Rapid, non-isotopic DNA probe assays for the detection of infectious disease, cancer and genetic disease

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SUMMARY. We present a rapid, non-isotopic DNA probe-based diagnostic assay format for the detection of a variety of diseases. The assay format utilizes short oligonucleotide probes which are covalently labeled with the highly chemiluminescent acridinium ester. A completely homogeneous assay format referred to as the hybridization protection assay has been developed which is quantitative, simple to perform, rapid, and capable of detecting all single-base mismatches. Using chemically modified acridinium esters, multiple analytes can be simultaneously detected and quantitated in the same reaction tube. Low copy number targets are amplified using "transcription-mediated amplification", an isothermal method that rapidly amplifies RNA or DNA targets in excess of one billion-fold.

A variety of targets have been detected using the DNA probe technology described above, including bacteria, viruses, cancer and genetic disorders. A number of these assays are commercially available for use in the clinical laboratory and a fully integrated instrument system is currently under development.

INTRODUCTION

The use of nucleic acid probe-based assays in the clinical laboratory for the detection of disease has grown significantly over the last decade. The technology has advanced rapidly, resulting in higher sensitivity and specificity, improved ease of use (including automation), and decreased assay times. Diverse applications have been established, and the number of commercially available assay kits has increased.

The field, however, is still young, and many practical and useful applications remain to be developed. The breadth of potential application of nucleic acid probe technology is very wide, including bacterial and viral pathogen detection in clinical medicine, food testing, dentistry, veterinary medicine, agriculture, *etc.*, genetic disease screening and diagnosis (including cancers), mutational analysis, drug resistance, susceptibility testing, pharmacogenetic analysis, gene detection and quantification and forensic analysis. To apply nucleic acid probes successfully to such diverse fields, the technology must continue to advance and provide the tools with which to build effective and useful assays.

We will present an overview of technologies developed at Gen-Probe, and will describe some of the DNA probe tools currently available with which to build clinical assays. Examples of application of these technologies will also be given.

TECHNOLOGY OVERVIEW

The hybridization protection assay

DNA probe-based assays are centered around Watson-Crick duplex formation, a high affinity, specific chemical binding reaction that is predictable and reproducible. The hybridization protection assay utilizes short oligonucleotide probes that hybridize specifically to the nucleic acid targets of interest. These probes are covalently labeled with acridinium ester, a highly chemiluminescent molecule which is utilized as a reporter group in a number of assay systems.[1–3] The acridinium ester is attached at any position of the oligonucleotide backbone using a non-nucleotide linker arm chemistry,[4–5] as shown in Figure 1.

Chemiluminescence of acridinium esters is triggered by reaction with alkaline peroxide, yielding an electronically excited N-methyl acridone which then decays back to the ground state with the emission of light (Figure 2).[1,5,6]

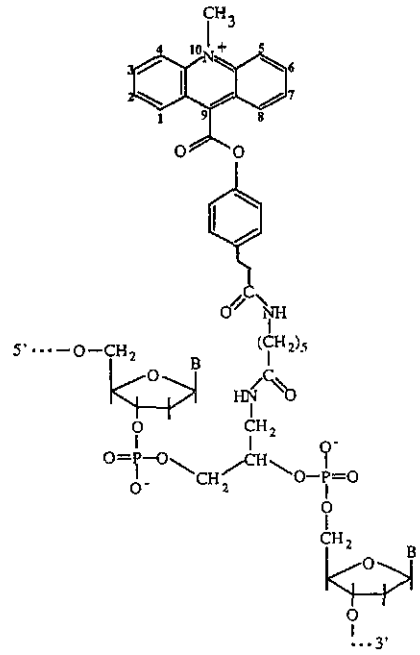

FIGURE 1. *Structure of acridinium ester attached to an oligonucleotide through a non-nucleotide linker arm. The linker arm is added to the growing oligonucleotide chain at any position using standard phosphoramidite chemistry. The acridinium ester is coupled to the linker arm post DNA synthesis, and the labeled probe is purified using high performance liquid chromatography.*

Another important reaction of acridinium esters is hydrolysis of the ester bond (Figure 2). This reaction yields N-methyl acridinium carboxylic acid, which is permanently non-chemiluminescent.[1] When acridinium ester is attached to an oligonucleotide probe, this hydrolysis reaction is rapid in mild alkaline solution. However, when the acridinium ester-labeled probe hybridizes to its perfectly complementary target, the acridinium ester is protected from hydrolysis and the rate is therefore slow (Figure 3).

This difference in acridinium ester hydrolysis rates allows discrimination between hybridized and un-hybridized acridinium ester-probe molecules with no physical separation step. The hybridization protection assay method[2] utilizes this differential hydrolysis process for the detection of target nucleic acid and consists of the

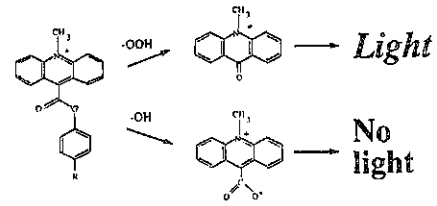

FIGURE 2. *Chemiluminescence and hydrolysis reactions of acridinium ester. Acridinium ester reacts with alkaline hydrogen peroxide to generate light. In a different reaction pathway, the phenyl ester is hydrolyzed in mild base to yield the non-chemiluminescent acridinium carboxylic acid.*

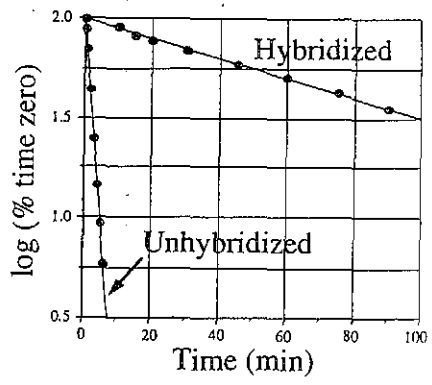

FIGURE 3. *Ester hydrolysis kinetics of hybridized and unhybridized acridinium ester-labeled oligonucleotide probe. Loss of chemiluminescence is plotted vs time for hybridized and unhybridized acridinium ester-labeled probe, demonstrating the difference in ester hydrolysis rates between the two species.*

following steps, all of which are performed in solution:

1. Addition of acridinium ester-probe to sample and hybridization (typically 15–30 minutes).
2. Addition of a mild alkaline solution and hydrolysis of acridinium ester linked to un-hybridized probe (5–10 minutes).
3. Detection of remaining acridinium ester to provide a measure of the amount of target present (2–5 seconds).

Mismatch discrimination using the hybridization protection assay

In applications such as detection of genetic mutations correlated with disease, identification

of drug-resistant pathogens and discrimination of closely related organisms, targets which differ by as little as one nucleotide must be distinguished. The hybridization protection assay format can be used to detect all single-base mismatches as well as multiple mismatches, insertions, deletions and genetic translocations. As mentioned above, in a perfectly matched duplex the acridinium ester is protected and the hydrolysis rate is slow. However, when a mismatch is present in the duplex immediately adjacent to the site of acridinium ester attachment, protection of acridinium ester is disrupted and the acridinium ester hydrolysis rate is rapid. This difference in acridinium ester hydrolysis rate between matched and mismatched duplexes allows rapid, in-solution discrimination between matched and mismatched targets.[7] Under stringent conditions, matched-to-mismatched signal-to-noise ratios of 1000:1 or greater are commonly achieved. The steps of the assay are essentially identical to those described above for the general hybridization protection assay protocol with the exception that mild alkaline treatment now hydrolyzes acridinium ester associated with mismatched duplexes as well as un-hybridized probe. A schematic diagram summarizing the hybridization protection assay format for detection of mutations is shown in Figure 4.

Multiple analyte analysis using chemically modified acridinium esters

The simultaneous detection of two or more analytes *per* reaction yields significant advantages. For example, simultaneous detection of two or more pathogens in a clinical sample increases efficiency and utility and decreases cost. Detection of one or more analytes, and an internal control in amplified tests yields a positive control of amplification for every sample. Simultaneous quantitative detection of normal and mutant sequences in genetic testing allows precise determination of the normal/mutant ratio.

The use of chemically modified acridinium ester molecules allows simultaneous detection of multiple target nucleic acids with the hybridization protection assay format. Specific chemically modified acridinium ester molecules have been developed which exhibit not only distinct chemiluminescent properties but appropriate differential hydrolysis properties as well. These labels have been attached to oligonucleotide probes and utilized in the hybridization protection assay format to detect simultaneously two or more nucleic acid targets in the same reaction tube.[8] One mode utilizes differences in the kinetics of the light emitting reaction to distinguish labels. For example, a single methyl group attached to the acridinium ring reduces the rate of light emission, whereas the addition of electron withdrawing groups to the phenyl ring increases the rate of light emission.[8–9]

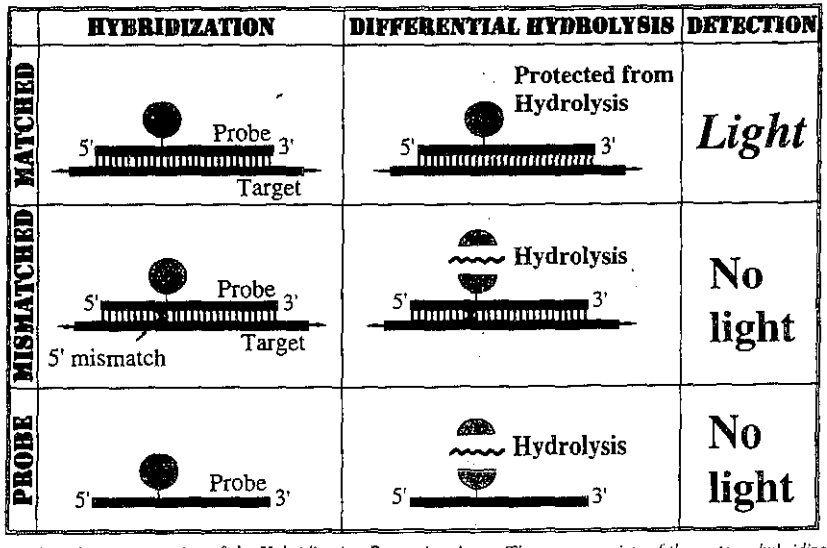

FIGURE 4. *Schematic overview of the Hybridization Protection Assay. The assay consists of three steps: hybridization (15–30 min), differential hydrolysis (5–10 min) and detection (2–5 sec).*

The emission profiles of these two labels is shown in Figure 5. In the hybridization protection assay format, oligonucleotide probes labeled with these modified acridinium esters are hybridized to their respective targets, and after the hydrolysis step, remaining chemiluminescent signal is collected over multiple time intervals and the individual signals are resolved mathematically to quantitate the amount of each target present.

This assay mode is referred to as the "Dual Kinetic Assay". Several chemically modified acridinium esters with distinct light emission 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 emission. The kinetic and pH modes can also be combined to discriminate several labels.

Amplification using transcription-mediated amplification

In some DNA probe applications, target nucleic acid is at high enough levels to be directly detected. However, in other applications target nucleic acid may be present in very small amounts, rendering it difficult or impossible to detect using direct methods. In these applications nucleic acid amplification is often employed to increase the target copy number to a detectable level.

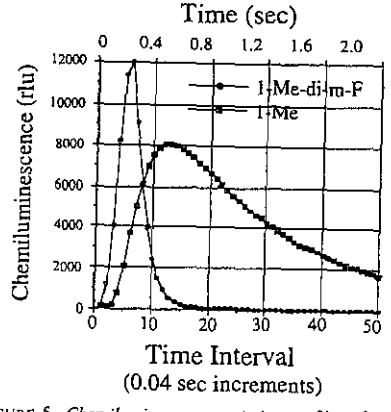

FIGURE 5. *Chemiluminescence emission profiles of two chemically modified acridinium esters. Chemiluminescence vs time is plotted for 1-methyl di-meta-fluoro acridinium (1-Me-di-m-F) and 1-methyl acridinium (1-Me) esters.*

Transcription-mediated amplification is an isothermal, autocatalytic process that amplifies RNA or DNA targets in excess of one billion-fold in less than one hour.[10–13] The steps of transcription-mediated amplification with an RNA target are shown in Figure 6.

Briefly, a primer which also contains a T7 RNA polymerase promoter region (promoter-primer) anneals to the target. Reverse transcriptase then extends this primer, producing a DNA: RNA hybrid. The RNA strand is degraded by ribonuclease H activities, and a second primer (Primer 2) anneals to the DNA strand and is extended by reverse transcriptase, yielding a double-stranded DNA template with an active T7 promoter region. T7 RNA polymerase then produces 100–1000 RNA copies of the template which are the opposite sense of the original target (RNA amplicon). Primer 2 anneals to each of these RNA copies, each is extended by reverse transcriptase resulting in DNA: RNA hybrids, and the RNA strands are degraded by ribonuclease H activities. Promoter-primer then binds to each of the DNA strands and extension with reverse transcriptase produces double-stranded DNA templates with an active T7 promoter. More RNA copies are made, and this cycle continues in an autocatalytic fashion.

Transcription-mediated amplification is rapid, simple to perform and amenable to automation. The basic steps of the assay (for which optimal temperatures vary depending on the application) are:

1. Addition of sample and amplification reagent (primers, buffers, and oil reagent which minimizes evaporation and acts as a barrier over the amplification reaction) and brief incubation at 60°C (primer annealing).
2. Addition of enzyme reagent (reverse transcriptase and T7 RNA polymerase) and incubation at 42°C for 30–60 minutes (amplification).
3. Analysis of amplicon using the hybridization protection assay format as described above (hybridization, differential hydrolysis, detection of chemiluminescence).

Transcription-mediated amplification can be used in a qualitative mode to determine rapidly the presence or absence of a target, or in a

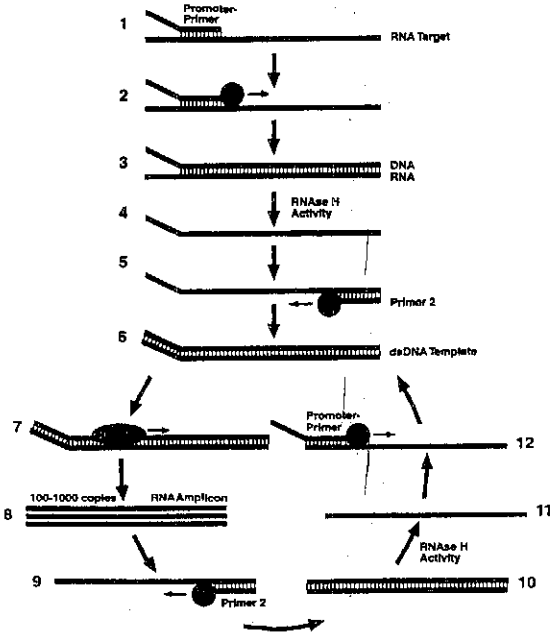

FIGURE 6. *Mechanism of Transcription-Mediated Amplification with an RNA target. [See text for details.]*

quantitative mode to determine accurately the amount of starting target over a concentration range of several orders of magnitude. Transcription-mediated amplification can be used to amplify a single target of interest, or configured in a multiplex mode to amplify simultaneously multiple targets of interest. Amplification and detection are performed entirely in solution in a single reaction tube with only a few reagent addition steps required (*i.e.*, no material is ever removed from the tube). This minimizes the risk of carry-over and cross-contamination.

APPLICATIONS

The technology described above has been applied to a number of nucleic acid targets in a variety of assay formats. Commercially available Food and Drug Administration (FDA) approved tests for use in the clinical laboratory include the PACE® assay kits[14–15] for direct detection of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* from urogenital swab specimens, ACCUPROBE® culture confirmation kits[16–17] for the positive identification of organisms following a culture step, the AMPLIFIED™ *Mycobacterium tuberculosis* Direct kit[11] for the detection of tuberculosis in sputum, and the AMPLIFIED™ *Chlamydia trachomatis* kit[12] for the detection of *Chlamydia* in urogenital/urethral swab specimens or urine (Gen-Probe Incorporated, San Diego, CA). The PACE® assays directly target rRNA in urogenital swab specimens and incorporate differential hydrolysis of acridinium ester-probes coupled with rapid separation on magnetic micro-spheres to yield low backgrounds and high sensitivity. ACCUPROBE® assays utilize a simple hybridization protection assay format to confirm the identity of organisms after a brief culturing period. These assays also directly target rRNA. The AMPLIFIED™ tests utilize transcription-mediated amplification to amplify the rRNA of the target organism and the resulting amplicon is detected using a hybridization protection assay.

The technology described here can also be used to detect cancer. In one application, the chimeric BCR/abl [t(9;22)] sequence associated with chronic myelogenous leukemia was detected using the hybridization protection assay format.[18,19] An acridinium ester-labeled probe was designed to bridge the junction of the chimeric product with the acridinium ester placed at the breakpoint. In this application, transcription-mediated amplification can be used to amplify the target if necessary.

Several applications of the transcription-mediated amplification and hybridization protection assay technologies for the detection of genetic mutations have also been demonstrated. In one example, an A to G point mutation in codon 181 of the reverse transcriptase coding region of human immunodeficiency virus (HIV-1), which causes broad resistance to non-nucleoside inhibitors such as nevirapine, was detected using the hybridization protection assay in the dual kinetic assay multiple analyte format. Acridinium ester probes were designed to be exactly complementary to the wild-type and mutant HIV sequences, with the acridinium ester attached at the site of the potential mismatch. The wild-type and mutant probes were labeled with chemically modified acridinium esters possessing rapid and slow chemiluminescence kinetics, respectively. A mixture of the probes was then used to assay simultaneously various concentrations of the wild-type and mutant sequences in the same reaction tube. The signals from the two different acridinium ester labels were resolved mathematically and the amounts of wild-type and mutant in each sample were quantitated.[8] Representative results are summarized in Table 1.

In another example, the ΔF-508 mutation, a three-base deletion associated with cystic fibrosis,[20] was detected using the hybridization protection assay format. Wild-type and mutant sequences were accurately detected (Table 2).

TABLE 1. *Simultaneous detection of wild-type and mutant HIV-1 sequences*

[Target] (fmol)		Calculated signal (rlu)	
wild-type	mutant	wild-type	mutant
0	0	273	0
0.1	5.0	1,010	102,308
0.1	0.5	1,113	10,504
0.1	0.1	1,570	1,750
5.0	5.0	79,812	116,314
5.0	0.5	68,507	10,832
5.0	0.1	77,682	765

rlu, relative light units.

In another example, HLA typing was performed using the hybridization protection assay format.[21–23] In one application, transcription-mediated amplification and hybridization protection assay were combined to perform HLA typing of the DRB, DQB and DPB loci. Genomic DNA was amplified using transcription-mediated amplification, and a mixture of acridinium ester-probes was utilized in a 96-well microtitre plate hybridization protection assay format to type each sample accurately.

A number of other applications are also under development. In one application, HIV-1 in plasma is amplified using a quantitative transcription-mediated amplification protocol, then detected and quantitated using the hybridization protection assay.[12] The assay can detect HIV RNA over a four log range and can distinguish two-fold differences in RNA levels in plasma samples. Applications of this assay include viral load determination and therapeutic monitoring.

Another project is the development of a screening assay for the detection of HIV-1 and hepatitis C virus (HCV) in donated blood. Typically, donated blood is tested for these pathogens using antibody-based tests which detect virus-specific antibodies or HIV-1 p24 antigen after seroconversion. The time between

TABLE 2. *Detection of the ΔF-508 cystic fibrosis mutation using the hybridization protection assay format.*

[Target] (fmol)	Normal target		Mutant target	
	nor probe	mut probe	nor probe	mut probe
0	1,450	1,330	1,384	1,343
0.5	32,170	1,490	1,284	17,185
2.0	60,757	4,087	1,264	26,457
5.0	152,967	7,597	1,215	58,895
20.0	540,470	2,858	1,425	286,018

initial infection of the donor and detectable seroconversion (commonly referred to as the window of infectivity) can result in units of blood containing undetected pathogen. The use of highly sensitive nucleic acid amplification-based tests, which directly target the virus and do not rely on seroconversion, decreases the amount of time between initial infection and detection, thus narrowing the window of infectivity. The assay under development consists of three basic steps:

1. Specific capture of viral RNA from serum or plasma using magnetic beads.
2. Amplification using transcription-mediated amplification.
3. Detection using the hybridization protection assay.

Three targets are captured, co-amplified and detected in a single reaction tube: HIV-1 RNA, HCV RNA and an internal control. Internal control signal is discriminated from HIV-1 and HCV signals using the dual kinetic assay format as described above. All major subtypes of HIV-1 and HCV are detected, including HIV-1 subtype O.

This assay will be automated on the TIGRIS™ instrument platform, which is also currently under development. The system will feature fully "walk-away" automation, with all steps of the assay (including sample preparation from primary specimens, amplification and detection) performed on the TIGRIS instrument. The instrument will yield a high throughput, completing 500 tests *per* 8 hours and 1000 tests *per* 12 hours. Time to first result will be 3.5 hours, and the instrument will have continuous specimen loading capabilities and bar code scanning for positive sample identification.

In another multiplex application, *Chlamydia trachomatis* and *Neisseria gonorrhoeae* are co-amplified in a single tube and then each is simultaneously detected and resolved using the dual kinetic assay format. This assay also utilizes specific target capture with magnetic microspheres, in this case to capture target rRNA from urine or urogenital swabs. This assay will also run on the TIGRIS instrument with the same throughput (500 specimens *per* 8 hours, 1000 specimens *per* 12 hours) as the blood screening assay described above. Currently *Chlamydia* and *Gonorrhoea* rRNA can be detected at the one cell level. Inhibitory substances, including 10% v/v blood, have been shown to be removed effectively by the sample processing method.

CONCLUSIONS

Nucleic acid probe technology has already been proven to be of great value in the clinical diagnostic laboratory. The technology is centered around the exquisite process of Watson-Crick duplex formation, a high affinity, specific chemical binding reaction that is predictable and reproducible. These features result in powerful, robust DNA probe diagnostic assays that are of high sensitivity and specificity. The technology is continuing to advance, as, for example, increases in binding affinity are achieved through the use of chemically modified nucleic acids. The future of nucleic acid probe technology looks as bright as it has ever been.

We have presented a number of nucleic acid-related technologies that we have used as tools to build various DNA probe assays. These assays are sensitive, specific, easy to run, rapid, robust and can be fully automated with the TIGRIS instrument system. They are useful in a number of areas, including the detection of bacterial and viral infectious disease agents, cancer and genetic diseases. These technologies and the associated test kits and instrumentation demonstrate some of the ways in which nucleic acid probes have contributed to developments in the clinical diagnostics industry.

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