Life beyond PCR- alternative target amplification technologies for the diagnosis of infectious diseases, part II

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Life Beyond PCR: Alternative Target Amplification Technologies for the Diagnosis of Infectious Diseases, Part II*

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Abstract

Non-PCR-based target amplification technologies, including transcription-mediated amplification (TMA), nucleic acid sequence-based amplification (NASBA), and strand displacement amplification (SDA) are currently the basis for a broad range of clinical infectious disease molecular diagnostics. These amplification technologies are very sensitive and specific and can be used in combination with traditional end-point or “real-time” detection formats. For several nucleic acid targets, TMA, NASBA, and SDA have certain advantages over PCR-based applications. This two-part article will review the molecular basis of each technology and how the technology has been applied to clinical diagnostic systems. The articles will describe the current testing platforms available, U.S. Food and Drug Administration (FDA)- and non-FDA-approved assays, and availability of analyte-specific reagents. In addition, an open platform system is described that utilizes standardized reagents and methods and allows the user to develop in-house protocols. Finally, applications for the future are discussed.

Gen-Probe Technologies

The first transcription-mediated amplification (TMA)-based system developed by Gen-Probe (Gen-Probe Incorporated, San Diego, CA) consisted of three steps: sample processing, target amplification, and target detection (8). In the sample preparation phase, the nucleic acids are released by chemical and/or mechanical means, such as sonication, as in the Mycobacterium Tuberculosis Direct Test (AMTD). Target nucleic acids are denatured at 95° C, and infectious agents are inactivated. The resulting lysates contain free total nucleic acids, which are used in the amplification process. Gen-Probe has developed a second-generation nucleic acid amplification system called APTIMA, which utilizes target capture specimen processing (8), TMA, and dual kinetic assay (DKA) detection technology (23). Target capture technology (Fig. 6) was developed to improve the performance of the first-generation TMA assays by removing inhibitory or interfering substances that may cause false-negative results (24,25). Target capture technology is less labor-intensive than first-generation assays and results in highly purified nucleic acids prior to TMA. The samples are lysed, and the target nucleic acids are released and hybridized to an intermediate capture oligomer. The target capture-oligo complex is captured by oligo-dT oligomers that are bound to the surfaces of paramagnetic particles. Magnets are used to draw the particles to the sides of tubes for wash procedures that remove inhibitors and purify and concentrate the target nucleic acids. TMA is performed directly on the captured target sequences while they are still bound to the surfaces of the magnetic particles.

The detection of TMA-amplified products can be performed by using either of two different technologies, the hybridization protection assay (HPA) technique (26) or DKA. In HPA (Fig. 7), acridinium ester (AE)-labeled DNA probes are hybridized to specific target sequences internal to the amplicons. Hybridized and unhybridized probes are separated by the addition of a selection agent that hydrolyzes the AE on the unhybridized probes. The AE on the hybridized probes is protected within the double-stranded DNA-probe complex and therefore is not hydrolyzed. After the addition of a detection reagent, light is emitted by

the hybridized probes and detected by a luminometer.

In the DKA (Fig. 8), two different targets can be detected by using two target-specific DNA probes that are each labeled with different AE molecules. One AE molecule (flasher) has fast light-off kinetics, and a very brief flash of light is detected. The other AE molecule (glower) has slower light-off kinetics. The luminometer is able to distinguish between the two different signals and an intermediate signal that is generated if both targets are present.

Currently, two Gen-Probe TMA assays, AMTD and the APTIMA Combo 2 (AC2) assay for the dual detection of Chlamydia trachomatis (CT) and Neisseria gonorrhoease (GC), are FDA approved. A TMA assay for hepatitis C virus (HCV) (Bayer, Inc., Tarrytown, NY) is available as an analyte-specific reagent (ASR). Additional TMA tests that use target capture are being developed for the detection of Chlamydophila pneumoniae, Mycoplasma pneumoniae, and Mycobacterium tuberculosis (Mtb).

AMTD

The first FDA-cleared clinical assay to use TMA was developed for the detection of Mtb directly from respiratory specimens that stained positive with the acid-fast bacillus (AFB) stain (27). The FDA-approved use of the test was later expanded to include AFB-stained, smear-negative respiratory specimens (28). Samples are processed according to standard mycobacterial decontamination procedures, and an aliquot is lysed, sonicated, and heat inactivated. The assay targets rRNA and can detect rRNA equivalent to less than one cell of Mtb. Many studies have demonstrated that the analytical sensitivity correlates with a higher clinical sensitivity when compared to both AFB-stained smears and solid-medium culture. The clinical sensitivity is equivalent to that of liquid media culture techniques. The assay is specific for Mtb complex, including Mtb, M. bovis, M. africanum, M. microti, and M. canetti. The assay has also been validated in laboratories for a variety of other sample types, including tissue and cerebrospinal fluid.

AC2 assay for CT and GC

The AC2 test was FDA cleared in May 2001 for the detection of CT and GC in male and female urogenital swabs and urine specimens from both symptomatic and asymptomatic individuals (29). Recently, the assay has also been cleared for testing on self- or physician-collected vaginal swabs. The procedure can be fully automated or semi-automated, and up to 500 samples can be processed in 9 h, or 1,000 samples in less than 14 h. The assay uses a target capture specimen-processing procedure that removes most amplification inhibitors, particularly those that interfere with urine testing (24,25). Studies have demonstrated an inhibition rate of 0.48% for spiked urine samples (29,30).

The analytical sensitivity of the assay is approximately 0.01 inclusion-forming unit/assay and 50 cells/assay for CT and GC, respectively (29). The AC2 assay was demonstrated to be more sensitive than the BD ProbeTecET assay (BD Diagnostic Systems, Sparks, MD), the Amplicor CT and GC assay (Roche Diagnostic Systems, Branchburg, NJ), and the former LCx assay for CT and GC (Abbott Laboratories, Abbott Park, IL) (29-32). A combination of target capture, rRNA targeting, and a lack of amplification inhibition contributes to the enhanced sensitivity of the assay. The AC2 is also very specific, with no cross-reactivity detected with closely related Neisseria species, a problem described for other molecular amplification methods, such as the Roche Amplicor CT/GC assay (30,31). The performance of the AC2 assay was evaluated in several clinical trial studies, and overall, the assay demonstrated sensitivities and specificities of $\geqslant$96 to 97% and $\geqslant$95 to 99%, respectively (29-32).

Procleix HIV-1/HCV assay

The Procleix HIV-1/HCV assay was developed by Gen-Probe Incorporated and is distributed by Chiron Corporation (Emeryville, CA) as a multiplex nucleic acid amplification test (NAAT) using TMA for screening denoted blood products for HIV-1 and HCV (33). The assay uses a combination of target capture, TMA, HPA, and DKA. The assay is internally controlled, with a demonstrated failure rate of 0.8%, which was reduced to 0% upon retesting. The sensitivity of the assay is approximately 100 copies of HIV-1 or HCV RNA/ml. The assay can detect all known subtypes of both viruses with comparable sensitivities, including HIV-1 groups O and N. Phase II clinical trials conducted by the American Red Cross and the Association of Independent Blood Centers have demonstrated that the specificity of the assay is >99.95%. In addition, the

data indicated that, on average, the assay can detect HIV-1- and/or HCV-positive specimens 16.3 and 32.8 days, respectively, before antibody detection and positive HIV-1 specimens 7.5 days before p24 antigen detection. The Procleix HIV-1/HCV assay in combination with the Procleix instrument system was approved by the FDA in February 2002. This assay is currently in use worldwide and is used for screening 80% of all blood products collected in the United States. A triplex test (Procleix Ultrio) that simultaneously targets HIV-1, HCV, and hepatitis B virus (HBV) in the same sample is currently available internationally and is in clinical trials for FDA approval.

Automation for TMA-Based Assays

The TIGRIS DTS system, developed by Gen-Probe Incorporated, is the first fully automated instrument for NAAT testing (34). After the sample is loaded, the instrument handles it from specimen processing through to amplification and detection, with a throughput potential of up to 500 tests in 9 h and up to 1,000 tests in less than 14 h. The time to first result is 3.5 h, and there is continuous specimen-loading capability. Urine and swab samples are processed identically; there are no centrifugation or other sample manipulation steps required by the operator. The samples are aliquoted within the instrument through a penetrable cap on the specimen tubes that reduces the likelihood of cross-contamination. The chances of cross-contamination and false-positive results are also reduced by eliminating manual pipetting, the addition of an oil layer to the reaction tubes, and the deactivation of amplicon at the end of the assay. The instrument has unidirectional interface capabilities for integration with laboratory information systems. The TIGRIS was cleared by the FDA in December 2003 for use with the AC2 assay and is currently in clinical trials with the Procleix Ultrio assay.

Future TMA Assays

Currently, under development by Gen-Probe Incorporated are TMA-based assays that incorporate molecular beacons into the amplification reaction. A variety of fluorophores (edans, fluorescein [FAM], rhodamine [ROX], TAMRA, Cy3, and Cy5) and quenchers (daccyl, QSY7, and BH1, -2, and -3) are compatible with the TMA system. The fluorescent light emitted by the molecular beacons can be detected throughout the amplification process by using a variety of instruments, including the Rotor-Gene 2000, Opticon II, ABI Prism 7000, Mx 4000, and I-Cycler. Similar to other real-time assays, TMA is a closed system and is capable of multi-color/multi-analyte detection. Preliminary studies by the manufacturer have demonstrated that the assays have a wide dynamic range (8 orders of magnitude), a sensitivity for both RNA and DNA of approximately 50 copies/ reaction, and excellent precision (coefficient of variation as low as <1%). The TMA molecular beacon assays currently in development by Gen-Probe include HIV-1, HCV, HBV, West Nile virus, and a CT/GC multiplex assay.

Strand Displacement Amplification (SDA)

SDA is an isothermal (52.5° C) process consisting of two discrete phases (Fig. 9) (9,10). Phase I generates a single-stranded target of defined length, and phase II results in an exponential amplification of the sequence. SDA is based on the ability of an exonuclease-deficient DNA polymerase to initiate DNA synthesis at a single-stranded nick present in double-stranded DNA. The SDA primers contain target-specific sequences and a restriction endonuclease site. As depicted in Fig. 9, the primers serve as templates for DNA synthesis in the opposite direction and initiate DNA synthesis on the target strand. For the nicking and displacement to occur, the restriction endonuclease must generate site-specific nicks in only one strand of a double-stranded DNA molecule. To accomplish this, three deoxynucleoside triphosphates (dNTPs) and one alpha-thio-triphosphate (dNTP[αS]) are used to generate hemiphosphorothioated DNA restriction endonuclease sites that are refractory to double-stranded nuclease. The restriction endonuclease nicks one strand of the DNA, and the polymerase initiates synthesis from the exposed 3' end, displacing the downstream strand into solution. The displaced strands serve as templates for subsequent nicking, extension, and displacement reactions that ultimately result in the accumulation of large amounts of single- and double-stranded copies of the original target. Modifications to the original amplification technology have enabled this process to be used in clinical assays (BD ProbeTec ET System, Becton Dickinson, Sparks, MD).

Single-stranded DNA probes (Fig. 10 and 11A) are present in the reaction and can bind the displaced single-stranded products for real-time detection of amplified product. The DNA probes are stem-loop structures comprised of the recognition sequence for the BsoBI enzyme and a downstream target-specific sequence. A number of alternative probe formats are employed with the BD ProbeTec ET System, depending on the assay. Probes may be labeled at the 5' terminus with FAM (fluorophore) and internally with ROX (quencher). Alternatively, they may be labeled at the 5' end with FAM and internally with dabcyl (quencher) or with a dabcyl quencher moiety at the 5' terminus and internally with ROX as the fluorophore. In their native state and prior to SDA amplification, the two labels are in close proximity to each other. Any excitation of the 5' fluorescent label leads to a transfer of energy to the 3' dabcyl label or ROX label, resulting in very little emission from the excited fluorophore. During the SDA process, when the specific target has been amplified, the probe becomes double stranded and is cleaved by the BsoBI restriction enzyme. This process separates the two labels, at which point no energy transfer (ET) can occur, resulting in emission by the fluorescent label.

BD ProbeTec ET, CT, and GC amplified DNA assays

The BD ProbeTec ET system is based on SDA of the target nucleic acid coupled with homogeneous real-time detection using fluorescent ET (9,10,35). This assay format is currently FDA approved for the direct, qualitative detection of CT and GC from endocervical and male urethral swabs and in female and male urine specimens. Either dry swabs or swabs in BD CT/GC liquid transport diluent are acceptable for testing. For the amplification of CT, the primers target the multicopy cryptic plasmid, and for GC, the primers target a region within the multicopy pilin gene-inverting protein homologue (36). The assay can detect as few as five GC cells or five CT elementary bodies. Amplification and detection time is 1 h, with the potential to report 564 patient results per shift.

In comparisons to culture and the Abbott LCx CT/GC NAAT, a multi-center study determined that the sensitivities for CT and GC detection were 92.8 and 96.6%, respectively, for cervical swabs and 80.5 and 84.9% for urine from women (36). CT and GC sensitivities were 92.5 and 98.5%, respectively, for male urethral swabs and 93.1 and 97.9% for urine from men. Another study of 396 specimens compared the ProbeTec assay to enzyme immunoassay (EIA), culture, the Roche Amplicor CT/GC PCR assay, and the Abbott LCx assay (37). The sensitivities and specificities of GC culture, PCR, SDA, and LCx were 69.8, 95.2, 88.9, and 88.9% and 100, 99.4, 100, and 99.1%, respectively. The sensitivities and specificities for CT EIA, PCR, SDA, and LCx were 42.0, 98.0, 94.0, and 90.0% and 100, 98.0, 100, and 98.6%, respectively.

The reagents for SDA are dried in two separate disposable microwell strips. The processed sample is added to the priming microwell, which contains the amplification primers, fluorescently labeled detector probe (5' FAM, internal ROX label), and other reagents necessary for amplification. After incubation, the reaction mixture is transferred to the amplification microwell, which contains the two enzymes, Bst DNA polymerase and BsoBI restriction enzyme, required for the SDA reaction. The microwells are sealed to form a closed system and placed in an incubator/fluorescence reader for the 1-h assay time. The amount of emitted fluorescence is measured and compared to predetermined cutoff values.

The user has the option to include an amplification control to test for the presence of potential amplification inhibitors in the sample, which could cause false-negative results. Several studies have demonstrated rates of inhibition of <1% for endocervical swabs and from <1 to <3.5% for urine samples (37,38). These data suggest that the use of an amplification control may not be necessary for endocervical swabs but should be considered for urine samples.

BD ProbeTec assays for the detection of Mycoplasma pneumoniae, Chlamydiaceae family, and Legionella pneumophila

A panel of assays has been developed for the detection of the agents of atypical pneumonia, M. pneumoniae, Chlamydophila pneumoniae, and L. pneumophila (39-41). All three assays include an internal amplification control oligonucleotide containing the same priming sequences as the native target but with a mutated internal region. Target and internal control are detected using probes labeled with different fluorescent dyes (target, ROX; internal control, FAM). The dual-dye capabilities of the ProbeTec ET System permit the

detection of target and internal control within 1 h. Testing can be performed directly from processed throat swabs and lower respiratory tract specimens after Qiagen extraction (Qiagen, Valencia, CA). The M. pneumoniae assay amplifies a region within the P1 cytadhesin gene (39). The L. pneumophila assay targets the macrophage infectivity potentiator (mip) gene and detects isolates from serogroups 1 to 14 (40). The Chlamydiaceae assay detects the three most important pathogens of this family, C. pneumoniae, Chlamydophila psittaci, and CT, as well as other species within the group (41). The target for this assay is the ribonuclease P gene. The analytical sensitivity for all three assays is approximately 100 genomic equivalents or copies of target DNA per amplification (39-41). The Chlamydiaceae assay was shown to detect <1,000 elementary bodies of C. pneumoniae per ml of spiked sputum (41).

In 2003, clinical trial studies were completed for these ProbeTec ET assays for the detection of atypical pneumonia pathogens. Recently, the BD ProbeTec assay for the detection of L. pneumophila was approved by the FDA for use in clinical diagnostics.

BD ProbeTec Mycobacterium tuberculosis assay (DTB)

The BD ProbeTec M. tuberculosis Direct (DTB) assay is currently available outside the U.S. The assay amplifies and detects the Mtb complex-specific IS6110 DNA target (42). Included in the assay is an internal control that is simultaneously amplified by SDA and detected by fluorescent ET using two different labeled probes (target, ROX; internal control, FAM). Clinical studies evaluated the DTB assay in comparison to the Gen-Probe AMTD assay (42). The sensitivity and specificity of DTB and AMTD were 88 and 99.2% and 74.3 and 100%, respectively, for respiratory specimens. The sensitivity and specificity for DTB and AMTD were 94.5 and 99.6% and 92.3 and 100%, respectively, for extrapulmonary specimens. In comparison to culture, the overall sensitivity and specificity were 82.7 and 98.5%, respectively (43). Another study compared the results of the ProbeTec to the Amplicor-PCR M. tuberculosis assay (Roche Diagnostic Systems, Inc., Indianapolis, IN). The overall sensitivities of the two assays were comparable at 93.5%, and assay specificity was 99.7% for the ProbeTec and 100% for the Roche Amplicor assay (44).

Automation for BD ProbeTec ET assays

The BD Viper Sample Processor is a high-throughput system that can accommodate 552 patient samples per 8-h shift while reducing the hands-on time requirement. Swab and urine samples are processed and lysed by heat, and the sample rack is transferred to the Viper. The system performs the liquid transfer and plate-sealing functions for the ProbeTec assays. The plates are then transferred to the ProbeTec instrument for amplification and detection. Currently, the Viper is FDA cleared for use with all ProbeTec CT and GC assays, with and without the amplification control. Future upgrades to the BD Viper system include cap-piercing technology for primary tube sampling, on-board extraction, and incorporation of the 1-h homogenous amplification and real-time detection capability currently found in the BD ProbeTec instrument.

Applications for the Future

TMA, nucleic acid sequence-based amplification, and SDA are excellent technologies for the detection of infectious agents. The versatility of the assays permits their use with a variety of different types of nucleic acid targets, and the amplified products are amenable to detection by a number of different technologies. In keeping with the demand of today's medical practice, we look forward to the many new assays in development, whether they be available as FDA-approved assays or ASRs. Alternative platforms bring variety to the bench and expand the testing capabilities of the laboratory. As with all molecularly based assays, automation of the entire process is the next key step in refining our methods and expanding our test menus.

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