27. **Meinkoth, J. and Wahl, G.,** Hybridization of nucleic acids immobilized on solid supports, *Anal. Biochem.*, 138, 267, 1984.
28. **Elfassi, E., Romet-Lemonne, J. L., Essex, M., Frances-McLane, M., and Haseltine, W. A.,** Evidence of extrachromosomal forms of hepatitis B viral DNA in a bone marrow culture obtained from a patient recently infected with hepatitis B virus, *Proc. Natl. Acad. Sci. U.S.A.*, 81, 3526, 1984.
29. **Delius, H., Gough, N. M., Cameron, C. H., and Murray, K.,** Structure of the hepatitis B virus genome, *J. Virol.*, 47, 337, 1983.
30. **Ju, L. H., Wu, P. C., Ching-lung, L., and Leong, S.,** Molecular hybridization study of plasma hepatitis B virus DNA from different carriers, *J. Infectious Dis.*, 154, 983, 1986.
31. **Ono, Y., Onda, H., Sasada, R., Igarashi, K., Sugino, Y., and Nishioka, K.,** The complete nucleotide sequences of the cloned hepatitis B virus DNA; subtype adr and adw, *Nucleic Acids Res.*, 11, 1747, 1983.
32. **Hansen, P.,** Sandwich hybridization method for nucleic acid detection, European Patent Application 139489, 1985.
33. **Hartley, J. L., Berninger, M., Jessee, J. A., Bloom, F. R., and Temple, G. F.,** Bioassay for specific DNA sequences using a nonradioactive probe, *Gene*, 49, 295, 1986.
34. **Kolberg, J. A., Besemer, D. J., Stempien, M. M., and Urdea, M. S.,** The specificity of pilin DNA sequences for the detection of pathogenic *Neisseria*, *Molecular and Cellular Probes*, 3, 59, 1989.
35. **Leary, J. J., Brigati, D. J., and Ward, D. C.,** Rapid and sensitive colorimetric method for visualizing biotin-labeled DNA probes hybridized to DNA or RNA immobilized on nitrocellulose: Bio-blots, *Proc. Natl. Acad. Sci., U.S.A.*, 80, 4045, 1983.
36. **Ruth, J. L. and Bryan, R. N.,** Chemical synthesis of modified oligonucleotides and their utility as nonradioactive hybridization probes, *Fed. Proc. Fed. Am. Soc. Exp. Biol.*, 43, 2048, 1984.
37. **Kuritza, A. P., Getty, C. E., Shaughnessy, P., Hesse, R., and Salyers, A. A.,** DNA probes for the identification of clinically important *Bacteroides* species, *J. Clin. Microbiol.*, 23, 343, 1986.
38. **Zwadyk, P., Jr., Cooksey, R. C., and Thornsberry, C.,** Commercial detection methods for biotinylated gene probes: comparison with ^{32}P-labeled DNA probes, *Curr. Microbiol.*, 14, 95, 1986.
39. **Clyne, J. M., Running, J. A., Stempien, M., Stephens, R. S., Akharan-Tafpi, H., Schaap, A. P., and Urdea, M. S.,** A rapid chemiluminescent DNA hybridization assay for the detection of *Chlamydia trachomatis*, 4, 357, 1989.
40. **Sanchez-Pescador, R., Stempien, M. M., and Urdea, M. S.,** Rapid chemiluminescent nucleic acid assay for detection of TEM-1 β-lactamase-mediated penicillin resistance in *Neisseria gonorrhoeae* and other bacteria, *J. Clin. Microbiol.*, 26, 1934, 1988.
41. **Sanchez-Pescador, R., Running, J. A., Stempien, M. S., and Urdea, M. S.,** A rapid nucleic acid assay for the detection of TetM mediated tetracycline resistant bacteria, *Antimicrob. Agents Chemother.*, 1813, 1988.
42. **Lerman, L. S.,** Introduction, in *DNA Probes: Application in Genetic and Infectious Disease and Cancer*, Lerman, L. S., Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1986, 1.

In Luminescence Immunology and Molecular Applications, van Dyke, K. & van Dyke, R. (Eds.), CRC Press, Boca Raton, FL. 1990, pp 293-309.

Chapter 19

HOMOGENEOUS AND HETEROGENEOUS CHEMILUMINESCENT DNA PROBE-BASED ASSAY FORMATS FOR THE RAPID AND SENSITIVE DETECTION OF TARGET NUCLEIC ACIDS

Norman C. Nelson, Philip W. Hammond, Wendy A. Wiese, and Lyle J. Arnold, Jr.

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We describe the development of several hybridization assay formats employing acridinium ester-labeled DNA probes. The most significant such format is a homogeneous assay procedure which requires only three steps to complete, including a 5-s detection step. Using this format we have been able to detect target sequences in the 10^{-16} to 10^{-17} mol range, which translates to 3000 to 300 bacterial organisms when using rRNA as target. At the same time, the assay can be performed in less than 30 min. This is the first homogeneous DNA probe assay to be clinically practical, and it represents a major advance in the simplification of hybridization assays. 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 reduce backgrounds due to unhybridized probe to only a few parts per million. This enhances assay sensitivity approximately tenfold to a range of 10^{-17} to 10^{-18} mol of target. We are in the process of improving the performance of these assay.

I. INTRODUCTION

Over the last several years, a major focus has been on the simplification of DNA probe assays as well as on the 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 upon Forster nonradiative energy transfer,[2] enzyme channeling,[3] and enzyme-linked double probe systems.[4] None of these assays, however, have adequate sensitivities for use in the clinical laboratory because they cannot detect more than about 10^{-12} mol of target. A strand displacement-based homogeneous assay[5] was able to achieve a sensitivity of about 2×10^{-15} mol, but this was found only in a pure system void of any other polyribonucleotides. Thus, this strand displacement assay is of little clinical use. In addition, a number of homogeneous immunodiagnostic assays have been described including 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 they have sensitivities which are limited to the detection of 10^{-9} to 10^{-13} mol of analyte. Therefore, they have been restricted primarily for use in drug monitoring where higher amounts of analyte are available.

Chemiluminescent acridinium esters allow for the development of highly sensitive nonisotopic DNA probe assays due to their high chemiluminescent quantum yield and rapid chemiluminescent reaction kinetics.[9] Figure 1 shows the reaction mechanism for methyl acridinium esters in the presence of base and hydrogen peroxide, while Figure 2 shows the detection of acridinium ester, measured in relative light units (RLU)* at decreasing concentrations. A signal of 2X over background (machine and reagent background) was obtained at 5×10^{-19} mol, which demonstrates the high sensitivity with which the acridinium ester can be detected. The specific activity of the acridinium ester was determined to be 1×10^{20} RLU/mol (which would be 1×10^{21} rlu/mol without the tenfold reduction of raw RLU performed by the instrument*). Comparatively, based on a specific activity of Bolton-Hunter reagent (single iodine) of 2200 C$_i$/mmol, 2.2×10^{12} dpm/mol,[11] and a 90% counting efficiency, the working specific activity of ^{125}I is 4×10^{18} cpm/mol. Assuming a detection limit of 10 cpm, the limit of sensitivity for ^{125}I would be 2.5×10^{-18} mol. Therefore the detection of acridinium ester is approximately fivefold more sensitive than the detection of

* Relative light units are the number of photon counts measured by the instrument in the counting period (typically less than 5 s) *divided by 10* by a built-in suppression circuit that is currently in the instrument.

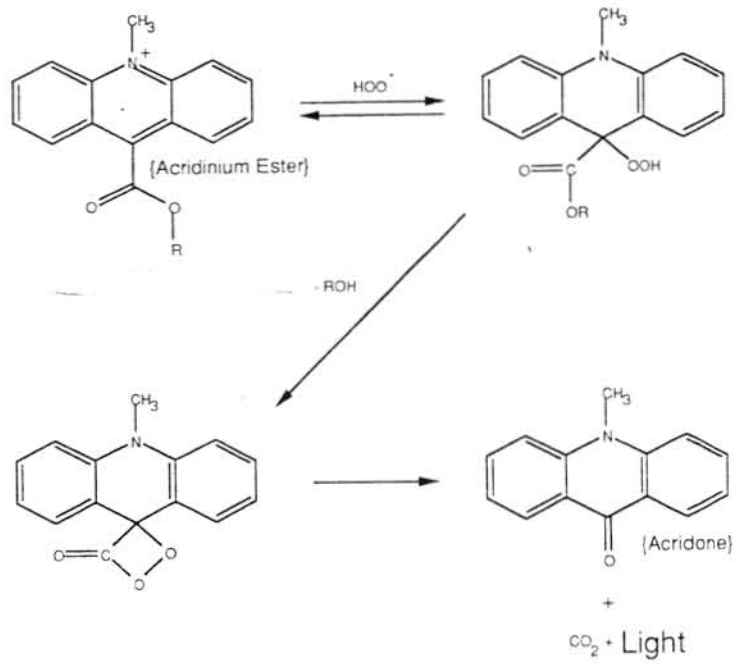

FIGURE 1. Chemiluminescent reaction mechanism for methyl acridinium esters. This figure depicts the chemiluminescent reaction pathway for methyl acridinium esters. The chemiluminescent reaction begins with attack by hydroperoxy anions on the 9 position of the acridinium ring. Under alkaline conditions a cyclooxetane ring intermediate is formed, followed by a rapid conversion to an excited N-methylacridone which emits light upon relaxation to the ground state.

^{125}I (even with the tenfold reduction circuit in the luminometer). Such sensitivity makes the acridinium ester an attractive substitute for the commonly used ^{125}I.

A label which can be detected with a high degree of sensitivity, however, does not assure that a highly sensitive assay can be developed. If background chemiluminescence cannot be reduced to the same levels as the acridinium ester detection limit, then assay sensitivity can be determined by background levels, i.e., the sensitivity of the assay will be background limited. Limits in sensitivity due to background are especially serious in hybridization assay formats because speeding the rates of hybridization causes the concentration of input probe to be one hundred thousand- to a million-fold or more higher than the amount of target one wishes to detect. This can be illustrated more clearly as follows: The rate at which hybridization between a probe and its corresponding target occurs can be described by the second order expression

$$\text{Rate} = k[\text{probe}][\text{target}] \quad (1)$$

where k is the kinetic rate constant (which is dependent on the conditions of the hybridization reaction). When the amount of probe is much greater than the amount of target, the hybridization kinetics become pseudo first order. The rate expression can then be written

$$\text{Rate} = k'[\text{target}] \quad (2)$$

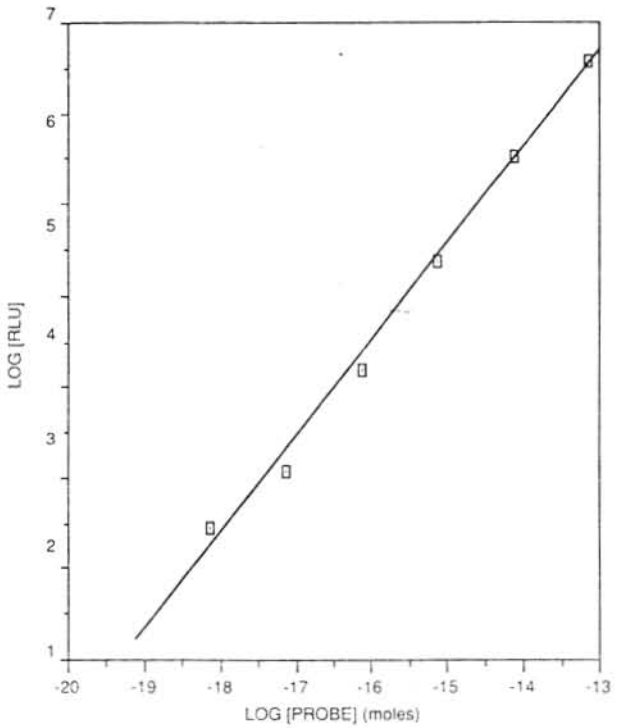

FIGURE 2. Sensitivity of detection of acridinium ester. A serial dilution of acridinium ester was measured for chemiluminescence as described in section II. The limit of detection was found to be 5×10^{-19} mol.

where $k' = k[probe]$. To determine the half-life of such a reaction, the following expression is used:

$$t_{1/2} = \ln 2/k' \quad (3)$$

Substituting 0.69 for ln 2 and k[probe] for k', and rearranging, we get

$$[probe] = 0.69/(t_{1/2} \times k)$$

Rate constants for our probes under standard conditions of hybridization are on the order of 2×10^6 l mol^{-1} s^{-1}. To achieve a hybridization half-life of 5 min (which would give approximately 94% completion in 20 min), the probe concentration would have to be

$$[probe] = 0.69/(300 \text{ sec} \times 2 \times 10^6 \text{ l mol}^{-1} \text{ s}^{-1})$$

$$[probe] = 1.15 \times 10^{-9} \text{ mol/l}$$

In a 100 µl hybridization reaction, this translates to 10^{-13} mol of probe. If the amount of target to be detected was 10^{-18} mol, probe would be 100,000-fold in excess over the target. This means that even a 1000-fold, or a 10,000-fold reduction in background would still be

10- to 100-fold higher than hybrid signal. Thus methods must be developed to reduce backgrounds into the part-per-million range (10 ppm would give background signal equal to target signal in this example). Either assay sensitivity for the detection of target sequences will be limited, or hybridization reactions will have to be slowed by reducing the amount of input probe. The background effects cause problems in the development of homogeneous hybridization assays due to an inadequate ability to discriminate between hybrid and unhybridized probe molecules in solution.

We have successfully developed a method to precisely discriminate between hybridized and unhybridized acridinium ester labeled DNA probes without using separation. This method is based on the selective chemical degradation of the acridinium ester label so that chemiluminescence associated with unhybridized probe is rapidly lost, while chemiluminescence associated with hybridized probe is minimally affected. Underlying this selective degradation process is a highly specific chemical hydrolysis reaction which is controlled by the local environment of the acridinium ester. This is the first time a diagnostic assay has been developed employing this principle.

In the course of conducting these studies we have developed chemical methods which allow us to attach protected alkylamine linker-arms at any location within synthetic DNA probes. The alkylamines are then used as labeling sites for acridinium esters as illustrated in Figure 3. Once purified, we have found that the acridinium ester-labeled DNA probes have the same chemiluminescent activity and limit of detection (5×10^{-19} mol) as free acridinium ester label. Again, this is equivalent or superior to the sensitivity with which single ^{125}I 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 employ the "capping", binding, and washing steps required when using indirect labels. At the same time, acridinium ester-labeled probes are both fully compatible with solution hybridization methods, and with a number of hybrid separation supports including those we have developed which employ cationic separation.[10]

In this study, we describe the use of a homogeneous hybridization assay format in the detection of both RNA and DNA target sequences. We also demonstrate the ability of this format to discriminate a single point mismatch and its use for improving the performance of more conventional separation assay formats.

II. MATERIALS AND METHODS

A. REAGENTS

The acridinium ester labeling reagent was synthesized as described previously;[8] magnetic microspheres were obtained from Advanced Magnetics, Inc. (MA) and modified by procedures described elsewhere;[9] 12 × 75 mm polystyrene or polypropylene tubes for assays and chemiluminescence determination were from Sarstedt (W. Germany); chemiluminesence was measured in a Gen-Probe Leader® I luminometer. All other substances were standard ultra-pure or reagent grade materials.

B. METHODS

1. Preparation of Acridinium Ester-Labeled DNA Probes

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

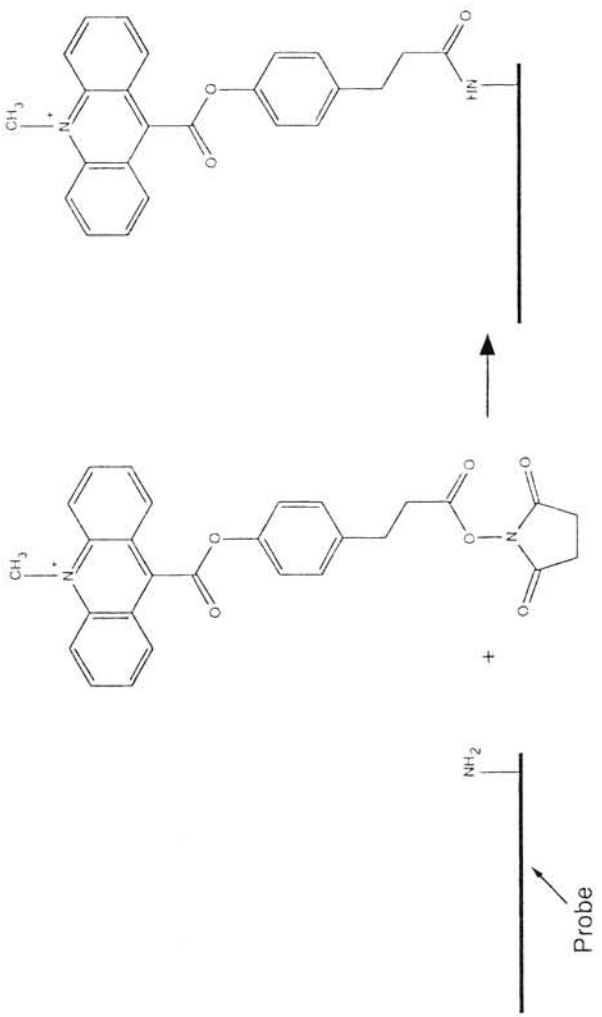

FIGURE 3. Reaction of a DNA oligonucleotide probe containing an alkylamine linker-arm with the acridinium N-hydroxy succinimide (NHS) ester. This figure shows the reaction of the NHS ester functionality of the acridinium ester with the primary amine on a DNA probe. After purification, a stable, fully hybridizable chemiluminescent DNA probe is obtained.

2. Hybridization Protection Assay (HPA)

Typically, hybridization reactions were performed in 0.1 *M* lithium succinate buffer, pH 5.2, 10% lithium laurel sulfate, 2 m*M* EDTA and 2 m*M* EGTA, at 60°C, and in volumes from 50 to 200 μl. Differential hydrolysis was performed at 60°C in sodium tetraborate buffer (0.15 to 0.20 *M*), 1 to 5% Triton X-100, at pH's ranging from 7.0 to 8.5.

In a typical HPA format, the sample containing the target was hybridized with the DNA probe in a volume of 100 μl at 60°C. Then, a 300-μl solution of tetraborate buffer (see above) was added and incubated further at 60°C. After cooling a few minutes at room temperature, chemiluminescence was measured in a Gen-Probe Leader™ I luminometer using one of two automatic reagent injection methods. *Method 1:* Injection of 200 μl of 0.1% H_2O_2; a 1-s delay; injection of 200 μl of 1 to 2 *N* NaOH; and integration of the resulting chemiluminescence for 2 to 5 s. *Method 2:* Injection of 200 μl of 0.1% H_2O_2, containing 1 to 2 *N* NaOH; and integration of the resulting chemiluminescence for 2 to 5 s. All steps in this process were carried out in a single 12- × 75-mm tube.

3. Differential Hydrolysis + Separation Assay

In a typical differential hydrolysis plus separation assay, hybridization was performed in a volume of 200 μl at 60°C. This was followed by the addition of 1 ml of tetraborate buffer containing 5% Triton® X-100 and further incubation at 60°C. A milliliter of separation solution was added which contained 0.4 *M* sodium phosphate buffer, pH 6.0, and 2.5 to 5.0 mg of Gen-Probe's magnetic polycationic microspheres. Then, the sample was vortexed and incubated for 5 min at 60°C. The spheres were pulled to the side of the tube using a Gen-Probe Pace-Mate™ magnetic separation rack, the supernatant decanted, and the spheres washed 1 to 3 times using 1 ml of 0.2 *M* phosphate buffer, pH 6.0. Hybrids were then eluted from the spheres by adding 300 μl of 0.2 *M* phosphate buffer, pH 6.0, 50% formamide, followed by vortexing and incubation at 60°C for 5 min. The spheres were magnetically pulled to the side of the tube, and the supernatant transferred to a new 12- × 75-mm tube. The samples were then read for chemiluminescence using Method 1 described above, except that injection 1 was 200 μl of 0.4 *N* HNO_3, 0.1% H_2O_2.

4. Separation Alone Assay

A typical separation alone assay was run similarly to the differential hydrolysis plus separation assay described above, except that the differential hydrolysis step was omitted and the separation step was performed by adding 2 ml of 0.2 *M* phosphate buffer, pH 6.0, 5% Triton® X-100 instead of the 1 ml of separation buffer.

III. RESULTS AND DISCUSSION

A. DEVELOPMENT OF A HOMOGENEOUS HYBRIDIZATION ASSAY

A primary goal of this research was to develop acridinium ester-labeled DNA probe assays which were fast, sensitive, and easy to use. Also we hoped to develop a homogeneous assay format which had low enough unhybridized probe backgrounds for viable use in the clinical laboratory.

With the knowledge that chemiluminescence associated with methyl acridinium esters could be completely eliminated by hydrolysis of the phenyl ester, we proceeded to design an acridinium ester-labeled DNA probe system based upon the selective protection of the acridinium ester to hydrolysis. Furthermore, we designed the system to selectively preserve chemiluminescence associated with hybrids because we wanted to use chemiluminescent output as a direct measure of target sequences. This principle is shown in Figure 4.

After extensive experimentation and modification, we succeeded in engineering acridinium ester-labeled DNA probes which show very pronounced differences in rates of

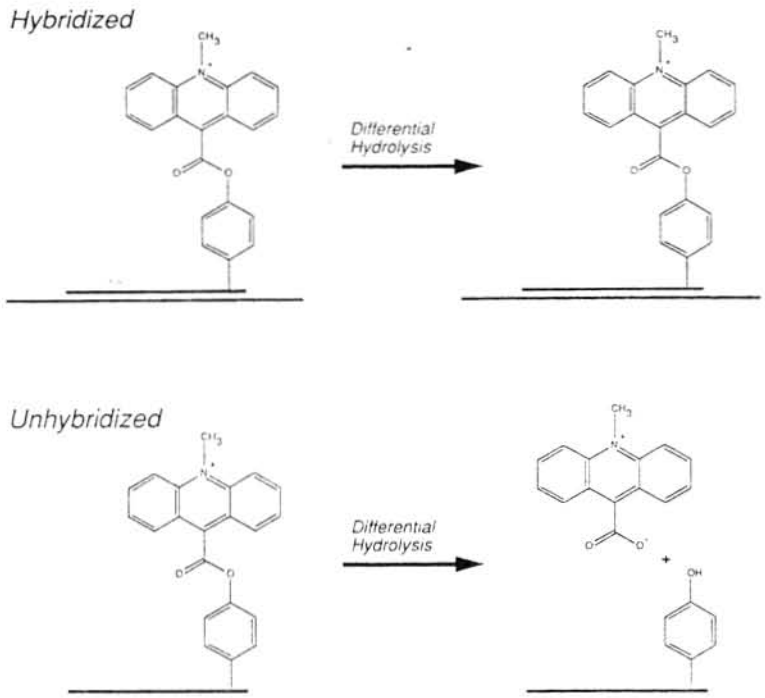

FIGURE 4. The chemical basis of the hybridization protection assay (HPA). Upon hydrolysis of the phenyl ester functionality of the acridinium esters, chemiluminescence is completely abolished. Based upon this fact, we have developed in-solution differential hydrolysis conditions (see Section II.B.) wherein the chemiluminescence associated with unhybridized DNA probe is rapidly lost, while chemiluminescence associated with hybridized DNA probe is minimally affected. This differential hydrolysis process is the basis of the homogeneous protection assay.

hydrolysis between their hybridized and unhybridized forms. An example of the differences in these hydrolysis rates is shown in Figure 5. These rates obey first order chemical kinetics and thus give linear plots of the log of the chemiluminescence with time. In the example shown, the differences in hydrolysis rates is more than 60 to 1. In practice we have obtained differential hydrolysis rates of about 20 to more than 200 depending upon the probe construction and assay conditions.

The impact of these differential hydrolysis rates is clear when one considers the amount of chemiluminescence which will remain for the hybridized and the unhybridized forms of the probe with time. This is easily illustrated using the following expression:

$$C_o \times (0.5)^{T/t_{1/2}} = \text{Remaining Chemiluminescence}$$

where C_o is the initial chemiluminescence, T is the elapsed time, and $t_{1/2}$ is the half-life of chemiluminescence loss. Using the half-lives derived in Figure 5, and a T of 15 min, the theoretical remaining chemiluminescence would be 80% for the hybrid and 0.00008% for the unhybridized probe.

This would provide a million-fold discrimination between hybridized and unhybridized probe. If T were longer, theoretical discrimination would be even better. In practice, we

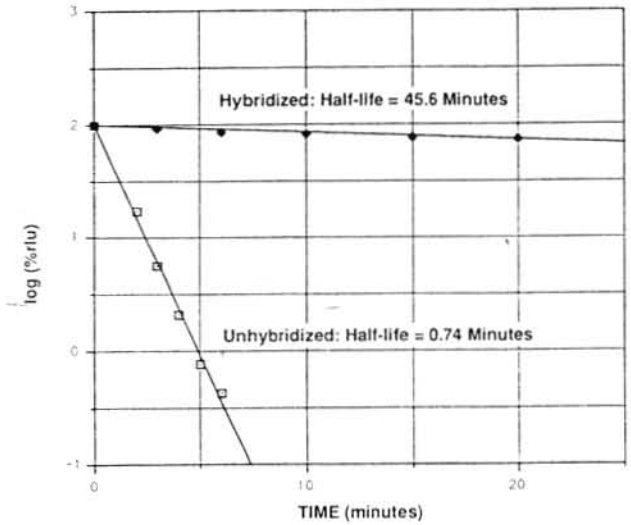

FIGURE 5. Kinetic analysis of the ester hydrolysis rates of hybridized and unhybridized acridinium ester-labeled probe. An acridinium ester-labeled probe specific for *Chlamydia trachomatis* was hybridized with 1 µg of purified target RNA as described in Section II.B. Aliquots of 15 µl containing approximately 100,000 RLU were placed in 100 µl of 0.2 *M* tetraborate, pH 7.6, 5% Triton® X-100 and incubated at 60°C. At various times separate tubes were removed and read for chemiluminescence (see Section II.B). The resulting chemiluminescent measurements were plotted as log % of initial chemiluminescence vs. time. The resulting slopes and associated half-lives were determined using standard linear regression analysis.

have not yet obtained background reductions associated with unhybridized probe to 0.00008% (0.8 ppm) using this method alone. We have, however, been able to achieve reductions to 0.002% (20 ppm). Theoretical performance has not yet been completely achieved due to other species present at very low concentrations which contribute some chemiluminescence. We have identified these species and are working to eliminate their effect on the assay. Regardless, the background reductions we currently achieve using "differential hydrolysis" is excellent and is superior to the performance of most conventional separation supports.

After developing this differential hydrolysis system, one of the first things which we tested was the effect of noncomplementary polynucleotide sequences on the hydrolysis rates. We added up to a ten thousand-fold molar excess of a *Candida albicans* nontarget rRNA sequence over a concentration known to give protection by a *Chlamydia* rRNA sequence and found no discernible protection of the acridinium ester. We have invariably found this to be the case. As will be shown below, the acridinium ester is even sensitive to regional instabilities near its site of attachment to the degree that it can discriminate single base changes in a target sequence. Employing the principle of "differential hydrolysis," we have developed an assay format which we term hybridization protection assay or HPA.

After sample preparation this assay is carried out according to the following three steps:

1. **Hybridization** — Add acridinium ester-labeled probe and incubate for 5 to 10 min at 60°C.
2. **Differential Hydrolysis** — Add the selective chemical hydrolysis reagent to eliminate the chemiluminescence due to unhybridized probe and incubate an additional 5 to 10 min at 60°C.

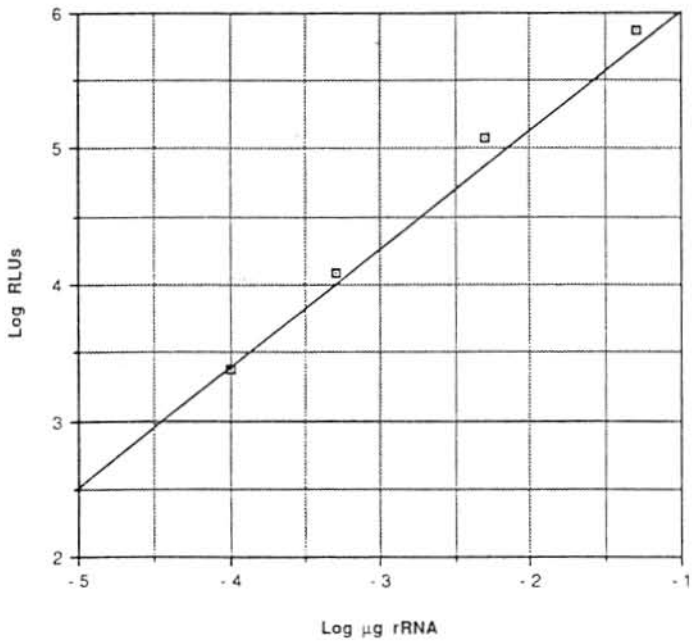

FIGURE 6. Homogeneous detection of *Chlamydia trachomatis* rRNA. Decreasing quantities of purified *C. trachomatis* rRNA were assayed using the HPA format (see Section II.B.).

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

Figure 6 shows the detection of a *Chlamydia* rRNA target over almost three orders of magnitude. An extrapolated concentration of 5×10^{-5} µg rRNA was found to give a signal which was twice control (labeled probe with no target present). This concentration of rRNA is equivalent to 3×10^{17} mol of target. This assay format obeys simple chemical stoichiometry consistent with solution hybridization and benefits from the broad dynamic range characteristic of luminescent detection, giving a linear response with the amount of "target" over several orders of magnitude. Thus, the HPA format is an accurate *quantitative* assay, which has important consequences in clinical diagnosis. For example, initial titer of disease organisms, the frequency of genetic abnormalities, and the monitoring of patient response to drug therapy are all easily and accurately achievable using the HPA format.

To be useful in the clinical laboratory, an assay format must be able to detect organisms directly out of clinical specimens. To demonstrate that the HPA format is directly applicable to the detection of organisms in clinical samples, the assay was used to detect *E. coli* cells which were "seeded" into and recovered from urine. The results were analogous to those shown in Figure 6 except that the sensitivity of detection was determined to be about 500 colony forming units (CFUs) of *E. coli* recovered from 1 ml of urine. This sensitivity is better than that described above for rRNA because there was only about a 30% plating efficiency for *E. coli* in culture. In contrast, the HPA assay detects all the organisms present regardless of viability.

B. STRINGENCY USING HPA

In order to test the ability of the HPA assay format to differentiate between two closely related organisms, we used *Neisseria gonnorhoeae* and *Neisseria menigitidis*. Using typical clinical diagnostic assays these are two of the most difficult organisms to discriminate. We employed a DNA probe, 24 nucleotides in length, containing a single acridinium ester which perfectly complemented *N. gonnorhoeae* and contained two mismatches if hybridized to *N. menigitidis*. Using the HPA format described above, we obtained perfect discrimination between these closely related species. The experiment was performed using 0.1 µg of *N. gonnorhoeae* or *N. menigitidis* rRNA as potential targets, as well as a single DNA probe labeled with a single acridinium ester. The entire assay time was 15 min. Signal from *N. gonnorhoeae* was 35,000 RLU while there was no detectable signal from *N. menigitidis* within experimental error (± 100 RLU). Thus, the ability of the HPA assay format can discriminate small differences between target sequences, even when the acridinium ester is placed distal to the site of mismatches.

C. DETECTION OF SINGLE-SITE MISMATCHES

In order to further examine the ability of the HPA assay format to discriminate mismatches, we synthesized an acridinium ester-labeled DNA probe 24 nucleotides in length which was specific for *E. coli*. In this case, we placed the acridinium ester at a site within the probe which would produce a single T/G mismatch if hybridized to *Citrobacter diversus*. The differential hydrolysis rates we observed as a result of this mismatch are shown in Figure 7. In this figure, the effect of the T/G mismatch is almost equivalent to the probe being fully unhybridized. In addition, the differential hydrolysis ratio of more than 23 is sufficient to allow for the complete discrimination of the single mismatch nontarget. To examine the degree to which the mismatch caused a local instability within the probe as opposed to the instability of the entire duplex, we alternatively bound the RNA/DNA hybrids to hydroxyapatite instead of performing the differential hydrolysis step. Under these conditions, we found that 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. Significantly, this experiment shows that it is possible to detect single-point mismatches using acridinium ester-labeled probes by engineering the probes such that the acridinium ester is placed in the region of the mismatch. We have extended these experiments to sequences associated with the *ras* oncogene mismatch at codon position 12 and found similar results. Further studies are necessary to determine if it will be possible to discriminate all or most mismatch combinations.

It should be mentioned that, regardless of the site of the label attachment, we have found that the HPA format gives better discrimination than hybrid separation supports. This improved discrimination is probably associated with the hydrolysis mechanism in the HPA format. Protection of the acridinium ester to hydrolysis is highly dependent upon the maintainence of local hybrid integrity. If the 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. Thus, the HPA format will be sensitive to subtle differences in hybrid stability which are not distinguishable by solid supports designed to bind hybrids while not binding unhybridized probes.

D. DNA AS TARGET

In addition to the RNA targets described thus far, the HPA format is also applicable to the detection of DNA targets. The procedure for DNA detection is the same as that for RNA detection, and the results are equivalent except that the differential hydrolysis rates (that is, the ratio of half-life of ester hydrolysis for hybridized probe to half-life of ester hydrolysis

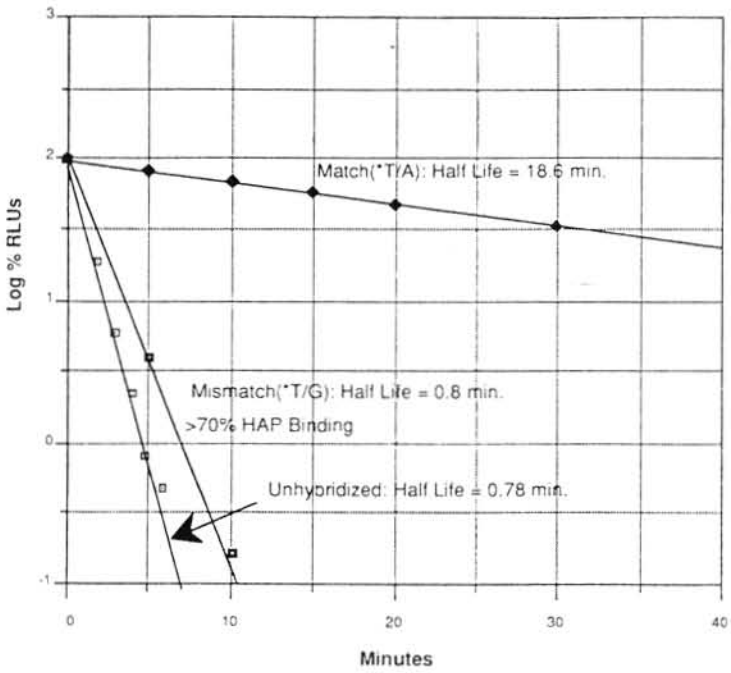

FIGURE 7. Single-site mismatch discrimination using differential hydrolysis. An acridinium ester-labeled probe specific for *E. coli* was hybridized with 1 μg of either *E. coli* RNA or *C. diversus* RNA (target containing a single T/G mismatch). The kinetics of differential hydrolysis of the two resulting hybrids, as well as unhybridized probe, were determined as described in Figure 4. In this case the acridinium ester was positioned at the site of the T/G mismatch in order for us to 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 gave poor discrimination of the mismatch. (See text for details.)

for unhybridized probe) are slightly lower (never more than twofold) in DNA:DNA hybrids than in DNA:RNA hybrids. Sources of target DNA we have evaluated include synthetic oligomers, cloned DNA, and DNA from target organisms. For example, a 28mer probe labeled with acridinium ester was hybridized with its exact synthetic DNA complement and assayed using the HPA format as described previously. The half-lives of hydrolysis were 23.7 min for hybridized probe and 0.66 min for unhybridized probe, giving a ratio of 35.9.

E. HPA FOR THE DETECTION OF PRODUCTS FROM TARGET AMPLIFICATION REACTIONS

The HPA format is also a very powerful tool for the detection of nucleic acid products from target amplification reactions. Target amplification is a recently introduced procedure for the enzymatic reproduction of target nucleic acid which produces a relatively large amount of target from a relatively small amount of target.[13-15] This methodology has wide applicability in the commercial, research, and public sectors. The nucleic acid products resulting from target amplification procedures have been detected using a wide variety of techniques.[16-22] All of these techniques, however, suffer from either lack of speed, lack of specificity, lack of sensitivity, or a combination of these factors. The HPA format, on the other hand, detects

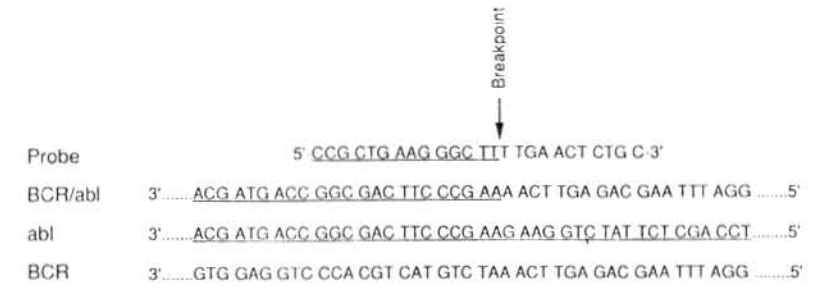

FIGURE 8. Chronic myelogenous leukemia (CML) "bridging" probe sequence. This figure shows the cDNA sequences which correspond to the normal BCR and abl mRNA sequences as well as the most common chimeric BCR/abl mRNA sequence. The BCR/abl chimeric sequence is the result of a translocation between chromosomes 9 and 22, and it is directly associated with the Philadelphia chromosome expressed in chronic myelogenous leukemia patients. The "bridging" probe was designed to span the breakpoint of the chimeric sequence, and at 60°C hybridizes only with the chimeric sequence and neither of the normal sequences.

the nucleic acid product of a target amplification reaction rapidly, specifically (via hybridization with a specific probe), and sensitively.

To show the use of the HPA format for the detection of target amplification products, we chose the detection of the chromosomal translocation associated with chronic myelogenous leukemia, commonly known as the Philadelphia Chromosome. Chronic myelogenous leukemia (CML) results from the reciprocal translocation of human chromosomes 9 and 22. As a result of this translocation, a chimeric mRNA sequence is produced which contains abl sequences obtained from chromosome 9 and BCR sequences which are obtained 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 8 represented in its cDNA form. We designed the acridinium ester-labeled probe to hybridize at 60°C to the desired chimeric cDNA target while not hybridizing to the "normal" sequences due to inadequate complementarity.

Then, we obtained a double stranded DNA product that had been amplified from the breakpoint region of the chimeric mRNA, which in turn had been obtained from K562 (CML) cells. The differential hydrolysis rates of the CML probe were determined with a synthetic DNA complement as well as with the amplified K562 cell cDNA (after it was denatured). In both cases, the differential hydrolysis ratios were 20:1 and there was no affect associated with either the normal abl or BCR sequences. A differential hydrolysis ratio of 20:1 is still fully adequate to clearly discriminate between hybrid and unhybridized forms. We proceeded to use the HPA assay format for the detection of CML sequences as a five-fold dilution series and to compare the sensitivity of the HPA assay format to that of a standard Southern hybridization procedure (Figure 9). Even at the 15,625-fold dilution the HPA assay format gave a signal which was 4 times higher than the signal generated by the No Target control. In comparison, Southern analysis gave sensitivity down to only the 125-fold dilution after a 2.5-h autoradiographic exposure. After an overnight exposure, the Southern procedure gave a faint band at the 625-fold dilution (data not shown). In addition, Southern analysis required more than ten steps to perform and required $2^1/_2$ days, even before autoradiography was initiated. In comparison, the HPA format was complete in less than 20 min (a 10-min hybridization, a 6-min differential hydrolysis, and a 5-s detection).

Importantly, the signal generated by the HPA format was not linear as we had seen when using rRNA as a target. This is because the target DNA strand and its complement are present in solution during hybridization. Consequently, the strands partially reanneal and reduce the amount of single stranded target available for hybridization. This process accel-

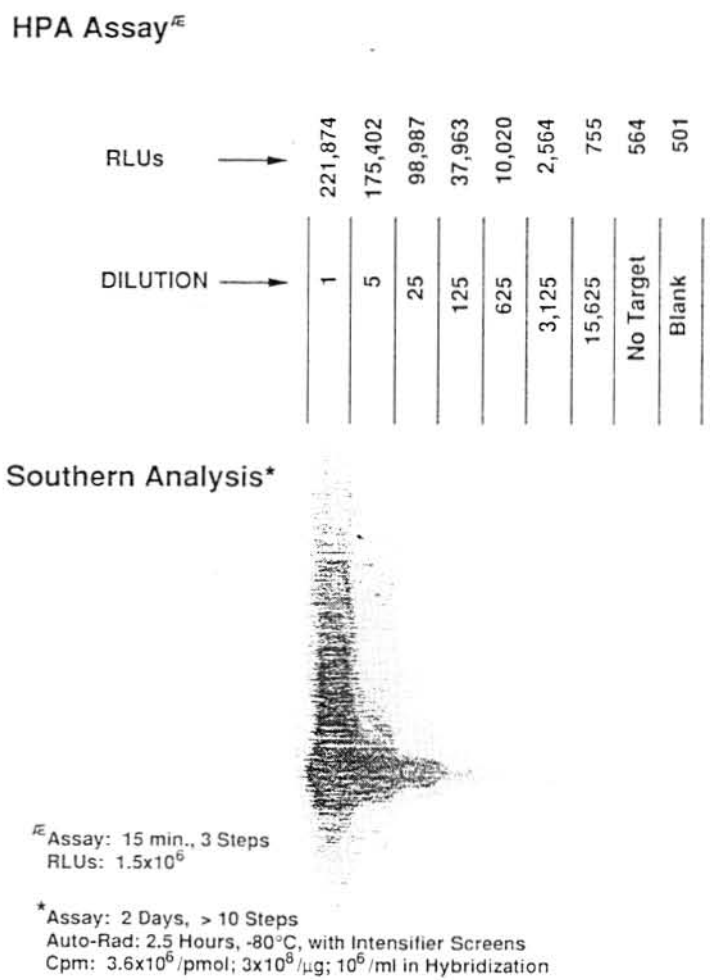

FIGURE 9. Comparison of the HPA format and the standard Southern analysis procedures for the detection of the BCR/abl sequence of CML. Chimeric mRNA transcript was purified from K562 (CML) cells and amplified into a double stranded DNA product by Mike Riggs, Gen-Probe, Inc., San Diego, CA. This DNA was assayed using the HPA format at pH 8.5 (see Section II.B.2) or with the standard Southern hybridization procedure. The raw RLU (relative light units) obtained from the HPA format as well as the autoradiogram from the Southern analysis are shown for increasing dilutions of the final amplification product. These results demonstrate that the HPA format is far more sensitive than the Southern method even when gels are autoradiographed for several hours.

erates as the concentration of target sequences increases and causes the HPA assay results to deviate from linearity. Even with this limitation the HPA assay format still gives excellent results when targeting duplex DNA in solution as long as the target is heat denatured prior to initiating the assay.

F. DIFFERENTIAL HYDROLYSIS WITH SEPARATION

In cases where even higher degrees of sensitivity are desired we have combined the differential hydrolysis technique with separation on solid supports. In this regard we have

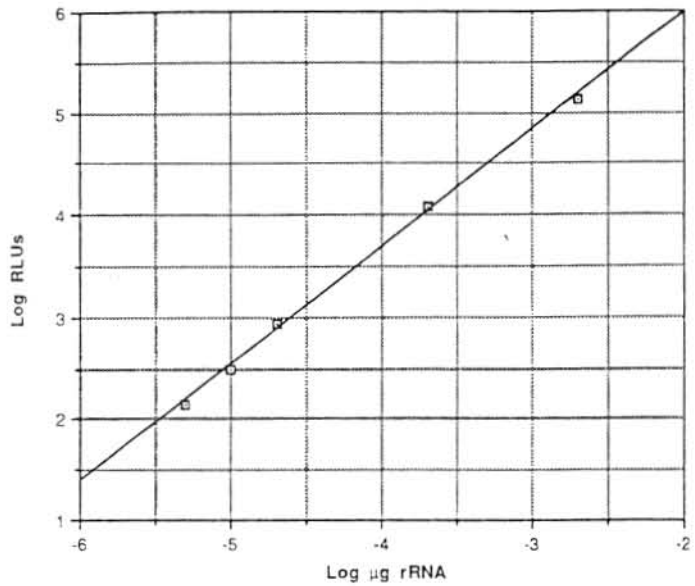

FIGURE 10. Detection of *Chlamydia trachomatis* rRNA "seeded" into clinical specimen using the differential hydrolysis + separation format. Purified *C. trachomatis* rRNA was seeded into cervical swab material and then assayed using the differential hydrolysis + separation format as described in Section II.B.3. The differential hydrolysis step was performed at pH 7.6 for 10 min.

developed a range of separation supports which employ 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-µ magnetic particle. Separation formats using these supports alone give backgrounds due to unhybridized probe of about 0.002 to 0.005% (20 to 50 ppm). Again, these systems give good linearity with the amount of target present (data not shown) because they employ solution hybridization.

We have developed an assay format which uses the same steps as those outlined for the HPA format except that the 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 Section II.B, Methods.) In this manner, we have been able to reduce backgrounds due to unhybridized probe an additional tenfold or more.

A typical dilution series is shown in Figure 10 using *Chlamydia* rRNA as target which had been "seeded" into cervical swabs. In this experiment we achieved a signal of twice background at a concentration of *Chlamydia* rRNA of 10^{-5} µg. This is equivalent to a concentration of 6×10^{-18} mol of target rRNA. In this experiment the number of input relative light units was 20×10^6 and the background due to unhybridized probe was only 43 RLU (2.2 ppm). When the assay was run using separation alone, the background was 496 RLU (24.8 ppm).

Backgrounds in the ppm range are unprecedented in simple diagnostic assay formats and allow us to use a large proportion of the sensitivity intrinsic to chemiluminescent acridinium ester labels. These low background levels are perhaps more thoroughly appreciated when compared to linear measurements. If the number of input RLU was made equivalent to 500 m, the residual background 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 directly attached chemiluminescent acridinium esters. Once purified these chemiluminescent probes, labeled with a single acridinium ester per probe, can be detected with sensitivities in the 5×10^{-19} mol range, which is approximately 5 times more sensitive than ^{125}I under similar conditions. With the photon count reduction circuit removed from the luminometer, acridinium ester detection would become tenfold more sensitive, and detection of acridinium ester-labeled probes has the potential to become even more sensitive if multiple acridinium esters are attached to each probe. In order to exploit this high degree of sensitivity we have designed a range of assay formats engineered to reduce chemiluminescent backgrounds to very low levels. The most novel of these assay formats is a highly sensitive homogeneous assay which we have termed HPA for hybridization protection assay. Employing this assay we have been able to obtain backgrounds due to unhybridized probe in the 0.002 to 0.005% (20 to 50 ppm) range.

The HPA format can provide results in less than 20 min with a minimum of "hands-on" time (usually $\leq$ 2 min). Such a format now makes it possible to perform hybridization assays which are even simpler than most immunoassay formats. Moreover, the HPA assay can be used to detect target molecules in the 10^{-16} to 10^{-17} mol range. This means that the HPA format is on the order of 10^4 to 10^5 times more sensitive than homogeneous immunodiagnostic assays currently in use. The high level of sensitivity makes the HPA format practical in the clinical laboratory, especially when coupled with the detection of rRNA which gives a 2×10^4 enhancement of sensitivity for organisms which contain ribosomes. Furthermore, the excellent linearity of the assay yields an accurate *quantitative* assay, which has beneficial implications in the field of diagnostic medicine.

Another useful application of the HPA format is the detection of target amplification products. Once target amplification has been performed, the nucleic acids produced can be specifically and sensitively detected within about 20 min with a minimum of effort by the HPA format. This combination of speed, ease of use, specificity and sensitivity render the HPA format a valuable component both commercially and noncommercially, in protocols involving target amplification.

The HPA format provides good stringency between closely related target sequences. It has also been useful in discriminating a single T/G mismatch. At the same time the HPA format can be combined with more conventional hybrid separation systems in order to improve their performance. This results in background reductions into the ppm range.

The ability to achieve the low backgrounds in both of these formats has allowed us to greatly shorten the time typically required for hybridization reactions. Historically, one of the dilemmas of hybridization assays was that in order to increase the rates of hybridization the amounts of unhybridized probe had to be increased to concentrations which were thousands to millions of times higher than the amount of target sequence. However because of an inadequate ability to achieve extremely low backgrounds, probe concentrations had to remain low, which resulted in long hybridization times. In contrast, the ability to achieve extremely low backgrounds has made it practical to increase probe concentrations to a point where solution hybridization reactions are complete in 10 min or less as opposed to hours as employed in many hybridization assay formats.

The highly chemiluminescent acridinium esters have enabled us to develop DNA probe assays which are fast, sensitive, and easy to use. Such assays will have a significant impact in the use of a wide range of DNA hybridization reactions.

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REFERENCES

1. **Matthews, J. A. and Kricka, L. J.**, *Anal. Biochem.*, 169, 1, 1988.
2. **Heller, M. J. and Morrison, L. E.**, in *Rapid Detection and Identification of Infectious Agents*, Kingsbury, D. T. and Falkow, S., Eds., Academic Press, New York, 1985, 245.
3. **Albarella, J. P., Anderson-DeRiemer, L. H., and Carrico, R. J.**, European Patent Application 146 039, 1985.
4. **Miller, J.**, U.S. Patent Number 4,670,379, 1984.
5. **Vary, C. P. H.**, *Nucleic Acids Res.*, 15, 6883, 1987.
6. **Litman, D. J., Harel, Z., and Ullman, E. F.**, U.S. Patent Number 4,318,707, 1982.
7. U.S. Patent Number 4,688,640.
8. **Henderson, D. R., Friedman, S. B., Harris, J. D., Manning, W. B., and Zoccoli, M. A.**, *Clin. Chem.*, 32, 1637, 1986.
9. **Weeks, I., Beheshti, I., McCapra, F., Campbell, A. K., and Woodhead, J. S.**, *Clin. Chem.*, 29, 1474, 1983.
10. **Arnold, L. J., Jr., Nelson, N. C., Reynolds, M. A., and Waldrop, A. A., III**, European Patent Application 88 301 839.2, 1988.
11. **Seevers, R. H. and Counsell, R. E.**, *Chem. Rev.*, 82, 575, 1982.
12. **Adams, J. M.**, *Nature*, 315, 542, 1985.
13. **Mullis, K. B. and Faloona, F. A.**, *Methods Enzymol.*, 155, 335, 1987.
14. **Kwoh, D. Y., Davis, G. R., Whitfield, K. M., Chappelle, H. L., DiMichele, L. J., and Gingeras, T. R.**, *Proc. Natl. Acad. Sci., U.S.A.*, 86, 1173, 1989.
15. **Chu, B. C. F., Kramer, F. R., and Orgel, L. E.**, *Nucleic Acids Res.*, 14, 5591, 1986.
16. **Nguyen, T. D.**, *BioTechniques*, 7, 238, 1989.
17. **Gyllensten, U. B. and Erlich, H. A.** *Proc. Natl. Acad. Sci., U.S.A.*, 85, 7652, 1988.
18. **Bugawan, T. L., Saiki, R. K., Levenson, C. H., Watson, R. M., and Erlich, H. A.**, *Bio Technology*, 6, 943, 1988.
19. **Kemp, D. J., Smith, D. B., Foote, S. M., Samaras, N., and Peterson, G.**, *Proc. Natl. Acad. Sci., U.S.A.*, 86, 2423, 1989.
20. **Crescenzi, M., Seto, M., Herzig, G. P., Weiss, P. D., Griffith, R. C., and Korsmeyer, S. J.**, *Proc. Natl. Acad. Sci., U.S.A.*, 85, 4869, 1988.
21. **Gorman, K. B. and Steinberg, R. A.**, *BioTechniques*, 7, 326, 1989.
22. **Sheffield, U. C., Cox, D. R., Lerman, L. S., and Myers, R. M.**, *Proc. Natl. Acad. Sci. U.S.A.*, 86, 232, 1989.