

Assay Formats Involving Acridinium-Ester-Labeled DNA Probes

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We describe the development of several hybridization assay formats involving acridinium-ester-labeled DNA probes. The simplest of these is a homogeneous assay procedure that requires only three steps to complete, including a 5-s detection step. Using this format, we have detected target sequences in the 10^{-16} to 10^{-17} mol range; when rRNA is the target, this translates to 3000 to 300 bacterial organisms. The entire assay can be carried out in <30 min. This is the first homogeneous DNA probe assay to be of practical use in the clinical laboratory, and it represents a major simplification of hybridization formats. We also demonstrate the use of this homogeneous assay format to discriminate single-base differences between two closely related target sequences and to detect DNA as well as RNA target molecules. By combining homogeneous hybrid discrimination with solid-phase separation, we have been able to decrease background readings from unhybridized probe to only a few parts per million. This enhances assay sensitivity about 10-fold, to a range of 10^{-17} to 10^{-18} mol of target. We are in the process of further improving the performance of these assays.

Additional Keyphrases: *homogeneous hybridization assay · rRNA hybridization · DNA hybridization*

During the last several years much effort has been focused on simplification of DNA probe assays and development of highly sensitive nonisotopic hybridization methods (1). An ultimate goal in the simplification of such assays is the development of homogeneous assays, which require no separation steps. Several different homogeneous hybridization assays have been described, including assays based on Forster nonradiative energy transfer (2), enzyme channeling (3), and enzyme-linked double-probe systems (4). None of these assays, however, has sensitivity adequate for use in the clinical laboratory, detecting no more than about 10^{-12} mol of target. A strand-displacement-based homogeneous assay (5) reportedly detected about 2×10^{-15} mol, but only in a pure system containing no other polynucleotides, thereby rendering it of little to no use in the clinical laboratory. In addition, several homogeneous immunodiagnostic assays have been described: the Syva EMIT system (6), the Abbott TDx fluorescent polarization assays (7), and the CEDIA methodology, which is based on the reassociation of β -galactosidase subunits (8). Typically, these immunodiagnostic assays are easy to use but detect only 10^{-9} to 10^{-13} mol of analyte; as a result, they have been restricted to drug monitoring.

Chemiluminescent acridinium esters, owing to their high chemiluminescent quantum yield and rapid chemiluminescent reaction kinetics, provide the opportunity for developing highly sensitive nonisotopic DNA probe assays (9). Figure 1 shows the chemiluminescent reaction mecha-

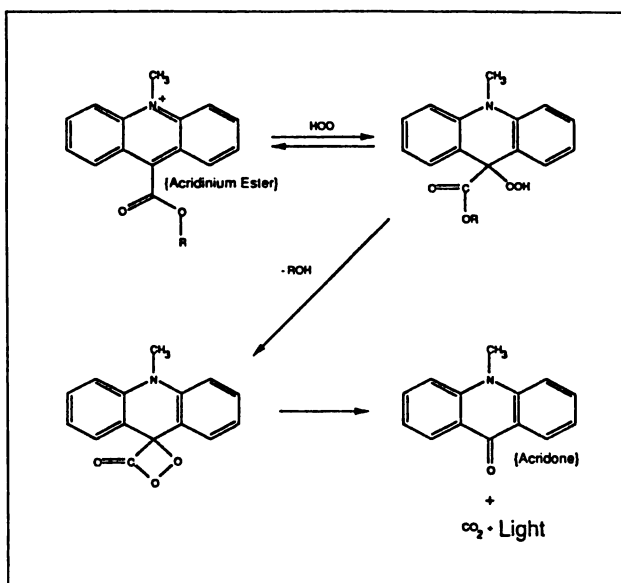


Fig. 1. Chemiluminescent reaction mechanism for methyl acridinium esters

The chemiluminescent reaction begins with attack by hydroperoxy anions on the 9 position of the acridinium ring. Under alkaline conditions a cyclooxetane ring intermediate is formed, followed by rapid conversion to an excited N-methylacridone, which emits light upon relaxation to the ground state

nism for methyl acridinium esters in the presence of base and hydrogen peroxide. Having a label that can be detected with a high degree of sensitivity, however, does not ensure that a highly sensitive assay can be developed. Also involved is the interplay between the amount of input labeled probe and the background readings, which must be kept low to fully exploit label sensitivity. Background-related limits in sensitivity are especially serious in hybridization assay formats because, to speed the rates of hybridization, the concentration of input probe may be 10^5 to 10^6 or more times greater than the amount of target one wishes to detect. Thus, unless methods are developed to reduce backgrounds into the 10^{-6} range, either the sensitivity of the assay for detecting target sequences will be limited or the hybridization reactions will have to be slowed by decreasing the amount of input probe. The effects of background are especially a problem in the development of homogeneous hybridization assays because of the difficulty in discriminating between hybrid and unhybridized probe molecules in solution.

We have overcome these difficulties by developing a method to discriminate precisely between hybridized and unhybridized acridinium-ester-labeled DNA probes without using separation steps. This method is based on selective chemical degradation of the acridinium ester label such that chemiluminescence associated with unhybridized probe is rapidly lost, whereas chemiluminescence associated with hybridized probe is minimally affected. Underlying this selective degradation process is a highly specific chemical hydrolysis reaction, controlled by the local environment of the acridinium ester. This is the first time a

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diagnostic assay has been developed that involves the selective chemical degradation of a detectable label, such degradation being dependent on the bound state of the label.

In the course of conducting these studies, we have developed chemical methods by which we can attach protected alkylamine linker-arms at any location within synthetic DNA probes. The exact nature of these linker-arms will be disclosed elsewhere. The alkylamines are then used as labeling sites for acridinium esters, as illustrated in Figure 2. Once purified, the acridinium-ester-labeled DNA probes have the same chemiluminescent activity as free acridinium ester label, with a limit of detection of about 5×10^{-19} mol. This is equivalent or superior to the sensitivity with which single ^{125}I -labeled molecules can be detected.

In addition, acridinium esters attached directly to DNA probes greatly simplify all types of assay formats, because it is not necessary to include the "capping," binding, and washing steps required when indirect labels are used. At the same time, acridinium-ester-labeled probes are fully compatible with solution hybridization methods, and also compatible with several hybrid separation supports, including those we have developed based on cationic separation (10).

Here we describe the use of a homogeneous hybridization assay format for detecting both RNA and DNA target sequences. We also demonstrate the ability of the homogeneous assay format to discriminate a single-point mismatch and to improve the performance of more-conventional separation assay formats.

Materials and Methods

Reagents

The acridinium-ester-labeling reagent was synthesized as described previously (9); magnetic microspheres from Advanced Magnetica, Inc., Cambridge, MA, were modified

by procedures described elsewhere (10); 12×75 mm polystyrene or polypropylene tubes for assays and chemiluminescence determination were from Sarstedt, Newton, NC; chemiluminescence was measured in a Leader I luminometer (Gen-Probe, Inc., San Diego, CA). All other substances were standard "ultra-pure" or reagent-grade materials.

Methods

Preparation of acridinium-ester-labeled DNA probes. Oligonucleotides were synthesized by Gen-Probe, Inc., by use of standard phosphoramidite chemistry. The chemical labeling of the DNA probes with acridinium ester was achieved by reacting alkylamine linker-arms, which were introduced during DNA synthesis, and an *N*-hydroxysuccinimide ester of a methyl acridinium phenyl ester. Once the acridinium-ester-labeled probes were purified and used in various assay formats, we detected their chemiluminescence with the Leader I luminometer as described below.

Hybridization protection assay (HPA).² Hybridization reactions were typically carried out at 60 °C in 0.1 mol/L lithium succinate buffer, pH 5.2, containing 100 g of lithium lauryl sulfate, 2 mmol of EDTA, and 2 mmol of [ethylenebis(oxyethylenitrilo)]tetraacetic acid (EGTA) per liter. Hybridization volumes ranged from 50 to 200 μL and contained from 0.05 to 0.5 pmol of probe. Differential hydrolysis was carried out at 60 °C in sodium tetraborate buffer (0.15–0.20 mol/L), containing 10–50 mL of Triton X-100 surfactant per liter, at pHs ranging from 7.0 to 8.5.

In a typical HPA format, the sample containing the target is hybridized with the DNA probe in a 100- μL volume at 60 °C. We then add 300 μL solution of tetrabo-

² Nonstandard abbreviations: HPA, hybridization protection assay; RLU, relative light units; and CML, chronic myelogenous leukemia.

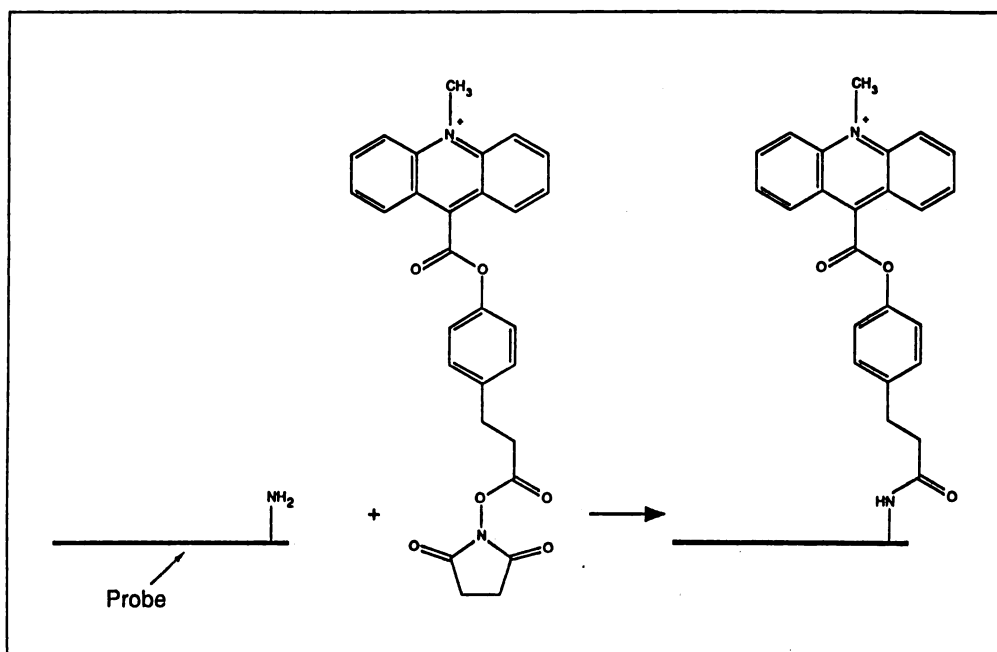


Fig. 2. Reaction of a DNA oligonucleotide probe containing an alkylamine linker-arm with the acridinium *N*-hydroxysuccinimide (NHS) ester. The NHS ester functionality of the acridinium ester with the primary amine is on a DNA probe. After purification, a stable, fully hybridizable chemiluminescent DNA probe is obtained.

rate buffer and incubate further at 60 °C. After the samples have cooled for a few minutes at room temperature, we measure the chemiluminescence in the luminometer, using one of two automated reagent-injection methods. In method 1, injection of 200 μ L of 1 mL/L H_2O_2 solution is followed, after a 1-s delay, by injection of 200 μ L of 1 to 2 mol/L NaOH reagent; the resulting chemiluminescence is integrated for 2 to 5 s. In method 2, 200 μ L of 1 mL/L H_2O_2 solution containing 1 to 2 mol of NaOH per liter is injected; the resulting chemiluminescence is integrated for 2 to 5 s. All steps in this process, including the hybridization and differential hydrolysis, are carried out in a single 12 \times 75 mm tube.

Differential hydrolysis + separation assay. In a typical differential hydrolysis plus separation assay, hybridization is carried out in 200 μ L at 60 °C, followed by the addition of 1 mL of tetraborate buffer containing 50 mL of Triton X-100 per liter and further incubated at 60 °C. We then add 1 mL of separation solution, which contains 0.4 mol/L sodium phosphate buffer, pH 6.0, and 2.5 to 5.0 mg of Gen-Probe's magnetic polycationic microspheres. After vortex-mixing the sample, we incubate it for 5 min at 60 °C. The spheres are pulled to the side of the tube with use of a Gen-Probe Pace-Mate magnetic separation rack, the supernate is decanted, and the spheres are washed one to three times, each time in 1 mL of 0.2 mol/L phosphate buffer, pH 6.0. Hybrids are then eluted from the spheres by adding 300 μ L of 0.2 mol/L phosphate buffer, pH 6.0, containing 500 g of formamide per liter, followed by vortex-mixing and incubating at 60 °C for 5 min. After pulling the spheres magnetically to the side of the tube, we transfer the supernate to a new 12 \times 75 mm tube and measure its chemiluminescence by method 1 as described above, except that injection 1 is 200 μ L of a 0.4 mol/L solution of HNO_3 containing 1 mL of concentrated H_2O_2 solution per liter.

"Separation-alone" assay. A typical separation-alone assay is run the same as the assay just described, except that the differential hydrolysis step is omitted and the separation step is performed by adding 2 mL of 0.2 mol/L phosphate buffer, pH 6.0, containing 50 mL of Triton X-100 per liter and 2 to 5 mg of the magnetic microspheres, instead of using the 1 mL of separation buffer described above.

Results and Discussion

Development of a Homogeneous Hybridization Assay

A primary goal of this research was to develop acridinium-ester-labeled DNA probe assays that would be fast, sensitive, and easy to use. It was also our goal to develop a homogeneous assay format for which backgrounds caused by unhybridized probe were sufficiently low that the assays would be useful in the clinical laboratory.

Knowing that chemiluminescence associated with methyl acridinium esters could be completely eliminated by hydrolysis of the phenyl ester, we designed an acridinium-ester-labeled DNA probe system based upon the selective protection of the acridinium ester to hydrolysis. This is done by positioning the acridinium ester in an interior portion of the probe to protect it upon hybridization. Interior placement stabilizes the acridinium ester to hydrolysis by providing it an intercalation site, once hybridization occurs, and gives it a location where hydrolysis catalyzed by the oxocyclic and cyclic amines on the nucleotide bases is minimized. In combination, these factors amplify the difference in hydrolysis rates of acridinium esters attached to hybridized as compared with unhybridized DNA probes.

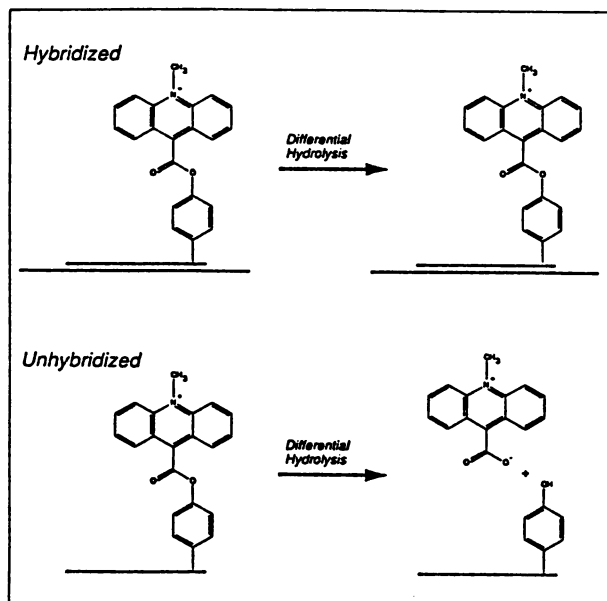


Fig. 3. Chemical basis of the hybridization protection assay (HPA). Upon hydrolysis of the phenyl ester functionality of the acridinium esters, chemiluminescence is completely abolished.

After hydrolysis, this makes it possible to use the chemiluminescence remaining as a direct measure of the amount of hybrid present. The general principle affecting this chemiluminescent loss is shown in Figure 3.

After extensive experimentation and modification we developed acridinium-ester-labeled DNA probes that show very pronounced differences in rates of hydrolysis between their hybridized and unhybridized forms (e.g., see Figure 4). These rates obey first-order chemical kinetics and thus

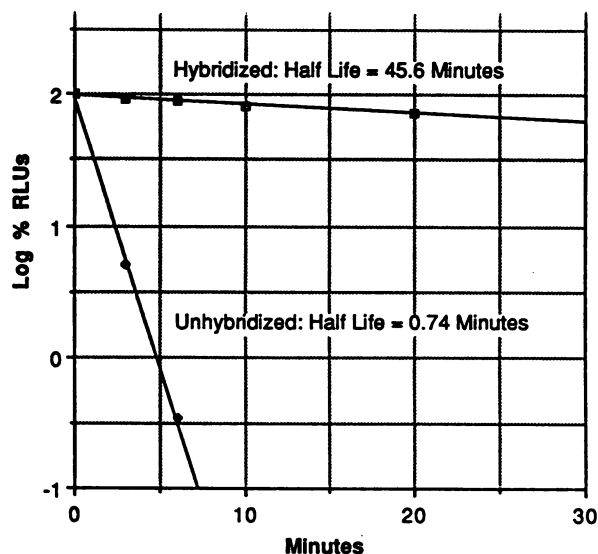


Fig. 4. Kinetic analysis of the ester hydrolysis rates of hybridized and unhybridized acridinium-ester-labeled probe.

An acridinium-ester-labeled probe (0.1 pmol) specific for *Chlamydia trachomatis* was hybridized with 1 μ g of purified target RNA, as described in Methods. Aliquots of 15 μ L containing approximately 100 000 RLU were placed in 100 μ L of 0.2 mol/L tetraborate buffer, pH 7.6, containing 50 mL of Triton X-100 per liter and incubated at 60 °C. At various times, separate aliquots in individual tubes were removed and their chemiluminescence was measured (see Methods). The resulting chemiluminescent measurements were plotted as log % of initial chemiluminescence with time. Slopes and associated half-lives were determined by standard linear-regression analysis.

give linear plots of the log of the chemiluminescence with time. In the example shown, the difference in the half-lives of the hydrolysis rates is more than 60 to 1. In practice, we have obtained hydrolysis half-life differences of about 20 to more than 200, depending on the probe construction and assay conditions.

The impact of these hydrolysis half-lives is clear when one calculates the amount of chemiluminescence that remains for the hybridized and the unhybridized forms of the probe with time.³ Consider the following expression:

$$C_0 \times (0.5)^{T/t_{1/2}} = \text{remaining chemiluminescence}$$

where C_0 is the initial chemiluminescence, T is the elapsed time, and $t_{1/2}$ is the half-life of chemiluminescence loss. With the half-lives derived in Figure 4, and a T of 15 min, the theoretical remaining chemiluminescence would be 80% for the hybrid and 0.00008% for the unhybridized probe, i.e., a million-fold (10^6) discrimination between hybridized and unhybridized probe. If T were longer, the discrimination would theoretically be even better. In practice, using this method alone, we have not yet obtained background reductions associated with unhybridized probe as low as 0.00008%. We have, however, been able to reduce such backgrounds to 0.002%. Theoretical performance has not yet been achieved, because other species present at very low concentrations contribute some chemiluminescence. We have identified these species, which are other acridinium ester compounds, and are working to eliminate their effect on the assay. Regardless, the background reductions we currently achieve using "differential hydrolysis" are excellent and give better discrimination than do most conventional separation supports.

After developing this differential hydrolysis system, one of the first things we tested was the effect of noncomplementary polynucleotide sequences on the hydrolysis rates. We added a molar excess of a nontarget *Candida albicans* rRNA sequence that is 10^4 fold higher than the concentration needed to stabilize the acridinium ester when a complementary *Chlamydia* rRNA target is used; under these conditions we saw no effect of the nontarget sequence on the rate of hydrolysis. Invariably, we have found this to be the case and, as will be shown below, the acridinium ester is even sensitive to regional instabilities near its site of attachment—so much so that it can discriminate single base changes in a target sequence.

Employing the principle of "differential hydrolysis," we have developed an assay format that we term "hybridization protection assay." After sample preparation this assay is carried out according to the following three steps:

1. **Hybridization.** Add acridinium-ester-labeled probe and incubate 5–10 min at 60 °C.

2. **Differential hydrolysis.** Add the selective chemical hydrolysis reagent to eliminate the chemiluminescence due to unhybridized probe and incubate for an additional 5 to 10 min at 60 °C.

³ The differences in half-life ratios are amplified exponentially. This means that even a ratio of 20/1 can easily give 10^6 discrimination. In this example, when the hybrid has undergone one half-life or retains 50% of its initial chemiluminescence, the unhybridized probe will have undergone 20 half-lives to produce 0.0001% of its beginning chemiluminescence. Another way to look at this effect is as the inverse or multiples of chemiluminescent loss: this becomes $2^1 = 2$ for one half-life vs $2^{20} = 1\,048\,576$ for 20 half-lives.

3. **Detection.** Detect the acridinium ester label associated with hybrid by using a luminometer that injects hydrogen peroxide and base. This takes 5 to 10 s.

Figure 5 shows the detection of a *Chlamydia* rRNA target over a concentration range of almost three orders of magnitude by use of this format. The assay is linear, because it obeys the chemical stoichiometry of solution hybridization and benefits from the broad dynamic range characteristic of luminescent detection. Thus, it gives a linear response with the amount of "target" over several orders of magnitude. In the dilution series shown in Figure 5 an extrapolated concentration of 5×10^{-6} μg of rRNA was found to give a signal that was twice that of the control (labeled probe with no target present). This concentration of rRNA is equivalent to 3×10^{-17} mol of rRNA, or about 1000 organisms, assuming 2×10^4 rRNA molecules per organism.

The HPA format is also directly applicable to the detection of organisms in clinical samples. An example is the detection of *Escherichia coli* that was "seeded" into urine, recovered by centrifugation, and detected by using the HPA format. The results of this experiment are analogous to those shown in Figure 5, except that the sensitivity of detection is about 500 colony-forming units of *E. coli* recovered from a milliliter of urine. This sensitivity is about the same as that described above for rRNA when the 30% plating efficiency we found for *E. coli* from urine is taken into consideration.

Stringency Using the HPA

We tested the ability of the HPA assay format to detect small differences between two closely related organisms by discriminating *Neisseria gonorrhoeae* from *Neisseria meningitidis*. These are two of the most difficult organisms to discriminate when using typical clinical diagnostic assays. We used an acridinium-ester-labeled DNA probe that is a perfect complement to *Neisseria gonorrhoeae* but contains two mismatches if hybridized to *N. meningitidis*. With use of the HPA format described above, discrimination between these two species is perfect. We did the experiment using

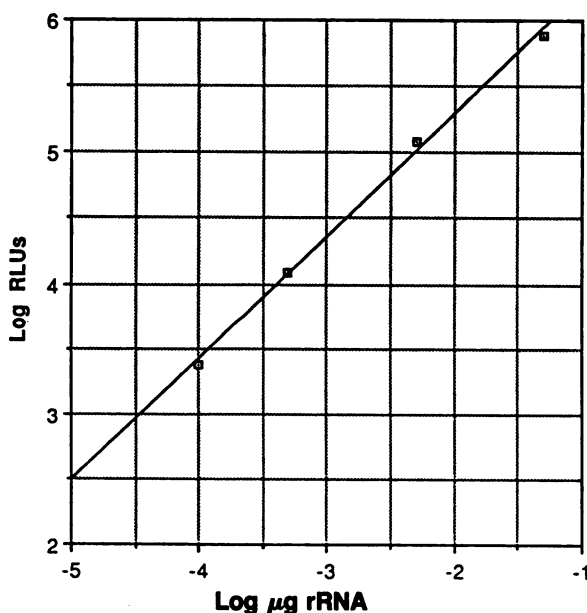


Fig. 5. Homogeneous detection of purified *Chlamydia trachomatis* rRNA by the HPA format

0.1 μ g of *N. gonorrhoeae* or *N. meningitidis* rRNA as potential targets, and a single DNA probe labeled with a single acridinium ester. The entire assay time was 15 min. Signal from *N. gonorrhoeae* was 35 000 RLUs, whereas there was no detectable signal from *N. meningitidis* within experimental error (± 100 RLUs). This shows the ability of the HPA assay format to discriminate small differences between target sequences, even when the acridinium ester is placed distal to the site of mismatches.

Further, to examine the ability of the HPA assay format to discriminate mismatches, we synthesized an acridinium-ester-labeled DNA probe 24 nucleotides in length, which is specific for *E. coli*. In this case, we placed the acridinium ester at a site within the probe that produces a single T/G mismatch, if hybridized to *Citrobacter diversus*. Figure 6 shows the differential hydrolysis half-lives, as a result of this mismatch. From this figure, it is apparent that the effect of the thymidine/deoxyguanosine mismatch is nearly equivalent to the probe being fully unhybridized. In addition, the differential hydrolysis ratio of more than 23 is sufficient to allow complete discrimination of the single mismatch nontarget and the target. We alternatively bound the RNA/DNA hybrids to hydroxyapatite instead of performing the differential hydrolysis step. This allowed us to examine the degree to which the mismatch caused a local instability as opposed to the instability of the entire duplex. As judged from binding by hydroxyapatite, between 70% and 90% of the probe was in the form of a hybrid with *Citrobacter diversus*.

This indicates that the acridinium ester can be used in an HPA format to detect unstable regions within a larger duplex. Most importantly, this experiment showed us that it is possible to detect single point mismatches by using

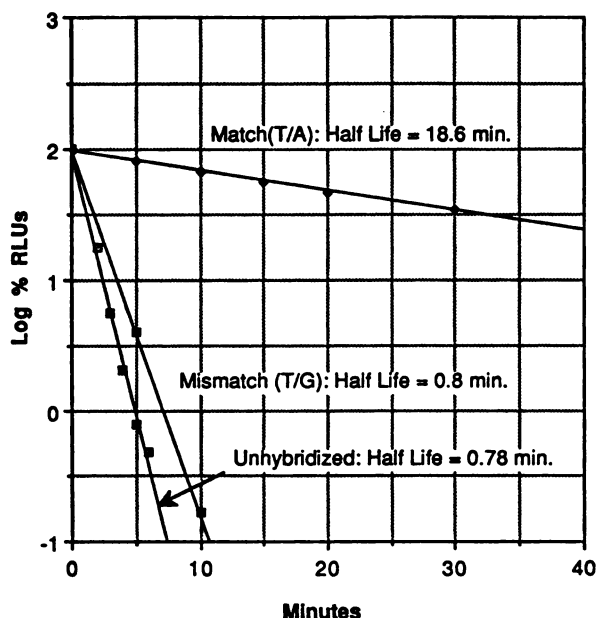


Fig. 6. Discrimination of a single site mismatch when using differential hydrolysis

An acridinium-ester-labeled probe specific for *E. coli* was hybridized with 1 μ g of rRNA from either *E. coli* or *Citrobacter diversus* (a target containing a single T/G mismatch). The kinetics of differential hydrolysis of the two resulting hybrids, as well as unhybridized probe, were determined as described in Figure 4. In this case, we positioned the acridinium ester at the site of the T/G mismatch so that we could test its ability to discriminate local changes in hybrid stability. The results show that differential hydrolysis can accurately discriminate the mismatch. Under these same conditions solid-phase separation methods bound 70–90% of the mismatch hybrid

acridinium-ester-labeled probes, engineering the probes to place the acridinium ester in the region of the mismatch. We extended these experiments to sequences associated with the *ras* oncogene mismatch at codon position 12, and we got similar results. Further studies are necessary to determine if it is possible to discriminate all or most mismatch combinations.

The HPA format gives better discrimination than hybrid separation supports, no matter where the label is attached. This improved discrimination is probably associated with the hydrolysis mechanism of the HPA format, whereby protection of the acridinium ester depends highly on maintenance of local hybrid integrity. If for some reason hybrid integrity is compromised, even transiently, in the region of the acridinium ester, hydrolysis will occur rapidly and the effect of the instability will be amplified through the loss of chemiluminescence. In this way the HPA format should be sensitive to subtle differences in hybrid stability that are not easily recognized by solid supports that bind hybrids selectively.

DNA as Target

We also explored the use of the HPA assay format to detect DNA target sequences. As an example, we chose the detection of the chromosomal translocation associated with chronic myelogenous leukemia (CML) that is commonly known as the "Philadelphia chromosome." CML results from the reciprocal translocation of human chromosomes 9 and 22 and produces a chimeric mRNA sequence that contains *abl* sequences obtained from chromosome 9 and *bcr* sequences from chromosome 22. Almost all CML patients possess one of two specific mRNA sequences (11). The most common of these two sequences is shown in Figure 7, where it is represented in its cDNA form. We designed the acridinium-ester-labeled probe shown to hybridize with the desired cDNA sequences at 60 °C while not hybridizing to either of the "normal" sequences.

An amplified double-stranded DNA product was then obtained from the breakpoint region of the chimeric mRNA associated with K562 (CML) cells. We then determined hydrolysis rates of the CML probe with amplified K562 cell cDNA (after denaturing it) and with a synthetic DNA complement. In both cases the differential hydrolysis half-life ratios were 20/1 and there was no effect of either the normal *abl* or *bcr* sequences that were run as controls. As discussed earlier, differential hydrolysis half-lives of 20/1 are fully adequate to clearly discriminate hybridized and unhybridized species. We then proceeded to use the HPA assay format to detect the cDNA as a fivefold dilution series and to compare the sensitivity of the HPA assay format with a standard Southern blot hybridization method (Figure 8). Even at the 15 625-fold dilution the HPA assay format gave a signal that was fourfold that of the signal generated by the "no target" control. In comparison, Southern blot analysis gave sensitivity to only the 125-fold dilution after a 2.5-h autoradiographic exposure. When exposed overnight the Southern blot procedure gave a faint band at the 625-fold dilution (data not shown). The Southern blot method also involved more than 10 steps and took two-and-a-half days to perform, even before starting autoradiography. In comparison, the HPA format was complete in less than 20 min (10 min for hybridization; 6 min for hydrolysis; 5 s for detection).

The duplex DNA used as target in this experiment did not give a linear response as did rRNA targets. This is

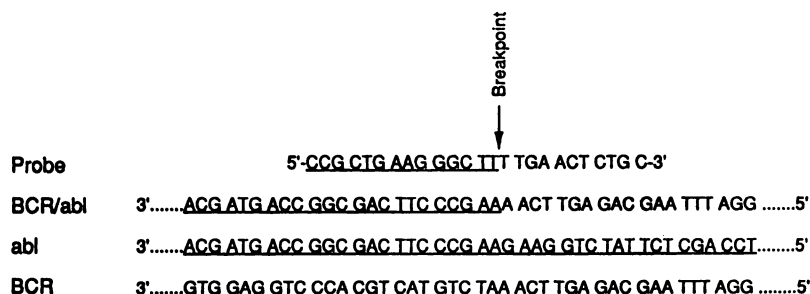


Fig. 7. Chronic myelogenous leukemia (CML) "bridging" probe sequence

Shown are the cDNA sequences corresponding to the normal *bcr* and *abl* mRNA sequences as well as the most common chimeric *bcr/abl* mRNA sequence. The *bcr/abl* chimeric sequence is the result of a translocation between chromosomes 9 and 22, and it is directly associated with the Philadelphia chromosome expressed in CML patients. The "bridging" probe was designed to span the breakpoint of the chimeric sequence, and at 60 °C the probe hybridizes only with the chimeric sequence and neither of the normal sequences

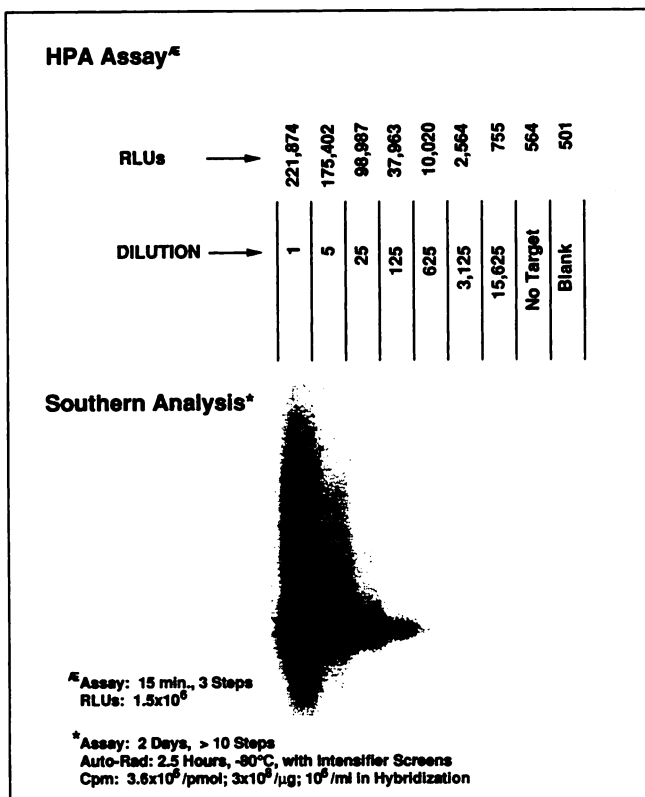


Fig. 8. Comparison of the HPA format and the standard Southern blot analysis procedures for the detection of the *bcr/abl* sequence of CML. Chimeric mRNA transcript was purified from K562 (CML) cells and amplified into a double-stranded DNA product by Mike Riggs (Gen-Probe, Inc). We assayed this DNA by using the HPA format at pH 8.5 (see *Methods*) or with the standard Southern hybridization procedure, using the same probe, 5' end-labeled with ^{32}P . The raw data for RLUs obtained from the HPA format and the autoradiogram from the Southern analysis are shown for increasing dilutions of the final amplification product. Clearly, the HPA format is far more sensitive than the Southern method, even when gels are autoradiographed for several hours

because the target DNA strand is present with its complement during the hybridization step and they have the opportunity to re-anneal, decreasing the amount of single-stranded target available for hybridization with the probe. The rate of re-annealing goes up as the concentration of target increases and causes the HPA assay to deviate from linearity. Even with this limitation, however, the HPA format gives excellent results when targeting duplex DNA in solution, as long as the target is heat-denatured before the assay is begun.

Differential Hydrolysis with Separation

For even higher sensitivity, we combined the differential hydrolysis technique with separation on solid supports. In this case, a range of separation supports were used that have cationic surfaces to separate hybridized from unhybridized probes. These supports function by binding DNA/RNA hybrids while not binding short unhybridized probes (10). One of the most useful of these supports is a 1-μm magnetic particle. Separation formats involving these supports alone give background signals owing to unhybridized probe of about 0.002–0.005%. Again, because these systems involve solution hybridization there is a good linear relation to the amount of target present (data not shown).

We developed an assay format in which the same steps are used as those outlined for the HPA format, except that hybrid is bound to magnetic microspheres after the differential hydrolysis step, followed by three wash steps, elution of the hybrid from the magnetic microspheres, and detection (see *Methods*). With this combination we have been able to decrease background signals associated with unhybridized probe an additional 10-fold or more.

A typical dilution series is shown in Figure 9 in which *Chlamydia* rRNA was used as target that was "seeded" into cervical swabs. In this experiment we achieved a signal of twice background at a concentration of *Chlamydia* rRNA of 10^{-6} μg. This is equivalent to a concentration of 6×10^{-18} mol of target rRNA or about 200 organisms (again assuming 2×10^4 rRNA molecules per organism). In this experiment, the number of input RLUs was 20×10^6 and the background signal from unhybridized probe was only 43 RLUs (0.00022%). When we ran the assay using separation alone, the background was 496 RLUs (0.0024%).

These extremely low background signals allow us to use a large proportion of the sensitivity intrinsic to chemiluminescent acridinium ester labels and to design assays with greater sensitivity. Such low background signals are perhaps more thoroughly appreciated when compared with distance measurements. If the number of input RLUs was made equivalent to 500 m, the residual background of 0.00022% due to unhybridized probe would be equivalent to only 1.1 mm.

In summary, we have successfully developed methods to label and purify synthetic oligonucleotide probes with chemiluminescent acridinium esters. Once purified, the chemiluminescent probes can be detected with sensitivities in the 5×10^{-19} mol range. To fully exploit this high sensitivity, we have designed a range of assay formats

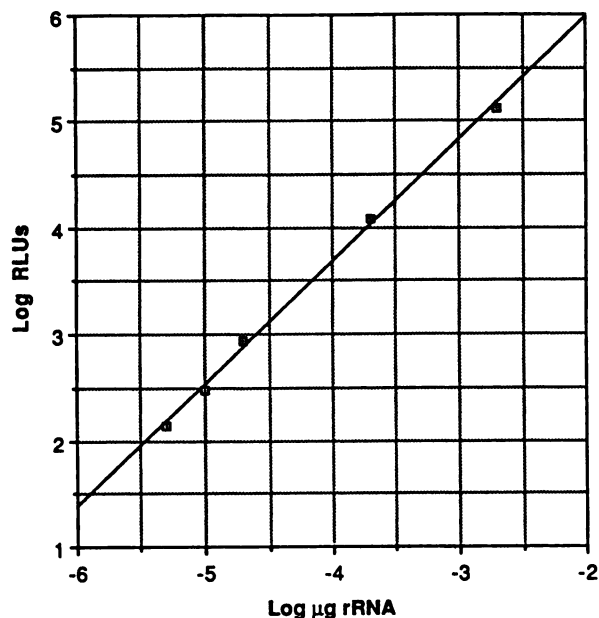


Fig. 9. Detection of *Chlamydia trachomatis* rRNA "seeded" into clinical specimen (cervical swab) with use of the differential hydrolysis + separation format

The differential hydrolysis step was performed at pH 7.6 for 10 min

engineered to minimize chemiluminescent background signals. The most novel of these assay formats is a highly sensitive homogeneous assay that we call HPA. Using the HPA format, we have been able to obtain background signals from unhybridized probe in the 0.002–0.005% range.

The HPA format can provide results in <20 min with a minimum of "hands-on" time (usually ≤ 2 min). This format now makes it possible to perform hybridization assays that are even simpler to do than most immunoassay formats. Moreover, the HPA assay detects target molecules in the 10^{-16} – 10^{-17} mol range. This means that the HPA format is on the order of 10^4 to 10^8 times more sensitive than homogeneous immunodiagnostic assays currently in use (6, 7). In addition, the sensitivity of the HPA format makes it of practical use in the clinical laboratory, especially when coupled with the detection of rRNA, which gives a 2×10^4 enhancement of sensitivity to about 10^{-20} – 10^{-21} mol for organisms that contain ribosomes (12, 13).

The HPA format also provides good stringency between closely related target sequences, and we have used it to discriminate a single T/G mismatch. At the same time, the HPA format can be combined with more-conventional hybrid separation systems to improve their performance. This reduces background signals from unhybridized probe into the 0.0002% range.

The ability to achieve the low backgrounds in both of these formats allows us to greatly speed hybridization

reactions by increasing the probe concentration. Historically, there was a dilemma, because, to increase the rates of hybridization, the amounts of unhybridized probe had to be increased to concentrations that were thousands to millions of times higher than the amount of target sequence present; however, because of an inadequate ability to achieve extremely low background signals from unhybridized probe, sensitivity was lost. Our ability to achieve extremely low backgrounds has made it practicable to increase probe concentrations to a point where solution hybridization reactions are complete in 10 min or less, as opposed to the hours that are typical of many hybridization assay formats.

The highly chemiluminescent acridinium esters have enabled us to develop DNA probe assays that are fast, sensitive, and easy to use. These types of assay formats should have a significant impact on the diagnostic detection of polynucleotide sequences. In this regard, Gen-Probe is currently developing more than 10 assays involving the HPA format.

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