platelets have limited shelf lives, and results of testing for viruses must be obtained rapidly so that units can be released. Some IVD manufacturers have adapted their detection systems to 96-well plate formats or other multiwell configurations and use automated or semiautomated processing systems to increase throughput. A fully automated system under development from Gen-Probe, the TIGRIS, allows 1000 specimens to be tested in a 12-hour period.[22]

One way to increase throughput is by multiplexing. Simultaneously testing for multiple analytes also lowers costs. However, multiplexing introduces a new set of considerations for probe design. For one, probes must not contain regions that are complementary to one another. Also, the background signal in an assay increases as the number of probes increases; thus, a probe must be evaluated for its contribution to the overall signal and background.

A High-Throughput Detection System

The discussion now focuses on the application of a homogeneous system for the detection of HIV-1 and HCV RNA in blood screening. This assay is a variation of the HPA. Named the Procleix HIV-1/HCV assay (Chiron; Emeryville, CA), it is currently being used as an investigational new drug (IND) to screen approximately 75% of the blood donations in the United States.

HIV-1/HCV Assay. The Chiron Procleix HIV-1/HCV assay is run on a semiautomated system called the eSAS that consists of a robotic sample pipettor by Tecan (Durham, NC), the Chiron Procleix target capture

system for removing plasma from the reaction tube after target capture, and the Chiron Procleix Leader HC+ luminometer for measuring chemiluminescent analyte signal. Procleix system software performs data analysis that includes tracking samples and reporting sample and run validity, sample reactivity, and analyte RLU values (see Figure 2).

Figure 2. The eSAS includes a robotic pipettor (Tecan; Durham, NC), the Chiron Procleix target capture system, and the Procleix Leader HC+ with the Procleix system software.

The Chiron Procleix assay uses an internal control molecule to control for the capture, amplification, and detection of viral RNA. Internal-control, HIV-1, and HCV RNA are simultaneously captured, amplified, and detected. The assay amplifies two regions of the HIV-1 genome (a region in the long terminal repeat (LTR) and a region in the pol gene) and a conserved region of the HCV 5' untranslated region (UTR), as well as the internal control. Thus, there can be as many as four separate amplicons in the detection step. One detection probe hybridizes to the internal control amplicon, three probes hybridize to amplicons generated from HIV-1, and two probes are complementary to the HCV amplicon.[23]

The assay uses a variant of HPA called the dual kinetic assay (DKA), which can differentially detect two targets simultaneously using two different types of acridinium ester.[24] One is an ortho-fluoro AE (o-f-AE) and the other a 2-methyl AE (2-MeAE); their chemical structures are shown in Figure 3. The ortho-fluoro label emits photons rapidly after hydrogen peroxide and sodium hydroxide are added and is called a "flasher," whereas 2-MeAE emits light much more slowly and is referred to as a "glower." In the Chiron Procleix assay, 2-MeAE is the label used on the probes that hybridize to the pol and LTR amplicons of HIV-1 and to the HCV 5' UTR amplicon, and the internal control probe is labeled with o-f-AE. The flasher signal of the control can be discriminated easily from the glower signal of the analyte in the reaction (see Figure 4).

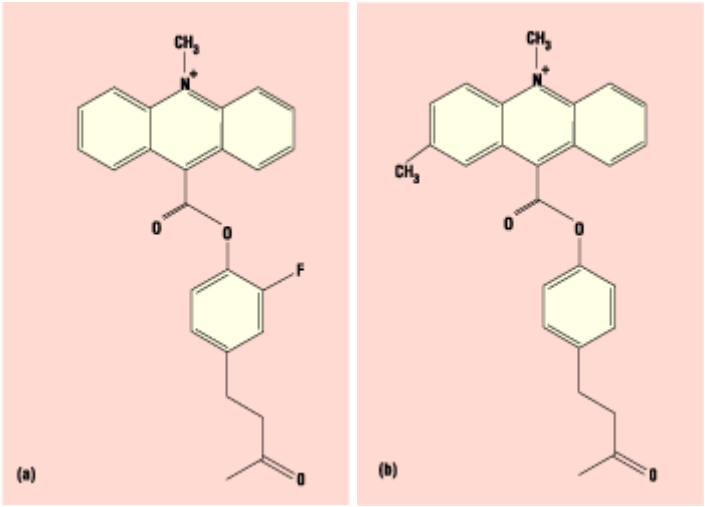

Figure 3. Structure of ortho-fluoro acridinium ester (a); structure of 2-methyl acridinium ester (b).

The Chiron Procleix HIV-1/HCV assay target-capture reagent contains the internal control; thus, internal control is present in all reactions. An internal control signal at the detection step indicates that capture, amplification, and detection steps were performed properly and that no inhibition of the assay reaction occurred.

At the completion of a run, any sample that has a glower signal above the calculated cutoff value is reactive for HIV-1 and/or HCV. HIV-1- and HCV-reactive specimens can be discriminated by running assays in which the multiplex probe reagent is replaced by only HIV-1-specific probes (an HIV-1 discriminatory assay) or only HCV-specific probes (an HCV discriminatory assay).

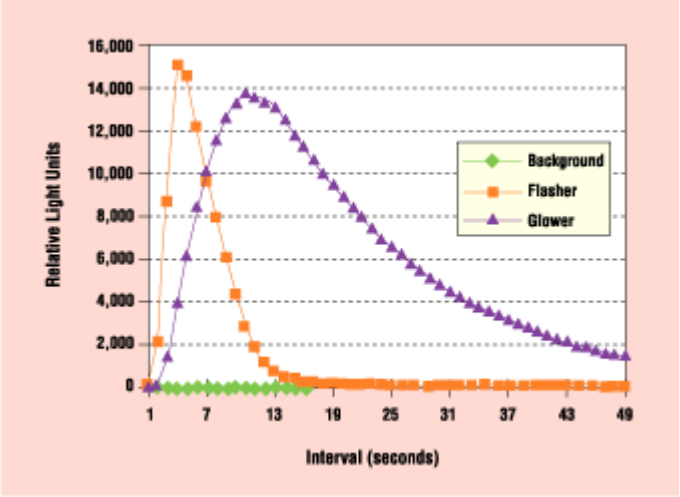

Figure 4. Comparison of light-off kinetics of ortho-fluoro AE (flasher) and 2-methyl AE (glower) probes in the Procleix HIV-1/HCV assay.

The Chiron Procleix assay was designed to detect all subtypes of HIV-1 and HCV. The use of multiple probes, targeting of multiple conserved regions of the virus, and the use of modified oligonucleotide derivatives in the detection probes so as to provide higher tolerance to mismatches than standard 2'-deoxyoligonucleotides are system elements devised for this purpose.

Several regions of each virus are targeted by the assay probes in order to ensure that all known variants and possible mutations are detected. The probes are designed to hybridize to highly conserved regions of the virus that are not under selective pressure.

Clinical Results. The Chiron Procleix HIV-1/HCV assay has been used in IND clinical trials at the American Red Cross, America's Blood Centers, and the Association of Independent Blood Centers since April 1999. To increase throughput, blood banks currently test pools containing 16 specimens. Individual units making up a reactive pool are identified and each is tested with the multiplex assay. All individual specimens testing reactive in the HIV-1/HCV assay are further tested with the HIV-1 and HCV discriminatory assays.

At the American Red Cross, 7.7 million units have been tested. Of those, 25 positive HCV RNA samples were identified that tested negative in HCV antibody–based assays. One HIV-1 sample found reactive by the Chiron Procleix assay produced a negative result in the HIV-1-antibody assays. Of the 2.96 million units tested by America's Blood Centers, 16 were HCV RNA reactive yet were found negative in HCV-antibody assays, and 2 were HIV-1 RNA reactive yet were negative in HIV-1-antibody assays. The

Association of Independent Blood Centers had two positive HCV RNA samples found to be negative in HCV antibody–based assays, out of 375,000 donations. Yield samples—that is, samples found positive for virus by nucleic acid testing but negative by antibody assays—were confirmed by tests using an alternative molecular technology or by follow-up testing of the donor (see Table II).[25] Specificity was greater than 99.95% at all sites.

Test Sites	Units Tested	HCV-Reactive Units	HIV-1-Reactive Units
American Red Cross	7.7 million	25 (1:308,000)	1 (1:7.7 million)
America's Blood Centers	2.96 million	16 (1:185,000)	2 (1:1.5 million)
Association of Independent Blood Centers	375,000	2 (1:187,500)	0

Table II. Yield of nucleic acid test screening with the Chiron Procleix HIV-1/HCV assay among U.S. blood donors.

Conclusion

A variety of homogeneous and heterogeneous methods for detection of amplification products based on chemiluminescence, colorimetry, and fluorescence have been commercialized. IVD manufacturers have made significant progress in overcoming problems traditionally associated with detection of these targets; throughput and contamination control are both improved. The future of amplification product detection in the IVD marketplace clearly lies in the automation of homogeneous detection and further test improvements in ease of use, time to first result, throughput, sensitivity, and specificity.

The Chiron Procleix HIV-1/HCV assay offers homogeneous detection. Multiplexing and the inclusion of an internal control are possible with a variation of the Hybridization Protection Assay on which the system is based, termed the dual kinetic assay. The use of modified oligonucleotide probes and multiple probes targeting conserved regions of the virus allows detection of all subtypes of HIV-1 and HCV. Data generated in clinical trials indicate that the Procleix HIV-1/HCV assay can improve the safety of the public blood supply.

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