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Molecular tools for building nucleic acid IVDs

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With technology advancing daily, the future of nucleic acid probe technology for IVDs looks as bright as it ever has.

Since the mid-1980s, researchers have repeatedly demonstrated the practical utility of DNA probe—based assays for clinical laboratory use. The technologies related to nucleic acid assays have advanced rapidly, resulting in improved sensitivity and specificity, streamlined protocols (including automation), and a wider selection of target analytes and commercially available test kits.

Potential applications for nucleic acid probe technologies are very broad, including pathogen detection in clinical medicine, genetic screening and diagnosis, dental and veterinary medicine, drug resistance and susceptibility testing, pharmacogenetic analysis, food testing, and forensic analysis. But to successfully apply nucleic acid probes to such diverse fields, the technology must continue to advance and provide the tools to build effective and useful assays. The industry is still young, and much remains to be accomplished.

This article describes technologies recently developed at Gen-Probe, Inc. (San Diego), and offers examples of some of the tools currently available with which to build DNA probe—based IVDs. It also discusses potential applications for these tools.

Transcription-Mediated Amplification

In some clinical specimens from infected individuals, target

nucleic acids are present in high enough concentrations to be detected directly. For instance, the ribosomal RNA (rRNA) of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* are often present in sufficient quantities to be detected without extensive processing.

In many cases, however, target nucleic acids may be present in very small amounts, making them difficult or impossible to detect using direct methods. In a sputum sample being tested for *Mycobacterium tuberculosis*, for example, a single cell is considered a positive indication of disease. Viral detection and genetic disease analysis are other examples of clinical applications in which the target copy number can be very low.

In cases where the amount of target is low, nucleic acid amplification is often employed to increase number of copies of the target to a detectable level. Current amplification techniques include polymerase chain reaction (PCR; Roche Molecular Systems), ligase chain reaction (LCR; Abbott Laboratories), nucleic acid sequence—based amplification (NASBA; Organon Teknika NV), strand displacement amplification (SDA; Becton Dickinson) and transcription-mediated amplification (TMA; Gen-Probe, Inc.).[1—3]

TMA is an isothermal, autocatalytic nucleic acid target amplification system that can produce more than a billion RNA copies of target RNA or DNA in an hour or less (see Figure 1). It can be used in a qualitative mode to rapidly determine the presence or absence of a target, or in a quantitative mode to accurately determine the amount of starting target over a concentration range of several orders of magnitude. TMA can be used to amplify a single target of interest, or configured in a multiplex mode to simultaneously amplify multiple targets of interest.

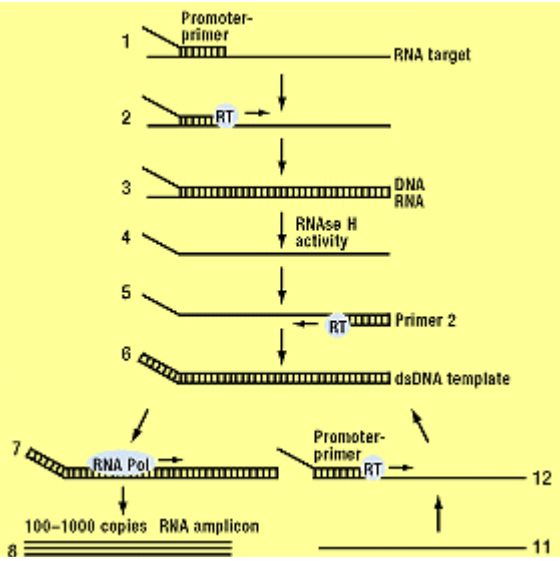

Figure 1. Mechanism of transcription-mediated amplification (TMA) reaction with an RNA target. The first primer (promoter-primer; contains a T7 promoter region) anneals to the target and is extended by reverse transcriptase (RT). The RNA strand is degraded by RNAse H activities, and a second primer (primer 2) anneals to the DNA strand and is extended by RT, yielding a double-stranded DNA template with an active T7 promoter region. T7 RNA polymerase binds to this promoter and transcribes the template strand, yielding 100 to 1000 RNA copies. Primer 2 anneals to each of these RNA copies and is extended by RT, and the RNA strand is degraded by RNAse H activities. The promoter-primer then binds to the DNA strand, and extension with RT produces the dsDNA template with an active T7 promoter. More RNA copies are made, and this cycle continues in an autocatalytic fashion.

Amplification by TMA is simple to perform, rapid, robust, extremely sensitive, and amenable to automation. Amplicon is detected using the hybridization protection assay (HPA) described below. Amplification and detection are performed entirely in solution in a single reaction tube with only a few reagent addition steps required (no material is ever removed from the tube). This minimizes the risk of carryover and cross-contamination.

Detection Using the Hybridization Protection Assay

To detect and identify specific nucleic acid targets, a wide variety of methods are currently used, including direct sequencing, hybridization with sequence-specific oligomers, gel electrophoresis, oligonucleotide arrays, and mass spectrometry. These methods can use heterogeneous or homogeneous formats, isotopic or nonisotopic labels, or no labels at all.

In the systems described in this article, target nucleic acid is detected using short sequence-specific oligonucleotide probes. These probes are covalently labeled with acridinium ester (AE), a highly chemiluminescent molecule that is used as a reporter group in a number of assay systems.[4—7]

This chemiluminescent method of detection works by directly attaching one AE molecule to each nucleic acid probe, using a non-nucleotide-based linker-arm chemistry that allows placement of the AE at any location within the probe.[8—9] Chemiluminescence is triggered by reaction with alkaline hydrogen peroxide, yielding an excited N-methyl acridone which collapses to ground state with the emission of a photon

(see Figure 2). Another important reaction of AE is ester hydrolysis, which yields the nonchemiluminescent N-methyl acridinium carboxylic acid.

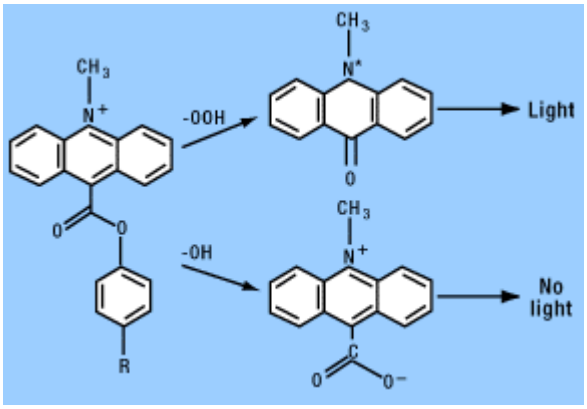

Figure 2. Acridinium ester (AE) reaction pathways. AE reacts with alkaline hydrogen peroxide to produce light. Ester hydrolysis in mild base yields the N-methyl acridinium carboxylic acid, which is nonchemiluminescent.

When the AE molecule is covalently attached to a nucleic acid probe, hydrolysis is rapid under mildly alkaline conditions. However, when the probe to which the AE is tethered binds to its exactly complementary target nucleic acid, the rate of AE hydrolysis is greatly reduced. By making use of this differential hydrolysis, researchers can discriminate between hybridized and unhybridized AE-labeled probe in solution—that is, without physical separation (see Figure 3).

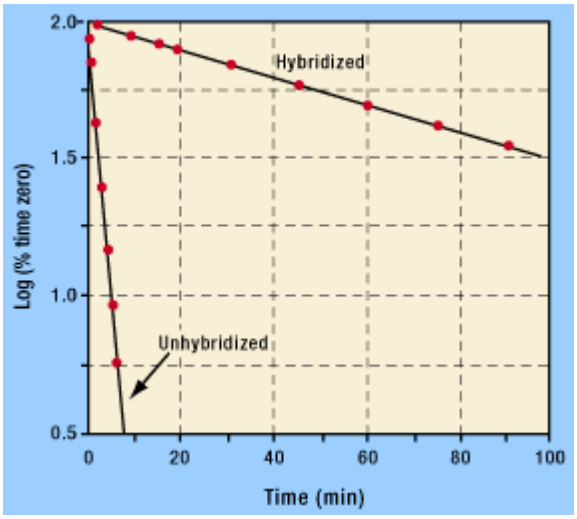

Figure 3. AE hydrolysis kinetics for hybridized and unhybridized AE-probe. Loss of chemiluminescence is plotted versus time for hybridized and unhybridized AE-probe, demonstrating the difference in AE hydrolysis rates between the two species.

Researchers have developed an assay format based on these differential hydrolytic properties of AE.[4,5] Referred to as the hybridization protection assay (HPA), it consists of the following steps.

- Hybridize the AE-probe to target nucleic acid in solution (typically 15—30 minutes).
- Add a mild alkaline solution and hydrolyze AE coupled to unhybridized probe (5—10 minutes).
- Detect the remaining hybrid-associated AE as a measure of the amount of target present (2—5 seconds).

Mismatch Discrimination Using HPA

In many applications, researchers must be able to discriminate targets that differ by as little as one nucleotide. Examples of this include detection of genetic mutations correlated with disease, identification of drug-resistant pathogens, and discrimination of closely related organisms. The HPA format can be used to detect such known sequence variations in nucleic acid targets.

As described above, hybridization of an AE-probe to an exactly complementary nucleic acid target protects the AE from alkaline hydrolysis. However, when a mismatch is present in the duplex immediately adjacent to the site of AE attachment, that protection is disrupted and AE once again hydrolyzes as rapidly as the unhybridized probe.[10] This phenomenon is observed with single-base mismatches as well as multiple mismatches, insertions, deletions, and genetic translocations. When conducted under stringent conditions, the assay based on this discrimination phenomenon commonly yields matched-to-mismatched signal-to-noise ratios of 1000-to-1 or greater.

The steps of the assay are essentially identical to those described above, except that mild alkaline treatment now hydrolyzes AE associated with mismatched duplexes as well as unhybridized probes. One AE-probe is constructed to be exactly complementary to the wild-type sequence and one AE-probe is constructed to be exactly complementary to the mutant sequence. The wild-type and mutant targets are then assayed with the appropriate probe—either in separate reactions or in the same reaction—using the method for multiple analyte analysis described below.

Multiplexed Analyte Analysis

Simultaneous detection of two or more pathogens in a clinical sample decreases laboratory costs and increases efficiency and utility. In tests requiring amplification, detection of one or more analyte and an internal control can provide a positive control of amplification for every sample. Simultaneous quantitative detection of normal and mutant sequences in a patient sample makes it possible to establish precisely the normal/mutant ratio.

One approach to detecting multiple analytes is to use chemically modified AE molecules that exhibit distinct chemiluminescence characteristics. When attached to DNA probes, these modified AE labels can be used in the HPA format for the simultaneous detection of multiple nucleic acid targets.[11]

One mode makes use of chemical modifications of AE that lead to differences in the light-off kinetics of the chemiluminescence reaction (see Figure 4). The light-off kinetics of 1-Me-di-m-F-AE are significantly faster than those of 1-Me-AE, enabling researchers to discriminate between the two labels. When these modified AEs are attached to DNA probes, two analytes can be simultaneously detected and quantitated using the HPA format. After the hydrolysis step, the chemiluminescent signals of the remaining hybrid-associated AEs are collected over multiple time intervals, and the signals of the individual labels are resolved mathematically to quantitate the amount of each target present. This assay mode is referred to as the *dual kinetic assay* (DKA).

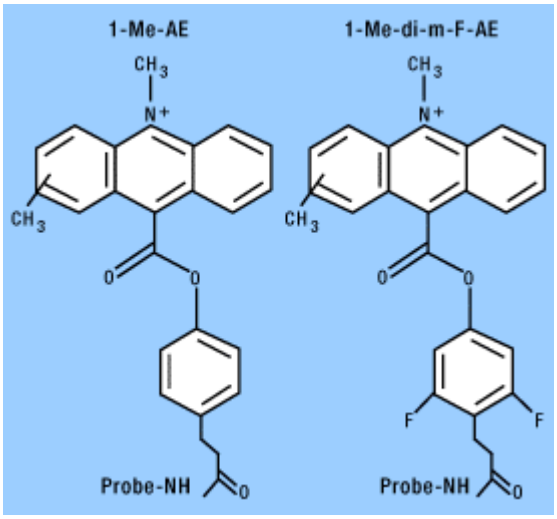

Figure 4. Chemically modified acridinium esters. The structure of two chemically modified acridinium ester molecules, 1-methyl-AE (1-Me-AE), and 1-methyl-di-meta-fluoro-AE (1-Me-di-m-F-AE), are shown.

Several chemically modified AEs with distinct light-off kinetics have been synthesized, and at least five labels can be clearly discriminated from one another in a single tube (data not shown). Selected labels can also be discriminated from one another on the basis of pH optimum for light-off. The kinetic and pH modes can also be combined to discriminate several labels.

Applications

The technologies described above have been applied to a number of nucleic acid targets in a variety of assay formats. For clinical laboratory use, Pace assay kits (Gen-Probe) are available for the direct detection of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* from urogenital swab specimens.[12,13] These assays take less than two hours to perform and can detect as few as 500 organisms.

Accuprobe kits (Gen-Probe) are available for positive identification of organisms following a culture step.[14,15] These tests take about 30 minutes to perform, and exhibit high sensitivity and specificity. Several such tests have become the gold standard for identification of such organisms as *M. tuberculosis* and *M. avium* in culture.[16—18]

In an amplified configuration, one assay now under development can detect both *Chlamydia trachomatis* and *Neisseria gonorrhoeae* in a single tube.[13] For this multiplexed assay, both organisms are coamplified in a single tube, and then each is simultaneously detected and resolved using the DKA format. To capture target rRNA from urine or urogenital swabs, this assay uses specific target capture with magnetic microspheres. Inhibitory substances, including 10% blood, have been shown to be effectively removed by the sample processing method. At present, chlamydia and gonorrhea rRNA can be detected at the level of a single cell.

Two tests that make use of transcription-mediated amplification (TMA) coupled with HPA detection are also commercially available. The Amplified Mycobacterium Tuberculosis Direct kit (Gen-Probe) for the detection of *M. tuberculosis* has been in routine use in Europe and Japan since 1993, and was approved for use in the United States in 1995.[19,20] The Amplified Chlamydia Trachomatis kit for the detection of *Chlamydia trachomatis* in male and female urogenital swabs and urine specimens from both symptomatic and asymptomatic individuals was approved in 1996.[21] Both of these assays take about three hours to complete.

Screening for HIV and HCV. Assays are under development for a number of other clinical applications. One project is a screening assay for HIV-1 and hepatitis C virus (HCV) in donated blood. This assay is being developed under a three-year contract with the National Heart, Lung and Blood Institute in response to the public pledge from the commissioner of FDA to minimize the risk of infectious disease transmission via transfusion. It is intended to detect all major subtypes of HIV-1 and HCV, including HIV-1 subtype O.

At present, the blood supply is tested for these viral pathogens using antibody-based tests that detect virus-specific
antibodies or HIV-1 p24 antigen after seroconversion. The time between the initial infection of a donor and detectable seroconversion—commonly referred to as the window of infectivity—can result in the use of donated blood that contains undetected pathogens. By using highly sensitive nucleic acid amplification—based tests, which target the virus directly and do not rely on seroconversion, it is hoped that the window of infectivity can be narrowed significantly.[22—24] The assay consists of three basic phases: capture of viral RNA from serum or plasma, amplification using TMA, and detection using HPA. Specific steps followed to perform the assay are as follows.

- Specimen (500 µl) is added to the reaction tube, and the virus is lysed with a buffer/detergent reagent.
- Viral RNA is specifically captured out of the solution onto magnetic beads. By collecting the beads magnetically, sample matrix—including potential amplification inhibitors—is washed away.
- TMA primers, an amplification reagent, and an oil reagent (which minimizes evaporation and acts as a barrier over the amplification reaction) are added, and the mixture is incubated at 60°C (10 minutes).
- Enzyme reagent is added, and the mixture is incubated at 41.5°C (60 minutes).
- Viral-specific AE-labeled probes are added and hybridization is carried out for 15 minutes at 60°C (15 minutes).
- Selection reagent is added to destroy AE associated with unhybridized probe, and the mixture is incubated at 60° C (10 minutes).

- Hybrid-associated AE is measured in a luminometer (2 seconds).

For the configuration of this assay currently under development, three targets (HIV-1 RNA, HCV RNA, and an internal control) are captured, coamplified, and detected in a single reaction tube. The internal control is added at the beginning of the assay, and a positive signal at the end of the assay verifies proper functioning of each of the assay steps. The internal control signal is discriminated from HIV-1 and HCV signals using the dual kinetic assay (DKA) format described above.

In this multiplex mode, current sensitivity is better than 200 copies/ml for both HIV-1 and HCV, with a dropout rate of less than 1% (i.e., samples that yield only background signal). This performance is better than or equal to that of any currently licensed PCR assay. All the steps of the assay are performed in a single tube with nothing removed from it after sample processing, thus reducing the risk of cross-contamination of samples.

Quantitative and Other Applications. In another application, HIV-1 in plasma is amplified using a quantitative TMA protocol, then detected and quantitated using HPA.[21] The assay can detect HIV RNA over a four-log range and can distinguish twofold differences in RNA levels in plasma samples (see Figure 5). Applications of this assay include viral load determination and therapeutic monitoring. Detection of the chimeric mRNA transcript of the BCR/abl oncogene [t (9;22)] associated with chronic myelogenous leukemia (CML) has also been demonstrated using quantitative TMA with HPA detection.

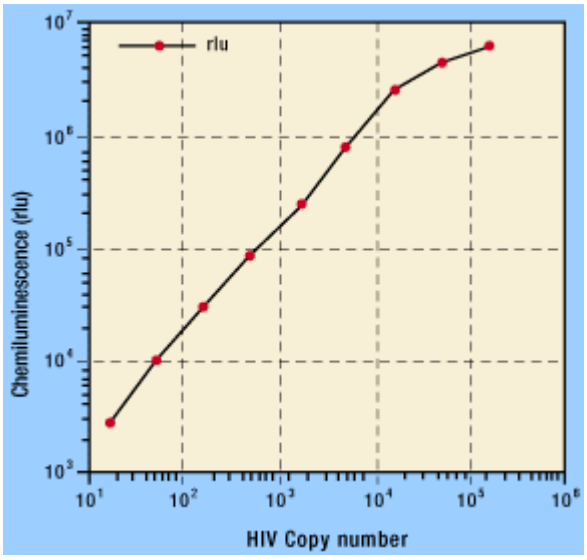

Figure 5. Quantitative TMA of HIV-1. Various amounts of HIV-1 in plasma were amplified using a quantitative TMA protocol, and amplicon was detected using HPA. The assay can distinguish twofold differences in RNA levels.

Other potential applications of the TMA and HPA technologies have also been demonstrated. In one example, the HPA mutational analysis technique was coupled with the DKA format to detect an A to G mutation in codon 181 of the reverse transcriptase coding region of HIV-1, which causes broad resistance to nonnucleoside inhibitors such as nevirapine.[25] AE probes were designed for wild-type and mutant HIV sequences, with the AE attachment site placed immediately adjacent to the mismatch site. Various concentrations of the normal and wild-type sequences were simultaneously assayed in the same reaction tube, and signals from the two analytes were resolved mathematically. Data from this assay demonstrate that the HPA/DKA mutational analysis technique can quantitatively detect both wild-type and mutant sequences in a single assay (see Table I).

Concentrations (fmol)		**Calculated Values**	
Wild-type	**Mutant**	**Wild-type**	**Mutant**
5.0	5.0	79,812	116,314
5.0	0.5	68,507	10,832
5.0	0.1	77,682	765
0.1	5.0	1010	102,308
0.1	0.5	1113	10,504
0.1	0.1	1570	1750
0.0	0.0	273	0.0

Table I. Simultaneous detection of wild-type and mutant HIV sequences. Wild-type and mutant (a single A to G base change in codon 181 of the reverse transcriptase coding region) HIV-1 sequences were simultaneously assayed using HPA mutational analysis in the DKA format. Values are expressed as relative light units.

In another example, the Delta-F-508 mutation—a three-base

deletion associated with cystic fibrosis—was detected using the HPA format (see Table II).[26] The assay showed good specificity, sensitivity, and accuracy. And TMA and HPA have also been used to perform HLA typing of the DRB, DQB, and DPB loci.[27] Genomic DNA was amplified using TMA, and a mixture of AE probes was used to accurately type each sample. HPA was performed in a 96-well microplate format, demonstrating the flexibility of the assay and the ease with which it can be automated.

	Normal Probe		Mutant Probe	
Target (fmol)	**Normal**	**Mutant**	**Normal**	**Mutant**
0.0	1450	1384	1330	1343
0.5	32,170	1284	1490	17,185
2.0	60,757	1264	4087	26,457
5.0	152,967	1215	7597	58,895
20.0	540,470	1425	2858	286,018

Table II. Detection of the Delta-F-508 cystic fibrosis mutation. Normal and mutant AE-labeled probes were constructed with the AE placed at the site of the mutation (a three-base deletion). Various concentrations of target were assayed with either the normal or mutant probes using the HPA format. Values are expressed as relative light units.

Conclusion

Nucleic acid probe technology continues to prove its value for the clinical diagnostic laboratory. The technology is centered around the exquisite process of Watson-Crick duplex formation, an extremely high affinity chemical binding reaction that is specific, reproducible, and predictable. These characteristics enable researchers to create powerful, robust DNA probe IVDs that are of high sensitivity and specificity.

Today, the future of nucleic acid probe technology for IVDs looks as bright as it ever has. But the technology is advancing daily, spurred by improvements such as ever-greater increases in binding affinity through the use of chemically modified nucleic acids. The nucleic acid—related technologies described in this article are but a few of the tools available to developers of DNA probe assays. Together with associated

test kits and instrumentation, such technologies provide a means for researchers to make breakthrough discoveries in a wide variety of fields, not the least of which are the medically important areas of mutational analysis and the detection of bacterial and viral infectious disease agents.

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