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I. INTRODUCTION

A. Background

Chemiluminescence—the generation of light via a chemical reaction—has gained widespread use as a detection mode over the last several years (Weeks *et al.*, 1986; McCapra and Beheshti, 1985; Hastings, 1987). Advantages of chemiluminescent labels include high sensitivity; ease of use, handling, and disposal; precise control of detection (the reaction chemistry is typically very simple yet very specific); a wide range of detection methods (from photographic film to sophisticated instrumentation); and long shelf-lives. Chemiluminescence detection has been utilized in a variety of formats, including immunodiagnostics (Berry *et al.*, 1988; Bronstein *et al.*, 1989; Weeks *et al.*, 1983b) and DNA probe-based assays (Arnold *et al.*, 1989; Bronstein *et al.*, 1990; Daly *et al.*, 1991; Dhingra *et al.*, 1991; Granato and Franz, 1989; Granato and Franz, 1990; Harper and Johnson, 1990; Heiter and Bourbeau 1993; Jonas *et al.*, 1993; Lewis *et al.*, 1990; Littig and Nieman, 1992; Ou *et al.*, 1990; Sanchez-Pescador *et al.*, 1988; Schaap *et al.*, 1989; Stockman *et al.*, 1993; Tenover *et al.*, 1990; Urdea *et al.*, 1988; Warren *et al.*, 1992).

Recently the acridinium ester (AE) depicted in Fig. 1 was developed for use as a chemiluminescent label in bioassays (Weeks *et al.*, 1983a). The AE molecule reacts rapidly (typically 1 to 5 sec) with hydrogen peroxide under alkaline conditions (Fig. 2) to produce light at 430 nm (McCapra, 1970, 1973; McCapra and Perring, 1985). These rapid reaction kinetics permit detection over a very short time frame, thereby minimizing background noise and improving overall sensitivity. Detection in a standard luminometer (see Section II,B) exhibits a linear response over an AE concentration range of more than 4 orders of magnitude, with a detection limit of approximately 5×10^{-19} mole (Arnold *et al.*, 1989).

Some other important reactions of the AE molecule are shown in Fig. 3. Hydroxide ion can catalyze the hydrolysis of the ester bond, rendering the AE permanently nonchemiluminescent. This property is utilized in an AE assay format, as described in Section I,B. Under the proper conditions hydroxide ion can also react with the carbon in the acridinium ring (C-9) that reacts with hydrogen peroxide (see Fig. 3), resulting in what is referred to as the *carbinol* or *pseudo-base* form of the AE (Weeks *et al.*, 1983a). This form is nonchemiluminescent since the hydroxide ion blocks reaction with peroxide. This inhibition can be easily reversed by incubation in acid, which rapidly reverts the hydroxic adduct, restoring the AE to its chemiluminescent form. Other nucleophiles can form adducts with the AE (Hammond *et al.*, 1991), and they also block chemiluminescence in

a reversible manner. Adduct formation can also protect the AE against ester hydrolysis under certain conditions (Hammond *et al.*, 1991).

The AE reagent shown in Fig. 1 can be covalently attached to primary amine-containing compounds via specific reaction of the *N*-hydroxysuccinimide (NHS) ester of the AE with the primary amine of the compound. DNA probes can therefore be directly labeled with AE by first incorporating a primary amine into the DNA, then reacting this amine with the NHS-AE (see Section III,A). Direct labels of this kind are simpler to use than indirect labels (those attached through an avidin/biotin interaction, for example), since the *capping*, binding of the label, washing, and substrate addition steps associated with indirect labels are unnecessary.

B. Principle of the Method

First, a chemiluminescent AE is attached directly to a synthetic oligonucleotide probe complementary to the target nucleic acid. An AE-labeled probe (AE-probe) displays the same chemiluminescence characteristics

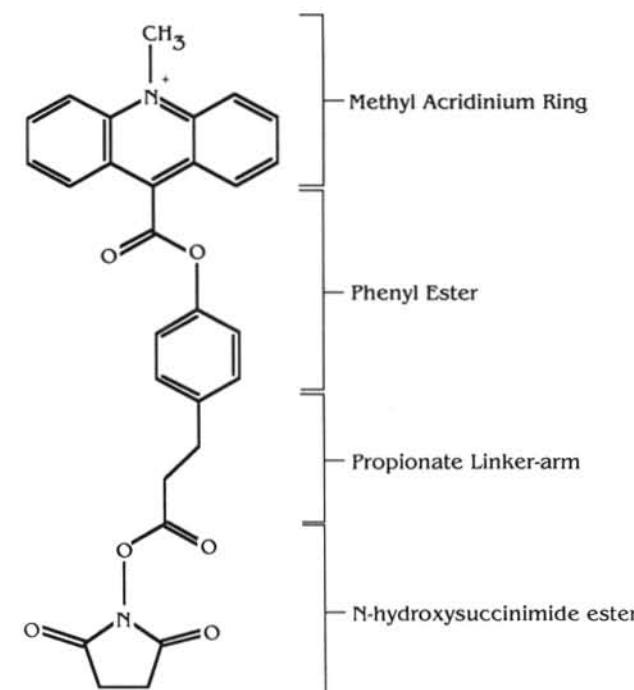


Fig. 1—The structure of the *N*-hydroxysuccinimide ester of the *N*-methyl acridinium ester.

(reaction kinetics, specific activity, and sensitivity of detection) as the free label, demonstrating that the detection properties of the label are not compromised by attachment to oligonucleotides. This is in part owing to the cleavage of the light-emitting species (namely, the acridinium ring) from the oligonucleotide during the chemiluminescence reaction (see Fig. 2), thus minimizing intramolecular quenching (Weeks *et al.*, 1983a). Furthermore, an AE-probe displays hybridization characteristics (rate and extent of hybridization, thermal stability, and specificity) essentially equivalent to its ^{32}P counterpart, demonstrating that attachment of the AE label also does not compromise hybridization performance of the probe.

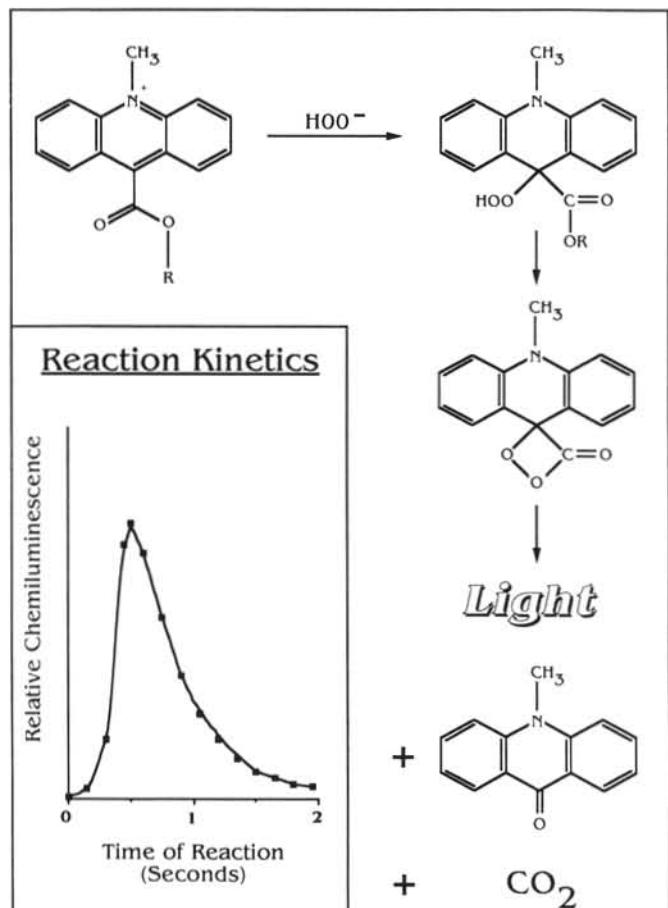


Fig. 2—The *N*-methyl acridinium ester reacts with the hydroperoxy anion (HOO^-) through the pathway shown above to produce light. The inset depicts the rapid kinetics of this reaction. The NHS phenyl ester (see Fig. 1) is represented as R.

The AE-probe can then be used in a variety of simple and rapid assay formats to directly detect target nucleic acid. All these formats utilize in-solution hybridization, which offers significant advantages (see Section I,C) over the more common solid-phase hybridization techniques (Keller and Manak, 1989). Various methods of separating hybridized from unhybridized AE-probe after the in-solution hybridization step have also been developed. In one approach (Arnold *et al.*, 1989), cationic submicron-sized magnetic microspheres are used to specifically capture the hybridized AE-probe from solution. After the spheres are magnetically collected on the side of the tube, the solution, which contains unhybridized AE-probe, is removed, the spheres are washed, and the chemiluminescence associated with hybrid is detected.

In another approach, discrimination between hybridized and unhybridized AE-probe is performed completely in-solution, thus eliminating the need for any physical separation steps. This method is based on chemical hydrolysis of the ester bond of the AE molecule, cleavage of which renders the AE permanently nonchemiluminescent (see Section I,A and Fig. 3). Conditions have been developed under which the hydrolysis of this ester bond is rapid for unhybridized AE-probe but slow for hybridized AE-probe. Therefore, after hybridization of the AE-probe with its target nucleic acid (under conditions that do not promote ester hydrolysis), the reaction conditions are adjusted so that the chemiluminescence associated with unhybridized AE-probe is rapidly reduced to low levels, whereas the chemiluminescence associated with hybridized AE-probe is minimally affected. Following this *differential hydrolysis* process, any remaining chemiluminescence is a direct measure of the amount of target nucleic acid present.

An example of this differential hydrolysis process is shown in Fig. 4, which depicts the loss of chemiluminescence due to ester hydrolysis as a function of time. From linear regression analysis, hydrolysis half-lives were determined to be 50.5 and 0.72 min for hybridized and unhybridized AE-probe, respectively. The theoretical percentage of remaining chemiluminescent label after a given hydrolysis time can be calculated using the equation

$$(0.5)^n \times 100 = \text{percentage remaining chemiluminescence} \quad (1)$$

where n is the ratio of elapsed time to the half-life of chemiluminescence loss (i.e., how many half-lives have transpired during a given incubation time). Using the half-life values given above, the calculated values for percentage remaining chemiluminescence after 15-min differential hydrolysis step would be 81% for hybridized probe and 0.00005% for unhybridized probe. This represents greater than a one million-fold discrimination between hybridized and unhybridized AE-probe, achieved in 15 min with

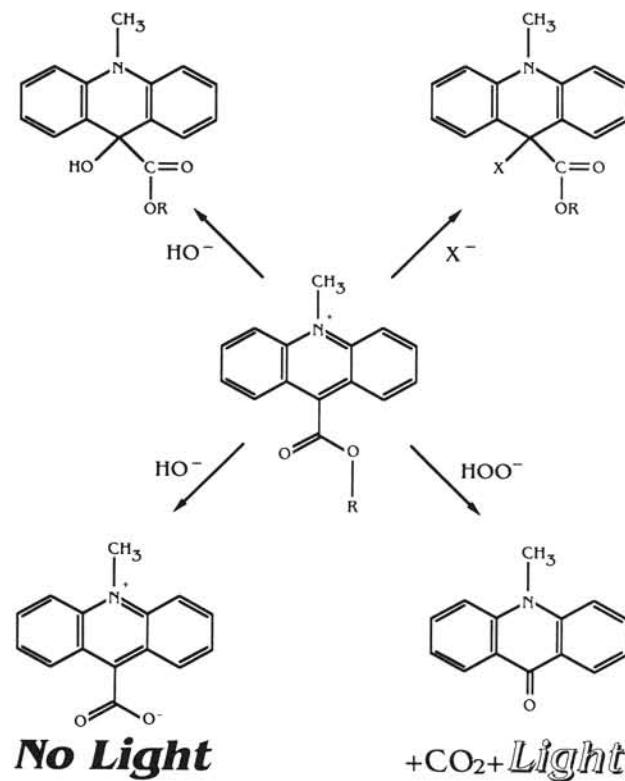


Fig. 3—The *N*-methyl acridinium ester (AE) reacts with hydroxide to form the reversible C-9 carbinal adduct (upper left) or to hydrolyze the ester bond, yielding the non-chemiluminescent acridinium carboxylic acid (lower left). Acridinium ester can also react with other nucleophiles (represented as X^-) to form reversible adducts as the C-9 position (upper right). As shown in Fig. 2, reaction with alkaline peroxide produces light (lower right).

the addition of a single reagent and without any physical separation. In practical use, background levels of 0.00005% have not yet been achieved because the rate of AE hydrolysis plateaus before reaching these levels. Nevertheless, background levels of 0.002% have been achieved in a homogeneous format, which is still a significant signal-to-noise discrimination.

A homogeneous assay format for the detection of target nucleic acids based on this differential hydrolysis process has been developed. This format, referred to as the hybridization protection assay, or HPA (Arnold *et al.*, 1989; Nelson and Kacian, 1990; Nelson *et al.*, 1990), consists of the following three basic steps (Fig. 5):

1. *Hybridization*. AE-probe is added to a solution containing the target nucleic acid, and the mixture is incubated. The length of time required

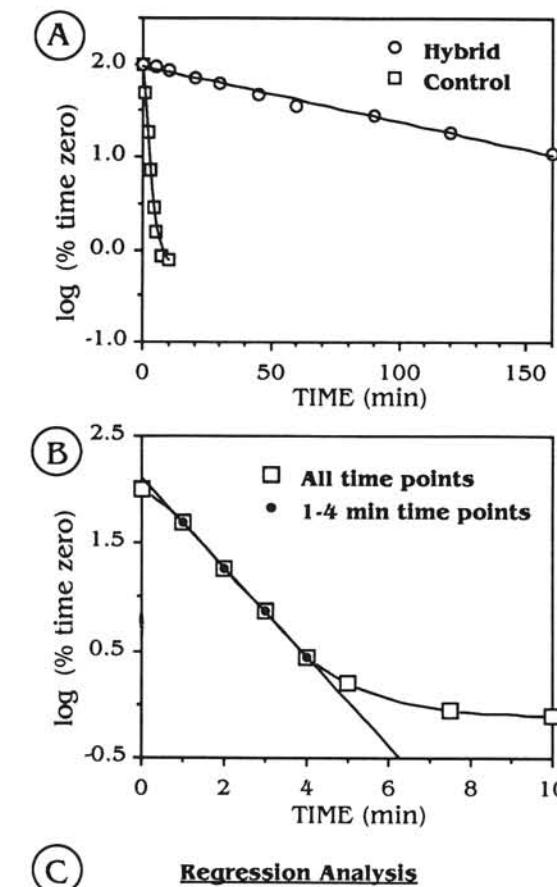


Fig. 4—(A) Ester hydrolysis kinetics of hybridized and unhybridized acridinium ester-labeled DNA probe (AE-probe) are shown as loss of chemiluminescence versus time. (B) An expanded view of the hydrolysis curve for the unhybridized AE-probe. The hydrolysis rate begins to slow at between 4 and 5 min of incubation, and plateaus at about 10 min. Additionally, the rate of the hydrolysis between 0 and 1 min of incubation is variable since the contents of the tube are going from room temperature to 60°C during that interval. Therefore, the rate of hydrolysis is measured between 1 and 4 min of incubation, during which interval the hydrolysis rate is constant. (C) The equations generated from linear regression analyses for the hybridized and unhybridized AE-probe are given along with the R^2 values for each fit.

for this step is dependent on many factors, including temperature, buffer and salt conditions, kinetics of the particular probe used and concentration of the hybridizing nucleic acid strands. At 60°C, hybridization times longer than 1 hr are uncommon, and times of 10 to 15 min are routine.

3. Detection

2. Differential Hydrolysis

1. Hybridization

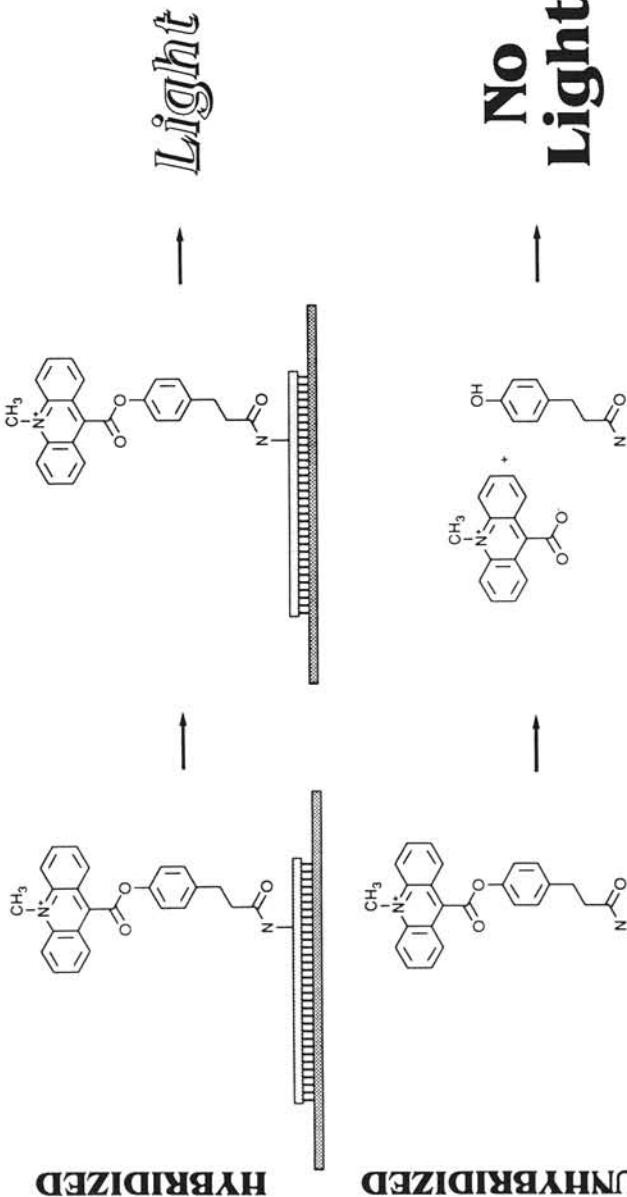


Fig. 5—Schematic representation of the hybridization protection assay (HPA) depicting the three basic steps of the assay. In step 1, acridinium ester-labeled probe (AE-probe) is hybridized with its target nucleic acid. In step 2, acridinium ester (AE) associated with unhybridized AE-probe is rapidly hydrolyzed, whereas AE associated with hybridized AE-probe is protected from hydrolysis. In step 3 (detection), hybridized AE-probe (intact AE) produces chemiluminescence, whereas unhybridized AE-probe (hydrolyzed AE) does not produce chemiluminescence. See text and Figs. 3 and 4 for details.

2. Differential hydrolysis. An alkaline differential hydrolysis buffer is added to the hybridization reaction, thereby effecting hydrolysis of AE associated with unhybridized probe. This step is very rapid (typically 6 to 12 min at 60°C).

3. Detection. The assay tubes are placed directly into a luminometer, and chemiluminescence is measured automatically. This process takes only 5 to 10 sec per sample.

The limit of sensitivity of the HPA format is approximately 6×10^{-17} moles (Arnold *et al.*, 1989), detection is quantitative and linear over a wide range of target nucleic acid concentrations, and the assay is rapid and easy to perform.

In yet another approach, the differential hydrolysis process is combined with separation using cationic magnetic beads, thereby achieving even greater discrimination between hybridized and unhybridized AE-probe. In this format, backgrounds as low as 0.0002% are achieved, and assay sensitivity is approximately 6×10^{-18} moles (Arnold *et al.*, 1989). This format is also very rapid and easy to perform because of the simplicity of the magnetic separation step.

Some properties of the AE detection system (both the unconjugated AE and the AE-probe assay formats) are listed in Table I.

C. Advantages of the Method

There are several advantages to using the chemiluminescent AE as a detection label, including high inherent sensitivity, low quenching, simplicity, ease of handling and disposal, precise chemical control, and a long shelf-life. Furthermore, these properties are not adversely affected by attachment of the AE to oligonucleotide probes, nor are the hybridization characteristics of the oligonucleotide probe compromised. There are also

Table I
Selected Properties of the Acridinium-Ester Detection System

Parameter	AE-probe assay formats		
	AE alone	HPA	DH + separation
Sensitivity	5×10^{-19} moles	6×10^{-17} moles	6×10^{-18} moles
Quantitation	Objective	Objective	Objective
Specific activity	1×10^8 RLU/pmol	1×10^8 RLU/pmol	1×10^8 RLU/pmol
Linear dynamic range	$\geq 10^4$	10^4	$\geq 10^4$
Time to result	2–5 sec	20–75 min	50–110 min

several advantages to the AE-probe assay formats presented here, including the following:

1. *In-solution approach.* Advantages of the in-solution approach include availability of all the target molecules (in target immobilization procedures, only a portion of the molecules are available for hybridization), faster hybridization kinetics, better quantitation, fewer steps and less complexity, and faster time-to-result.

2. *Sensitivity.* The assays are sensitive. This is in part owing to the high specific activity and inherent low background characteristics of the detection system as well as the low background levels achieved with the assay formats described.

3. *Specificity.* The assays retain the high specificity inherent in DNA probe-based formats. Furthermore, the differential hydrolysis assays described here afford an additional level of specificity not possible with other assay/detection systems. This is because the stability of the AE label in the hybridized form of the AE probe is very sensitive to duplex structure. Even small perturbations in structure, such as those caused by single base mismatches, can lead to large increases in the AE hydrolysis rate. Therefore, differential hydrolysis characteristics can be used to discriminate between nucleic acids with only small sequence differences (even a single base change), even when overall hybridization characteristics cannot. An example of this has been described previously (Arnold *et al.*, 1989; Nelson *et al.*, 1990); two nucleic acid targets that differed by only a single base were assayed using a single AE-probe perfectly complementary to one of the targets. Using a standard solid-phase separation method, no significant difference in hybridization was seen between the two targets. However, the differential hydrolysis method in an HPA format clearly discriminated between the two targets.

4. *Simplicity.* The assays are very easy to perform (there are only three basic steps; see Section I,B) and the *time-to-result* is very short. Assays utilizing the HPA format are typically complete in 30 to 60 min, of which only about 5 min is *hands-on* time. Also, the entire assay is performed in a single tube, which is simply placed in an automatic luminometer for detection. Furthermore, direct labels are simpler to use than indirect labels since the capping, binding of the label, washing, and substrate addition steps associated with indirect labels are unnecessary.

5. *Quantitation.* The assays are quantitative with a linear response over several orders of magnitude of target concentration (the limit of the linearity is typically the luminometer and not the assay itself).

6. *Precision.* The assay is reliable and reproducible.

7. *Versatility.* The assay can be used for the detection of both DNA and RNA targets. Several of the formats developed to date target ribo-

somal RNA (rRNA), which provides the further benefits of natural target amplification (there are approximately 10,000 rRNA copies per bacterial cell) and improved specificity (Enns, 1988; Kohne, 1986; Woese, 1987).

8. *Clinical compatibility.* The AE-probe retains its chemiluminescence and hybridization characteristics in the presence of relatively large amounts of clinical specimen material, a feature that allows the methods described here to be utilized for direct specimen testing in clinical diagnostic assays.

D. Limitations of the Method

Limitations of the AE-probe system that should be kept in mind when considering potential applications are as follows:

1. The ester bond of the AE is inherently unstable (this property is the basis for the differential hydrolysis process), and therefore AE-probes can be used only within certain pH and temperature ranges. For example, the AE will not withstand incubation at 95°C and therefore cannot be incorporated *during* polymerase chain reaction (PCR) amplification (although it can be readily used for the detection of the products from *in vitro* amplification procedures; see Sections I, E and III,C). Also, AE-probes cannot be run under standard polyacrylamide gel electrophoresis conditions (although other types or conditions of electrophoresis are feasible).

2. The AE will react with alkaline peroxide to produce light only once. Therefore, samples cannot be re-read for chemiluminescence.

3. Some target nucleic acid samples may contain inherent chemiluminescence. If these levels are high enough, sensitivity of the HPA format will be decreased owing to increased background. In these cases additional steps to eliminate the sample chemiluminescence are necessary.

E. Applications of the Method

The AE-probe method can be used for the in-solution detection of virtually any target nucleic acid, provided the target possesses a hybridizable probe region (the sequence must be known) and is available in sufficient quantity to be within the sensitivity range of the assay. One of the earliest reported uses of this method (Arnold *et al.*, 1989) was the detection and quantitation of purified ribosomal RNA (rRNA). More recently, applications ranging from single mismatch discrimination (Nelson *et al.*, 1990) and detection

of amplified human immunodeficiency virus (HIV) DNA (Ou *et al.*, 1990) to the detection of nucleic acids from clinically relevant pathogens (see the following) have been reported. Potential applications include the detection and identification of a wide variety of pathogens and other microorganisms in medicine, dentistry and veterinary; forensic science; therapeutic drug monitoring; genetic screening (including disease susceptibility) and typing; food science; the petroleum industry; and environmental testing; as well as a wide spectrum of basic research applications involving nucleic acids.

A particularly useful application of the HPA format is the detection of products from target amplification reactions such as the PCR (Mullis and Faloona, 1987; Nelson and McDonough, 1994), and the transcription amplification system, or TAS (Kwoh *et al.*, 1989). The HPA format retains the distinct advantage of specific probe hybridization to unequivocally identify the amplified sequence. Methods that assay simply for extension of primers, such as gel electrophoresis followed by ethidium bromide staining, are equivocal since nontarget species of similar size can be produced with some frequency (Innis and Gelfand, 1990). Detection of HIV DNA (Kacian *et al.*, 1990; McDonough *et al.*, 1989; Ou *et al.*, 1990), the Philadelphia chromosome (Arnold *et al.*, 1989; Dhingra *et al.*, 1991), hepatitis B virus (HBV) DNA (Kacian *et al.*, 1990), the genes that code for the rRNA of *Neisseria gonorrhoeae* (Gregg *et al.*, 1990) and *Chlamydia trachomatis* (Kranig-Brown *et al.*, 1990), and *Mycobacterium tuberculosis* RNA (Jonas *et al.*, 1993) using this procedure have been reported. Test kits utilizing this detection approach are commercially available (Gen-Probe, San Diego).

Another useful application of the AE-probe system is the detection and identification of pathogenic organisms in the clinical laboratory. Diagnostic assays for a variety of organisms have been reported, including test for *Chlamydia trachomatis* (Harper and Johnson, 1990; LeBar *et al.*, 1987; Warren *et al.*, 1993; Woods *et al.*, 1990; Yang *et al.*, 1991), *Neisseria gonorrhoeae* (Hale *et al.*, 1993; Lewis *et al.*, 1990; Granato and Franz, 1989; Granato and Franz, 1990; Harper and Johnson, 1990; Vlaspolder *et al.*, 1993), *Mycobacterium tuberculosis* (Jonas *et al.*, 1993), *Escherichia coli* (Davis and Fuller, 1991; Watson *et al.*, 1990), *Streptococcus agalactiae*, *Haemophilus influenzae*, *Enterococcus* species (Daly *et al.*, 1991), *Streptococcus pneumoniae* (Denys and Carey, 1992), *Streptococcus pharyngitis* (Heiter and Bourbeau, 1993), *Histoplasma capsulatum* (Stockman *et al.*, 1993; Sandin *et al.*, 1993) and *Campylobacter* species (Popovic-Uroic *et al.*, 1991; Tenover *et al.*, 1990). Kits for the detection of a variety of pathogenic organisms both directly in clinical specimen material and as culture confirmation assays are commercially available (Gen-Probe, San Diego).

II. MATERIALS

A. Reagents

1. Preparation of Acridinium Ester-Labeled DNA Probe (AE-Probe)

The *N*-hydroxysuccinimide (NHS) ester of AE is synthesized as described by Weeks and co-workers (Weeks *et al.*, 1983a). The dimethylsulfoxide (DMSO) used in the preparation of the stock solution of the AE used in the labeling reaction must be completely free of water (otherwise the NHS ester will be hydrolyzed, rendering the labeling reagent inactive). Therefore, DMSO is purchased as reagent grade (Aldrich, Wisconsin), distilled and stored over molecular sieves (activated, Type4A, 8–12 mesh; J. T. Baker). There is also DMSO added to the labeling reaction mixture in addition to that added as the AE labeling reagent (see Section III,A). This DMSO does not have to be perfectly dry, since it is mixed with water. Therefore, for this particular use, reagent grade DMSO (Aldrich) is adequate (although it certainly does not hurt if the dry DMSO is used). Ethanol used in nucleic acid precipitations is 200 proof from Quantum Chemical Corporation. For small quantities of DNA (less than about 10 µg), a carrier should be used in the ethanol precipitation steps to maximize recovery. Glycogen (Sigma) is used as the carrier in the procedures given here.

High-pressure liquid chromatography (HPLC) buffer compositions are as follows: Buffer A, 20 mM sodium acetate (pH 5.5), 20% (v/v) acetonitrile; Buffer B, 20 mM sodium acetate (pH 5.5), 20% (v/v) acetonitrile, 1M LiCl; all reagents used in the preparation of these buffers must be HPLC grade (Fisher Scientific or equivalent). After preparation, both of these buffers must be filtered through a 0.45 µm Nylon-66 filter (Rainin); a vacuum filtration apparatus designed for this purpose is useful (also available from Rainin). The column used is a Nucleogen-DEAE 60-7 ion-exchange column (also available from Rainin). See Section II,B below for a discussion of HPLC instrumentation. For all other chemicals, reagent grade is adequate (Fisher Scientific or equivalent).

2. Determination of Differential Hydrolysis Kinetics of AE-Probe

Lithium succinate, ethylenediaminetetraacetic acid (EDTA), ethylene glycol bis(β-aminoethyl ether) *N,N'*-tetraacetic acid (EGTA), sodium tetraborate (or boric acid), lithium lauryl sulfate and Triton X-100 are reagent grade (Fisher Scientific or equivalent). The borate buffer may be made

as 0.15 M tetraborate or 0.6 M boric acid (titrated to the correct pH with NaOH). The solubility of sodium tetraborate is approximately 0.2 M at pH 8. Hybridizations are performed in 1.5-ml screw-capped microcentrifuge tubes (Eppendorf); AE hydrolysis and chemiluminescence measurements are performed in 12 × 75 mm polystyrene tubes (Sarstedt, West Germany).

3. AE-Probe Assays

The formamide used in the differential hydrolysis + separation protocol is from Fluka; the magnetic microspheres are from Advanced Magnetics, Inc. (Cambridge, Massachusetts). The magnetic separation solution is made by adding a small volume of a concentrated stock solution of magnetic microspheres in water to 0.4 M phosphate buffer, pH 6.0 (1 to 50 µl of magnetic spheres per ml of buffer is used, depending on the amount of spheres required for a given application); this solution must be used within 8 hr of preparation. Reagents used in these assays that are also used in the determination of the differential hydrolysis kinetics are described in Section II,A,2; all other reagents used are reagent grade (Fisher Scientific or equivalent).

The magnetic particles can be separated from solution with virtually any magnet of adequate strength. Racks that hold the 12 × 75 mm assay tubes and that have built-in magnets are particularly convenient. Racks that are compatible with the assays described here are commercially available (Corning; Gen-Probe).

4. General

Chemiluminescence measurements are performed in 12 × 75 mm polystyrene tubes (Sarstedt, West Germany). Some lots of tubes possess chemiluminescent contamination; several empty tubes of each lot should therefore be read in the luminometer. Tubes should always be blotted with a mildly damp tissue or paper towel before chemiluminescence measurement to remove any static charge from the tubes (this charge can lead to errantly high readings). The tissue or towel should not be *too* wet, as this can potentially damage the interior of the luminometer. Blotting before reading will also remove any contamination that may be present on the outside of the tube, thus protecting the luminometer from contamination and/or damage.

The reagents required for the chemiluminescence reaction of AE are base and peroxide. Reagent grade NaOH and hydrogen peroxide (supplied as a 30% solution; Fisher Scientific) are used for this purpose. Typically, an automatic two-injection protocol is used on the luminometer to inject these reagents (see Section II,B), the first injection containing 0.1% perox-

ide and the second injection containing 1 M NaOH. Hydrogen peroxide is very stable at acidic pH and unstable at basic pH. Therefore if the 0.1% peroxide solution is acidified (1 mM nitric acid), it will be stable at room temperature for weeks to months. In some assay modes, the peroxide solution contains a higher concentration of acid (e.g., 0.4 M nitric acid) in order to reverse carbinol formation of the AE (see Section III,B). This solution is also stable over extended periods. On occasion, the peroxide and the base are mixed and injected as a single reagent. The peroxide is stable in this basic reagent for only about 10 hr at room temperature; this reagent must therefore be prepared fresh daily.

Standard pipettors (Gilson Pipetman) are used in these protocols; standard vortex mixers are purchased from Fisher Scientific; samples are evaporated in a Savant Speed-Vac rotary/vacuum drying apparatus.

B. Instrumentation

1. Luminometer

The reaction of the AE with alkaline peroxide to produce chemiluminescence is very rapid (see Section I,A and Fig. 2). Therefore a luminometer that automatically injects the alkaline peroxide into the sample and immediately measures the chemiluminescence is required for accurate detection of the AE described here. Furthermore, the assay protocols described here are based on a 12 × 75 mm reaction tube, and the luminometer selected must therefore be able to accommodate such tubes. Luminometers that fulfill both these requirements are commercially available from Gen-Probe, Berthold (Germany), and Turner. A luminometer that is capable of automatically injecting the alkaline peroxide but is based on a microtiter well format is available from Dynatech. All procedures described here were developed using a LEADER I or a LEADER 50 luminometer (Gen-Probe).

2. DNA Synthesizer

Any DNA synthesizer capable of standard phosphoramidite solid-phase chemistry is appropriate for synthesis of the DNA probes. All procedures described here were developed using a Biosearch Model 8750 or an ABI Model 380A synthesizer.

3. High-Performance Liquid Chromatograph

Any of a number of instruments currently available would be appropriate for the chromatography described herein. The instrument must be capable

of performing automatic gradient elution and must be equipped with a detector capable of measurement at 260 nm; a dual-wavelength detector capable of measurement at 260 and 325 nm is preferable. The chromatography described here was performed on a Beckman System Gold HPLC.

III. PROCEDURES

A. Preparation of an Acridinium Ester-Labeled DNA Probe (AE-Probe)

An oligomer of virtually any sequence can be labeled, and selection of this sequence is obviously dependent on the target nucleic acid to be detected. The oligonucleotide is synthesized using standard phosphoramidite solid-phase chemistry (Caruthers *et al.*, 1987) on a commercially available automated DNA synthesizer. A primary amine is introduced into the oligomer during synthesis using the nonnucleotide-based linker-arm chemistry described previously (Arnold *et al.*, 1988). This chemistry allows incorporation of the linker-arm at any position in the growing oligonucleotide chain, including the 3' terminus, the 5' terminus, or at any position within the oligonucleotide sequence. The resulting primary amine-containing oligomer is purified using standard polyacrylamide gel electrophoresis and desalting techniques (water is the final solvent). Labeling of this oligomer with AE-NHS proceeds as follows:

1. Evaporate to dryness the oligomer to be labeled in a 1.5-ml screw-capped microcentrifuge tube using a Savant Speed-Vac drying apparatus.
2. After drying, add to the tube 8 μ l of 0.125 M HEPES buffer (pH 8.0)/50% DMSO and 2 μ l of 25 mM AE-NHS in DMSO. Cap the tube, gently mix the contents, and then incubate for 20 min at 37°C.
3. Add an additional 3 μ l 25 mM AE-NHS in DMSO, mix gently, then add 2 μ l 0.1 M HEPES buffer (pH 8.0). Recap, mix gently, and then incubate an additional 20 min at 37°C.
4. Quench the reaction by adding 5 μ l 0.125 M lysine in 0.1 M HEPES buffer (pH 8.0)/50% DMSO. Mix gently.
5. Add 30 μ l M sodium acetate buffer (pH 5.0), 245 μ l water, and 5 μ l glycogen (40 mg/ml); vortex to mix. Add 640 μ l chilled (-20°C) absolute ethanol; cap tube, and vortex to mix. Incubate on dry ice for 5 to 10 min, then centrifuge at 15,000 rpm using the standard head in a refrigerated microcentrifuge (Tomy or Eppendorf).

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the standard head in a refrigerated microcentrifuge (Tomy or Eppendorf).

6. Carefully aspirate the supernatant, making sure not to disturb the pellet. Resuspend the pellet in 20 μ l 0.1 M sodium acetate (pH 5.0) containing 0.1% SDS.

The labeled oligomer (AE-probe) is then purified by gradient elution ion-exchange high-performance liquid chromatography (IE-HPLC) using a 4.0 \times 125 mm Nucleogen-DEAE 60-7 column as follows:

1. Pre-equilibrate the IE column in 60% Buffer A, 40% Buffer B at a flow rate of 1 ml/min.
2. To the 20 μ l resuspended AE-probe pellet from step 6 of the labeling reaction, add 190 μ l of the pre-equilibration buffer (i.e., 60% Buffer A, 40% Buffer B). Vortex to mix.
3. Load 100 μ l of the AE-probe sample into a 100- μ l injection loop.
4. Inject the AE-probe onto the IE column equilibrated as described in step 1. Elute the AE-probe using a linear gradient from 40 to 70% Buffer B in 30 min at a flow rate of 1 ml/min; monitor absorbance at 260 nm as well as 325 nm (if possible); collect 0.5-ml fractions.
5. If absorbance at 325 nm is not monitored, or the amount of AE-probe being purified is too small to give a discernable absorbance at this wavelength, measure the chemiluminescence of each fraction as follows: Pipette 2 μ l of each fraction into corresponding 12 \times 75 mm polystyrene tubes containing 100 μ l 0.1% SDS; vortex to mix. Measure the chemiluminescence of each tube with the automatic injection of 200 μ l 0.4 M HNO₃, 0.1% H₂O₂, then 200 μ l 1 M NaOH, followed by measurement of signal for 5 sec. Plot chemiluminescence (measured as relative light units, or RLU) versus fraction number. The fractions may need to be diluted if the chemiluminescence contained in 2 μ l is too high for the luminometer to measure. If so, dilute in 0.1% sodium dodecyl sulfate (SDS) and vortex thoroughly to ensure a homogeneous sample.
6. Precipitate fractions containing the purified oligomer (see Fig. 6) as follows: Add 5 μ l glycogen (40 mg/ml) and 1 ml chilled (-20°C) absolute ethanol; cap the tube, and vortex to mix. Incubate on dry ice for 5 to 10 min, then centrifuge at 15,000 rpm using the standard head in a refrigerated microcentrifuge (Tomy or Eppendorf).
7. Carefully aspirate the supernatant, making sure not to disturb the pellet. Resuspend the pellet in 20 μ l 0.1 M sodium acetate (pH 5.0) containing 0.1% SDS.

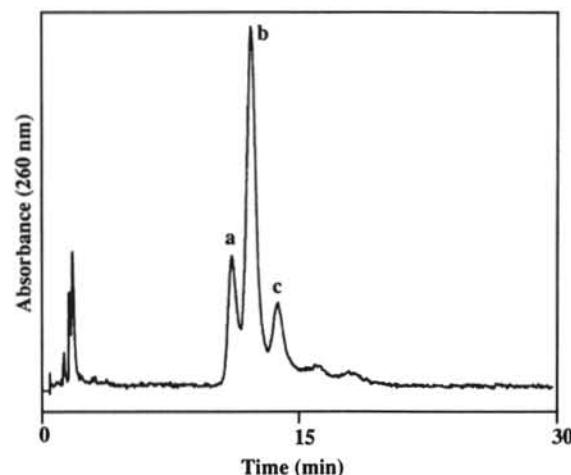


Fig. 6—A DNA probe was labeled with acridinium ester (AE) and purified by ion-exchange HPLC, as described in the text. The resulting chromatogram is shown. Peak a represents unlabeled probe; peak b represents AE-labeled probe; and peak c represents AE-probe that has undergone ester hydrolysis (see Fig. 3).

Purification of an AE-labeled 26-mer is shown in Fig. 6. The specific activity of the AE-labeled probe is determined as follows:

1. Perform serial dilutions of the AE-probe stock in 0.1 M sodium acetate (pH 5.0) containing 0.1% SDS; the final measurement of chemiluminescence should be in the 50,000 to 250,000 RLU range.
2. Pipet 10 μ l of the final dilution of AE-probe into 200 μ l 5% Triton X-100 (3 to 5 replicates is recommended). Measure the chemiluminescence of each tube with the automatic injection of 200 μ l 0.4 M HNO₃, 0.1% H₂O₂, then 200 μ l of 1 M NaOH, followed by measurement of signal for 5 sec. Based on the dilution factor from (1), calculate the RLU/ μ l of the stock AE-probe.
3. Measure the absorbance at 260 nm of AE-probe in 0.1 M sodium acetate (pH 5.0) containing 0.1% SDS; the final measurement should be in the 0.2 to 1.0 optical density (OD) range (depending on the amount of probe labeled, dilutions may or may not be necessary to achieve this concentration).
4. Calculate the concentration of DNA probe in the stock solution. Since the AE as well as the oligomer contributes to the OD at 260 nm, first calculate the contribution of the oligomer to the OD reading. The extinction coefficient of the oligomer is roughly 10,000 M⁻¹ times the number of nucleotides in the oligomer (the

exact extinction coefficients for each base can be used if desired). The extinction coefficient of the AE is 78,800 M⁻¹. The total extinction coefficient of a 20-mer, for example would be

$$[10,000 \text{ M}^{-1} \times 20] + 78,800 \text{ M}^{-1} = 278,800 \text{ M}^{-1}$$

The contribution from the oligomer will therefore be

$$200,000 \text{ M}^{-1}/278,800 \text{ M}^{-1} = 71.7\%$$

For an AE-labeled 20-mer, the OD reading will have to be multiplied by .717 to calculate the OD reading of the oligomer.

To calculate the concentration of the oligomer from the OD, the following two conversions must be used: 1 OD unit \times 40 μ g of DNA (approximate value; other values may be used if preferred), and

$$\text{molecular weight of an oligomer} = 330 \text{ g/mol nucleotide} \times \text{the number of nucleotides}$$

For example, if the OD/ml reading of the AE-labeled 20-mer is 1.0, the concentration is calculated as follows:

$$\text{MW of oligo} = 330 \text{ } \mu\text{g}/\mu\text{mol nucleotide} \times 20 \text{ nucleotides} = 6600 \text{ } \mu\text{g}/\mu\text{mol} \quad (2)$$

$$1 \text{ OD/ml} \times .717 = 0.717 \text{ OD/ml (contribution from the oligomer)} \quad (3)$$

$$0.717 \text{ OD/ml} \times 40 \text{ } \mu\text{g}/\text{OD} = 28.68 \text{ } \mu\text{g}/\text{ml} \quad (4)$$

$$28.68 \text{ } \mu\text{g}/\text{ml} \times 1 \text{ } \mu\text{mol}/6600 \text{ } \mu\text{g} = 4.35 \text{ nmol}/\text{ml} \\ = 4.35 \text{ pmol}/\mu\text{l} \quad (5)$$

[Note: this value will have to be multiplied by the dilution factor if any dilutions of the stock AE-probe were made.]

5. Determine the specific activity based on the results of 1 through 4 above:

$$(RLU/\mu\text{l})(pmol}/\mu\text{l}) = \text{specific activity in RLU/pmol} \quad (6)$$

Specific activities of AE-labeled probes are typically in the range of 0.5 to 1.0 \times 10⁸ RLU/pmol.

Procedural Notes/Troubleshooting

1. Oligomers as long as 40 bases can be purified using the IE-HPLC protocol given. For oligomers longer than 40 bases, alternative ion-exchange columns can be utilized (data not shown). Also, reverse-phase

chromatography is an excellent alternative for the purification of AE-labeled oligomers of a wide variety of sizes (Arnold and Nelson, 1993).

2. HPLC resolution can be somewhat improved by extending the duration of the gradient. Additionally, the initial buffer conditions should be slightly adjusted for different length oligomers to optimize elution time—a lower percentage Buffer B for shorter oligomers and a higher percentage B for longer oligomers. The exact conditions can be determined empirically.

3. Absorbance at 325 nm is used to monitor the AE without any interference from the DNA.

4. Various amounts of oligomer can be labeled using this protocol. Approximately 10 nmol can be labeled using the volumes described above; proportionally larger amounts of oligomer can be labeled using proportionally larger amounts of each reagent (the limiting factor is solubility of the AE-probe).

5. An acceptable alternative to the labeling protocol described above is as follows: to the dried oligomer, add 15 μ l of a mixture containing 0.1 M HEPES buffer (pH 8.0), 60% DMSO, and 5 mM AE; mix gently; incubate 90 min at 30°C.

6. Be extremely careful when using the stock solution of AE not to contaminate your buffers, pipettors, workspace, etc. (a 1 μ l aliquot of the 25 mM stock contains approximately 10¹¹ RLU of chemiluminescence).

7. After precipitation of the AE-probe, be careful to remove as much of the ethanol as possible. However, avoid drying in the Speed-Vac (or other similar drying device) as this can lead to breakdown of the AE on the oligomer.

8. If labeling efficiency is poor, the AE-NHS may be hydrolyzed (this reagent is very water sensitive, and once it is broken down, it will no longer react with the primary amine or the oligomer). Prepare a fresh stock of the AE-NHS in freshly distilled DMSO and repeat the procedure.

9. If peak "c" in the HPLC chromatogram (hydrolyzed AE-probe; see Fig. 6) is large, the pH of the reaction mixture may be incorrect. Measure the pH of the existing HEPES buffer as well as a mock reaction cocktail, and/or prepare fresh buffer and repeat the procedure.

10. If the labeling reaction did not proceed to a high extent (peak "a" high and peaks "b" and "c" low; see Fig. 6), the pH of the reaction mixture may be incorrect. Measure the pH of the existing HEPES buffer as well as a mock reaction cocktail, and/or prepare fresh buffer and repeat the procedure. Alternatively, the NHS ester of the AE labeling reagent may be hydrolyzed, in which case the labeling procedure should be repeated using a freshly prepared stock AE-NHS.

11. Mixing HEPES and DMSO produces heat; therefore, these reagents should be pre-mixed and allowed to cool before adding the AE-NHS labeling reagent.

12. See Section III,D, Procedural Notes/Troubleshooting, General, for tips that are applicable to all AE-based procedures.

B. Determination of the Differential Hydrolysis Kinetics of an AE-Probe

Once an oligomer is labeled and purified, the AE hydrolysis kinetics of the hybridized and unhybridized oligomer are measured during the protocol given. These data are then utilized to evaluate the differential hydrolysis performance characteristics of an AE-probe and to determine optimal hydrolysis times to be used in an assay.

-
1. In 1.5-ml microcentrifuge tubes, prepare three 30 μ l reaction mixtures to contain the following: Hybrid—0.1 M lithium succinate buffer, pH 5.2, 2 mM EDTA, 2 mM (EGTA), 10% (w/v) lithium lauryl sulfate, 0.1 pmol AE-probe, and 0.5–1.0 pmol of the target nucleic acid (i.e., nucleic acid complementary to the AE-probe); Control (i.e., unhybridized AE-probe)—same as Hybrid, except containing no target nucleic acid; Blank—same as Control except containing no AE-probe. Incubate all three reactions in a water bath 30 min at 60°C.
 2. Remove reactions from water bath, add 270 μ l 0.1 M lithium succinate buffer, pH 5.2, 2 mM EGTA, 10% (w/v) lithium lauryl sulfate to each, and vortex thoroughly.
 3. To measure the AE hydrolysis rate for hybridized AE-probe, pipette 10- μ l aliquots of the diluted Hybrid reaction into each of seven 12 × 75 mm assay tubes. Add 100 μ l 0.15 M sodium tetraborate buffer, pH 7.6, 5% (v/v) Triton X-100 to each tube, and vortex each 3 sec. Measure the chemiluminescence of two of the replicate tubes immediately (see detection protocol in step 5); these will be the *time zero* values. Cover the remaining five replicates and place them in a water bath at 60°C; remove a single tube at 5, 10, 15, 20, and 30 min. Immediately after removal from the water bath, place each tube in an ice/water slurry for 15 sec, remove to room temperature and let stand for 30 sec, then measure the chemiluminescence as described in step 5.
 4. To measure the AE hydrolysis rate for unhybridized AE-probe, the protocol in step 3 is repeated with the Control reaction from step 1, with the exception that tubes are removed from the water bath at 1, 2, 3, 4, and 5 min. The Blank reaction is also an-

- alyzed in this manner (to be run concurrently with the Control analysis).
5. Detect chemiluminescence in a luminometer by the automatic injection of 200 μl 0.4 M HNO_3 , 0.1% H_2O_2 , then 200 μl of 1 M NaOH, followed by measurement of signal for 5 sec. Remember to blot the tubes with a moist tissue or paper towel before measuring the chemiluminescence (see Section II,A,4).
 6. Analyze the data as follows: First, subtract the average Blank value from each Hybrid and Control value. Then plot the data for Hybrid and Control as the log of the percentage of time zero chemiluminescence versus time. Finally, determine the slopes of the linear portions of the hydrolysis curves (and therefore the rates of AE hydrolysis), using standard linear regression analysis.

An example of the data generated using this protocol is given in Fig. 4. From the slope of the line the half-life of hydrolysis can be calculated (half-life in min = 0.3 log units per half-life divided by the slope in log units per min), and the ratio of the hybrid to control half-lives determined. This differential hydrolysis ratio (or DH ratio) is a useful parameter for describing AE-probe performance. Obviously the higher the DH ratio, the higher the discriminating power achieved in an HPA format.

Procedural Notes/Troubleshooting

1. Acid reversion is used in this protocol to revert any pseudo-base that formed during the DH step. If acid reversion is not used, the protocol becomes a measurement of the *combined* rates of ester hydrolysis and pseudo-base formation.

2. In one variation of the method for determining AE hydrolysis rate, the acid hydrolysis is achieved via the manual addition of an acid/peroxide solution to the reaction mixture prior to placement in the luminometer for chemiluminescence measurement. This stops the hydrolysis reaction at any given time, and circumvents the need to place the samples on ice. The procedure is run essentially as described earlier, with the following modifications: For the time zero samples, after addition of the sample (hybrid, control, or blank), 200 μl 0.4 M HNO_3 , 0.1% H_2O_2 is added to the tubes, followed by addition of 100 μl sodium tetraborate buffer. The chemiluminescence is measured in the luminometer by the automatic injection of 200 μl 1 M NaOH, followed by a 5-sec read. For the hydrolysis time points, after addition of the sample (hybrid, control, or blank), 100 μl sodium tetraborate buffer is added, the tubes are placed in the water bath at 60°C, and then at various time points 200 μl 0.4 M HNO_3 , 0.1% H_2O_2 is added and the chemiluminescence is measured, as described.

The tubes can be read immediately, or they can sit for hours or even days, since the AE is stable in the acid.

3. Since hydrolysis rate is temperature sensitive, it is very important that the temperature used in this procedure be accurately maintained during the hydrolysis time course. It is also very important that the temperature be very consistent from tube to tube. For these reasons a circulating water bath is recommended for this procedure.

4. It is very important that half-lives of hydrolysis be calculated from the linear portions of the kinetic curves. Small differences in slope can lead to relatively large differences in half-lives and DH ratios. Values for R^2 in the regression analysis of 0.98 or greater are recommended. Errant values for half-life of hydrolysis are particularly likely in the Control since hydrolysis rates are much faster in this case. More data points in the early portion of the curve are useful in improving accuracy. In the first 15 to 30 sec, the contents of the tubes are coming to temperature, and therefore data points at 30 sec and beyond are typically more significant.

5. The Blank reaction is necessary to factor out chemiluminescence inherent in the reagents. This is especially important for the later time points of the Control sample. The lower of this amount of chemiluminescence, the more accurate the analysis; therefore, care in preparing reagents without introducing contaminating chemiluminescence is very important.

6. Hydrolysis-rate analysis is used to determine optimal differential hydrolysis time in a full assay format. This is described in Section III,C under Procedural Notes/Troubleshooting for general HPA protocol.

7. For the Hybrid sample, the y-intercept of the line determined using regression analysis represents the percentage hybridization of the AE-probe being tested. In most cases the extent of hybridization will be approximately 100% (the reaction is performed in target excess), and the y-intercept will be 2 (i.e., the log of 100%). In some cases, however, the extent will not be 100%. In these cases the hydrolysis curve will be biphasic—an initial rapid phase representing hydrolysis of the unhybridized AE-probe, and a second, slow phase representing the hydrolysis of the remaining hybridized AE-probe. Back extrapolation of the slow phase to the y axis is then used to determine the percentage of AE-probe that is hybridized.

8. The DH characteristics of an AE-probe can also be determined at different pH values using basically the same protocol given. However, hydrolysis rates will be faster at higher pH and slower at lower pH, and the time points must be chosen accordingly. It is difficult to measure accurately ester hydrolysis rates at 60°C for unhybridized AE probes at pH higher than about 8.5 since hydrolysis is so rapid at these pH values. Preheating the hydrolysis buffer and using very short intervals between time points helps the accuracy of the analysis in these cases.

9. If the data points to use for the regression analysis are unclear, or if the R^2 value is less than 0.98, the analysis should be repeated.
10. If necessary or desired, more data points can be collected than recommended.
11. A small square of standard laboratory label tape (*Time tape*, VWR Scientific) pressed snugly onto the assay tubes is an ideal covering during incubation at 60°C.
12. See Section III,D, Procedural Notes/Troubleshooting, General, for tips that are applicable to all AE-based procedures.

C. AE-Probe Assay Formats

The Hybridization Protection Assay can be formatted in a number of ways depending on the exact application. However, each format contains the same three basic steps: hybridization, differential hydrolysis, and detection. There are also quite a large number of ways a sample can be prepared for assay depending on the type of sample and the form and concentration of the target nucleic acid(s). If rRNA is to be assayed from a clinical specimen suspected of containing an infectious microorganism, the organism must be lysed and free rRNA released into solution. If PCR-amplified DNA is to be assayed, the sample must first be denatured since the product of the reaction is double-stranded. If single-stranded nucleic acid is to be quantified after a purification procedure, a sample need simply be pipetted into the assay tube. It is beyond the scope of this chapter to describe all the possible methods by which a sample of nucleic acid can be prepared for assay using the HPA protocol. The general requirement is that the target be available for hybridization in solution at a concentration within the sensitivity limit of the assay. Furthermore, it is preferred that the target nucleic acid be contained in a relatively small volume so the components in the sample mixture will not contribute significantly to the final hybridization conditions.

1. General HPA Protocol

1. *Hybridization.* In a 12 × 75 mm assay tube, prepare a 100 μ l hybridization reaction containing 0.1 M lithium succinate buffer, pH 5.0, 2 mM EDTA, 2 mM EGTA, 10% (w/v) lithium lauryl sulfate, 0.1 pmol AE-probe and the nucleic acid target to be detected. Also prepare negative controls (all components except the target nucleic acid) and positive controls (all components including a *known* amount of target nucleic acid; this can be a

- purified and quantitated sample of the target, a synthetic complement to the AE-probe, etc.). Vortex to mix; cover; incubate 5 to 60 min at 60°C.
2. *Differential hydrolysis.* Add 300 μ l 0.15 M sodium tetraborate buffer, pH 7.6, 5% (v/v) Triton X-100. Vortex 3 sec; cover; incubate at 60°C for 10 min.
3. *Detection.* Immediately after removal from the water bath, place the tubes in an ice/water slurry for 15 sec, remove to room temperature and let stand for 30 sec, then measure the chemiluminescence in a luminometer by the automatic injection of 200 μ l 5 mM HNO₃, 0.1% H₂O₂, then 200 μ l of 1 M NaOH, followed by measurement of signal for 5 sec. Remember to blot the tubes with a moist tissue or paper towel before measuring the chemiluminescence (see Section II,A,4).

As mentioned in Section I, this procedure has been used for the detection of a wide variety of target nucleic acids. As little as 6×10^{-17} moles of target can be detected, and chemiluminescence response is linear over three to four orders of magnitude. This basic format can be applied to the assay of almost any nucleic acid if, as mentioned above, the target is available for hybridization in solution at a concentration within the sensitivity of the assay. This general procedure can also be varied somewhat.

Procedural Notes/Troubleshooting

1. Incubation time for step 1 is dependent on the hybridization kinetics of the particular probe used. These kinetics can be measured and the hybridization time determined accordingly. This can most easily be done in the assay format described previously by setting up replicate hybridization reactions, incubating them all at 60°C, and assaying tubes (steps 2 and 3) at various time points. We have found that, under the conditions described above, hybridization is essentially complete for most AE-probes in 10 to 15 min.
2. The amount of probe used in an assay can be varied; a higher probe concentration will accelerate hybridization, but it will also increase background signal at the end of the assay, whereas a lower probe concentration will lower background signal but potentially lower hybrid signal due to slower hybridization kinetics.
3. The pH of hybridization is mildly acidic to protect the AE from hydrolysis. A range of pH 4.7 to pH 5.5 is acceptable, with pH 4.8 to pH 5.0 optimal.
4. The lithium lauryl sulfate contained in the hybridization mixture can range from 2 to 10% (10% is best).

5. Differential hydrolysis (DH) time is a critical factor in assay design. The optimal time for the DH step can be determined by measuring the AE hydrolysis kinetics of the AE-probe used in the assay as described in Section III,B. The point at which the AE hydrolysis rate of the unhybridized probe reaches a plateau (see Fig. 4) is the key factor in determining the DH time. For example, the optimal DH time for the AE-probe used for the hydrolysis rate study depicted in Fig. 4 is 10 min (under the conditions used in that experiment). After the ester hydrolysis of unhybridized AE-probe plateaus, reduction of signal from unhybridized AE-probe (i.e., background) is minimal, whereas signal from hybridized AE-probe is slowly but continually declining. Therefore assay sensitivity will slowly decrease beyond this point. It should be kept in mind, however, that the AE hydrolysis is rate and therefore the DH time is condition dependent (pH, temperature, etc.) and must therefore be measured for each condition used. Ideally, optimal DH time should be determined in the exact assay format to be used.

6. An assay temperature of 60°C was chosen to give optimal performance of the system in regard to multiple factors, including hybridization kinetics, specificity of reaction, stability of the AE, and differential hydrolysis characteristics. However, other temperatures can be used if the particular application warrants the change. At temperatures lower than 60°C, hybridization times as well as differential hydrolysis times will have to be increased (if the pH is constant). Hybridization kinetics and differential hydrolysis rates should be measured, and the appropriate incubation time selected accordingly.

7. Since the ester hydrolysis rate is directly proportional to the pH of the solution, the differential hydrolysis time can be manipulated by the pH of the differential hydrolysis buffer. A range of pH from 7.5 to 8.5 yields convenient differential hydrolysis times (6 to 12 min) at 60°C. Other pH values can be used if desired, especially if alternate temperatures are also used (for example, a higher pH may be used at a lower temperature in order to keep the duration of the differential hydrolysis step relatively short).

Maintaining an accurate pH during the differential hydrolysis step is obviously very important. The composition of the hydrolysis buffer is designed to maintain an accurate pH, but the samples must be thoroughly mixed after addition of this buffer to assume proper and consistent performance.

8. Since this assay is completely homogeneous, all of the liquid that is put into the tube during the course of the assay remains in the tube during the detection step. Since there is a volume limitation in the luminometer (beyond a certain volume efficiency of *capture* of chemiluminescent signal rapidly diminishes), there is also a volume limitation for the assay itself.

17. Acridinium Esters/Chemiluminescence

For the 12 × 75 mm tube format, a typical volume maximum is 800 µl (this depends on the exact luminometer being used). A typical injection volume for a luminometer is 200 µl. Therefore, if a two-injection regimen is being used for chemiluminescence detection, the volume maximum for all the steps of the assay before detection is 400 µl. If the peroxide and base are added in the same injection, the assay volume can be increased to 600 µl. One disadvantage of this approach is that the peroxide is stable in the base only for about 10 hr. A single-injection regimen also precludes an acid reversion step as described in Section II,B, although acid reversion is not *typically* used in the HPA format (see following sections).

9. Acid reversion is typically not used in the HPA format. This is because a portion of the background component in the assay is in the pseudo-base form, and acid reversion would revert this component and thus raise backgrounds. Hybridized AE-probe, on the other hand, does not require acid reversion to recover all available chemiluminescence. Therefore maximal signal-to-noise ratios can be achieved using a detection regimen without acid reversion.

10. Double-stranded target nucleic acid must be denatured before hybridization with the AE-probe. This is typically accomplished by a short incubation period at 95°C (see HPA Protocol for the Detection of Products from *in vitro* Amplification Techniques).

11. A small square of standard laboratory label tape (Time tape, VWR Scientific) pressed snugly onto the assay tubes is an ideal covering during incubation at 60°C.

12. Possible explanations of high background signals include the following:

- The pH of the differential hydrolysis step may be too low. This can result if the pH of the differential hydrolysis buffer is errantly low, but can also result if the sample being assayed contains buffer components or is acidic such that the borate in the DH buffer is insufficient to maintain the proper pH. If this is the case, lower sample volumes should be used, or the sample should be neutralized, semipurified, other buffer concentrations selected, etc., before being assayed.

- The sample may not be mixed thoroughly after addition of the DH buffer (the solution is fairly viscous because of the high detergent concentrations), leading to an inconsistent pH throughout the mixture.

- The temperature of hydrolysis may be incorrect. For example, when racks of tubes are assayed, the centrally located tubes may not come to the proper temperature during the incubation period.

- The AE-probe may have been splashed up the sides of the tube during one of the steps before the hydrolysis step. This material may be high enough that it does not come into contact with the hydrolysis buffer and

therefore remains unhydrolyzed, but low enough that it comes into contact with the detection reagents and therefore contributes to the final chemiluminescent signal. Special care in pipetting and mixing will help to eliminate this problem.

- The sample may contain components that reduce the rate of AE hydrolysis of the unhybridized probe. The most likely candidates in this regard are strongly nucleophilic compounds that can form adducts with the AE, thus protecting it from hydrolysis (see Fig. 3 and Section I,A). For example, certain buffers and reducing agents, such as Tris and dithiothreitol (DTT), will form adducts at millimolar concentrations at basic pH. Solutions to this problem include lower sample volumes per assay, prepurification of the target nucleic acid and avoiding use of such components in sample preparation. Another approach is to react such components with compounds that render them inert, as described by Hammond and co-workers for the reversion of adduct formation in AE (Hammond *et al.*, 1991).

- Samples to be tested, especially those that are biological in nature, may exhibit inherent chemiluminescence, which can lead to elevated background values. The best way to examine this parameter for a given sample type is to perform the assay normally with the exception that AE-probe is omitted.

13. Possible explanations of low hybridization signals include the following:

- The pH of the differential hydrolysis step may be too high. This can result if the pH of the differential hydrolysis buffer is errantly high, but can also result in the sample being assayed contains buffer components or is basic such that the borate in the DH buffer is insufficient to maintain the proper pH. If this is the case, lower sample volumes should be used, or the sample should be neutralized, semipurified, other buffer concentrations selected, etc., before being assayed.

- The AE-probe may not have hybridized to the target nucleic acid completely. If this is suspected, retest the hybridization kinetics under the exact assay conditions that resulted in low signals.

- The temperature of hydrolysis may be incorrect. Low signal is indicative of too high a temperature.

- The melting temperature (T_m) of the AE-probe may be too near the operating temperature. The T_m of the AE-probe should be determined under the exact assay conditions (probe concentration, salt concentration, etc.).

- The target nucleic acid may not be available for hybridization with the AE-probe. The sample may need to be denatured before hybridization (see HPA Protocol for the Detection of Products from *In Vitro Amplification Techniques*).

- The sample may contain components that alter the normal structure of duplex DNA. This can have a negative effect on the protection of the AE since this protection is very structure dependent. Examples of such compounds include polyamines and strong intercalating compounds. Solutions to this problem include lower sample volumes per assay, prepurification of the target nucleic acid, and avoiding use of such components in sample preparation.

2. HPA Protocol for the Detection of Products from *In Vitro Amplification Techniques*

A typical protocol follows for the assay of a target amplification reaction such as PCR and TAS (see Section I,E). A denaturation step is often required, and a convenient sample size at this step is 50 μ L. However, it is not typically necessary to assay 50 μ L directly from the amplification reaction, and in fact 50 μ L may be too much to assay (see Procedural Notes/Troubleshooting for the general HPA protocol described). In this case the sample (5 to 10 μ L of the amplification reaction, for example) should be diluted with the appropriate amount of water to bring the volume to 50 μ L. This dilution will also help prevent the strands from reannealing after denaturation. For convenience the AE-probe is typically premixed into a 2X hybridization buffer such that 50 μ L contains the proper amount of AE-probe to be used per assay. Therefore, addition of 50 μ L of this AE-probe reagent to 50 μ L of the sample yields an ideal hybridization mixture.

- Denaturation.** Pipette 50 μ L of sample to be tested into a 12 \times 75 mm assay tube; cover. Incubate at 95°C min to denature the target nucleic acid (this step can be omitted if the target is single stranded). After 5 min immediately place the tubes into an ice/water slurry.
- Hybridization.** Cool the tubes in the ice/water slurry for 1 min, then pipet 50 μ L prepared AE-probe reagent [0.2 M lithium succinate buffer, pH 5.0, 4 mM EDTA, 4 mM EGTA, 17% (w/v) lithium lauryl sulfate and 0.1 pmol AE-probe] into each tube. Vortex to mix; cover; incubate the tubes in a water bath at 60°C for 10 min.
- Differential hydrolysis.** Remove the tubes from the water bath, add 300 μ L 0.15 M sodium tetraborate buffer, pH 8.5, 5% (v/v) Triton X-100. Vortex 3 sec; cover; incubate at 60°C for 6 to 7 min.
- Detection.** Immediately after removal from the water bath, place the tubes in an ice/water slurry for 15 sec, remove to room temper-

ture, and let stand for 30 sec, then measure the chemiluminescence in a luminometer by the automatic injection of 200 μl 5 mM HNO₃, 0.1% H₂O₂, then 200 μl of 1 M NaOH, followed by measurement of signal for 5 sec. Remember to blot the tubes with a moist tissue of paper towel before measuring the chemiluminescence (see Section II,A,4).

As mentioned in Section I,E, this protocol can be used for the detection of a variety of amplification products. In one application an overlapping pair of AE-probes is used to detect specific sequences within the *gag* gene of human immunodeficiency virus-1 (HIV-1) amplified by PCR. Ou and co-workers (Ou *et al.*, 1990) have reported the detection of HIV-1 proviral DNA amplified in the presence of cell lysate. They were able to detect as little as 4 copies of HIV DNA, and the assay was linearly quantitative over about 3 orders of magnitude. They also compared the HPA format with an isotopic method utilizing a ³²P-labeled probe and found the HPA method to be at least as sensitive as the ³²P-method. (The isotopic method consisted of solution hybridization, restriction enzyme digestion, gel electrophoresis, and autoradiography.) In another application the HPA format is used to detect the presence of the Philadelphia chromosome (Adams, 1985), the chromosomal translocation product associated with chronic myelogenous leukemia (CML). In one report (Dhinga *et al.*, 1991), chimeric mRNA was amplified using PCR and detected using the HPA format described. Samples from 60 different patients were analyzed, and the HPA format gave equivalent results to standard Southern analysis of the PCR reactions, in a fraction of the time. The HPA format also gave complete correlation with the results of karyotype or Southern blot analysis of genomic DNA. The HPA format has also been used to detect amplified nucleic acid from *Mycobacterium tuberculosis* (Jonas *et al.*, 1993), hepatitis B virus (Kacian *et al.*, 1990), the genes that code for the rRNA of *Neisseria gonorrhoeae* (Gegg *et al.*, 1990) and *Chlamydia trachomatis* (Kranig-Brown *et al.*, 1990), the 7.5 kb cryptic plasmid of *Chlamydia trachomatis* known as pCHL1, the HIV-1 envelope region, and the HIV-2 viral protein x and LTR regions.

Procedural Notes/Troubleshooting

1. The procedural notes and troubleshooting guidelines given for the general HPA protocol apply to this protocol as well.
2. Never include the AE-probe in the denaturation step, as it will be hydrolyzed. Make sure the sample is cooled to 70°C or below before adding the AE-probe (using the protocol described, the temperature will be well below 70°C).

3. The denaturation protocol can also be used for double-stranded nucleic acid targets other than those from amplification reactions.
4. See Section III,D, Procedural Notes/Troubleshooting, General, for tips that are applicable to all AE-based procedures.

3. Differential Hydrolysis + Magnetic Separation Protocol

Following is a protocol that combines the differential hydrolysis process with a magnetic separation step to further reduce backgrounds. The protocol given is one variation on this theme, and represents only one particular way this general procedure can be performed.

1. *Hybridization.* In a 12 × 75 mm assay tube, prepare a 200 μl hybridization reaction containing 0.1 M lithium succinate buffer, pH 5.0, 2 mM EDTA, 2 mM EGTA, 10% (w/v) lithium lauryl sulfate, 0.1 pmol AE-probe, and the nucleic acid target to be detected. Vortex to mix; cover; incubate 10 to 60 min at 60°C.
2. *Differential hydrolysis.* Add 1 ml 0.15 M sodium tetraborate buffer, pH 7.6, 5% (w/v) Triton X-100. Vortex 3 sec; cover; incubate at 60°C for 10 min.
3. *Magnetic separation.* Add 1 ml 0.4 M sodium phosphate buffer, pH 6.0 containing 0.1 to 5 mg of magnetic amine microspheres (see Section II,A,3). Vortex thoroughly; cover; incubate 5 min at 60°C. Place tubes in a magnetic separation unit (see Section II,A,3) at room temperature and allow the microspheres to be separated from the solution (about 5 min). With the tubes still in the magnetic separation unit, decant the supernatant and blot the tops of the tubes. Wash the spheres one to three times by removing the tubes from the rack, adding 1 ml 0.2 M phosphate buffer, pH 6.0, vortexing, placing the tubes back into the magnetic rack for 5 min and decanting the supernatant.
4. *Elution of AE-probe from magnetic microspheres.* After the last wash from step 3 is decanted and the tubes are carefully blotted, remove the tubes from the magnetic separation unit and add 300 μl of a solution containing 0.2 M sodium phosphate buffer, pH 6.0, and 50% (v/v) formamide to each tube. Vortex thoroughly; cover. Incubate the tubes at 60°C for 5 min, place tubes in magnetic separation unit (see Section II,A,3) at room temperature, and allow the microspheres to be separated from the solution (about 5 min). Transfer the supernatant (which now contains the AE-probe) to a new 12 × 75 mm assay tube.

5. **Detection.** Detect chemiluminescence in a luminometer by the automatic injection of 200 μl 0.4 M HNO₃, 0.1% H₂O₂, then 200 μl 1 M NaOH, followed by measurement of signal for 5 sec. Remember to blot the tubes with a moist tissue or paper towel before measuring the chemiluminescence (see Section II,A,4).

The limit of sensitivity of this procedure is as low as 6×10^{-18} moles of target, and chemiluminescence response is linear over four or more orders of magnitude (the limiting factor in range of linearity is usually the luminometer and not the assay itself). As mentioned, there are alternate protocols to perform a differential hydrolysis + separation assay. For example, a clinical diagnostic assay (*PACE 2*, Gen-Probe) utilizes a differential hydrolysis + separation protocol that includes only one wash step and omits the elution step (chemiluminescence is measured in the presence of the magnetic microspheres) (Vlaspolder *et al.*, 1993; Yang *et al.*, 1991).

Procedural Notes/Troubleshooting

1. The procedural notes and troubleshooting guidelines given for the general HPA protocol apply to this protocol as well.
2. In high enough concentrations, the magnetic spheres will quench the chemiluminescence from the AE-probe. That is why the AE-probe is eluted from the spheres before the detection step.
3. The elution step melts the hybrid and elutes the free AE-probe from the spheres. Since it is no longer protected by the duplex, the AE can now once again hydrolyze fairly rapidly. Therefore the 60°C portion of the elution step should be no longer than the recommended 5 min, and the samples should be measured for chemiluminescence as soon as they are transferred to new assay tubes.
4. Acid reversion is used in the detection step in this particular protocol since the AE is detected as unhybridized AE-probe and not hybridized AE-probe.
5. Freezing of the magnetic microspheres can lead to clumping of the spheres, which leads to low capture efficiency and errantly low assay readings. Therefore do not freeze this reagent.
6. See Section III,D, Procedural Notes/Troubleshooting, General, for tips that are applicable to all AE-based procedures.

D. General Procedural Notes/Troubleshooting

Following are some procedural notes and troubleshooting tips that are generally applicable to handling AE and AE-labeled DNA probes. Some

of these suggestions have been repeated in selected sections for emphasis.

- The AE is a hydrophobic molecule, and as such has a propensity to stick to surfaces such as test tube walls and pipet tips. For this reason the AE must always be handled in solutions that contain agents that disrupt hydrophobic interactions and keep the AE in solution. Detergents and organic solvents fulfill this criterion very well. For example, lauryl sulfate, DMSO, or acetonitrile are commonly used to keep the AE in solution. Never try to transfer AE from water, as you will invariably leave some behind.

- For optimal storage stability, AE-probes should be stored in a pH 5 buffer (sodium acetate, lithium succinate, etc.) with a small amount of detergent (e.g., 0.1% SDS) present to keep the AE in solution (see 1 above). In this form the AE-probe is stable for months at -20°C and years at -70°C. However, multiple freeze-thawing cycles will reduce the shelf-life of AE-probes, which should therefore be stored in small aliquots.

- When thawing aliquots of AE-probe, the solution must be heated to 45 to 60°C for about 1 min and then vortexed to thoroughly resolubilize the AE. If this is not done, poor recoveries of AE-probe from the stock tube(s) can result. Solutions with high concentrations of detergents may have to be heated for 2 to 3 min in order to fully solubilize all components. For example, AE-probe in 2X hybridization buffer (see Section III,C) can form a gel when stored in the cold. Using the reagent like this can result in improper delivery of AE-probe and hybridization buffer, leading to errantly low hybridized values. This gel can be easily redissolved by heating at 60°C for 2 min, followed by thorough vortexing.

- Due to the "sticky" nature of the AE and the relatively high viscosity of many of the solutions used in the AE protocols, all pipetting steps should be done very slowly and carefully. Pipet tips should just barely pierce the surface of the liquid that is being pipetted from or to, since a relatively large amount of AE will otherwise stick to the outside of the tip. All AE solutions should be delivered as close to the bottom of the tube into which you are pipetting as possible. A fresh disposable pipette tip should be used for *each* sample and positive and negative controls to avoid carryover. Repeating pipettors may be used for the addition of the AE-probe in hybridization buffer (including the aliquots used in determination of the AE hydrolysis kinetics), differential hydrolysis buffer, and the separation and wash solutions in the differential hydrolysis + separation format. When using these devices, make sure the liquid is dispensed with a smooth, gentle delivery to avoid splashing of reagent and sample already present onto the sides of the tube.

- Chemiluminescence is typically expressed as relative light unit (RLU).

- Be careful not to contaminate reagents, pipettors, workspace, etc., as any contaminating chemiluminescence can lead to errant results. Be especially careful when using the stock solution of AE, since a 1- μ l aliquot of the 25 mM stock contains approximately 10¹¹ RLU of chemiluminescence. The use of disposable polystyrene or polypropylene labware is strongly recommended for reagent preparation.
- Before insertion into the luminometer, tubes should be gently blotted with a lightly dampened tissue or paper towel. This removes any residue from the outside of the tubes (thus keeping the luminometer free of contamination and protecting against instrument damage due to moisture build-up in the measurement chamber, etc.) and also reduces static charge (a build-up of this charge can lead to errant readings).
- Any AE-probe that becomes deposited on the sides or the rim of an assay tube may not come into proper contact with the hydrolysis buffer, which will lead to high background values. This can be avoided by always pipetting to the bottom of the tube and avoiding touching the sides and the rim of the tube. Thorough vortexing of the tubes after the addition of the hydrolysis buffer will also minimize this problem.
- Temperature is a very important parameter in AE-probe assays. Specificity of hybridization is temperature dependent, and AE hydrolysis rate is very temperature dependent. Therefore hybridization and differential hydrolysis temperatures should be accurately maintained to within a degree of the desired operating temperature. A consistent temperature from tube to tube and a rapid equilibration of the sample tubes to the correct temperature is also important. For these reasons, incubation in a circulating water bath is the recommended procedure, although a noncirculating water bath or a heating block can be used if they fulfill the requirements just described.
- Nucleophilic compounds can form adducts with the AE (Hammond *et al.*, 1991; see also Fig. 3 and Sections I,A and III,C). If formed during the differential hydrolysis step, the AE hydrolysis rate of unhybridized AE-probe may be reduced, leading to elevated negative control values.

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