Simultaneous Detection of Multiple Nucleic Acid Targets in a Homogeneous Format

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ABSTRACT: The acridinium ester 4-(2-succinimidyloxycarbonylethyl)phenyl-10-methylacridinium 9-carboxylate trifluoromethane sulfonate (AE), which reacts rapidly with alkaline hydrogen peroxide to produce light, has been used as a detection label in a number of assay procedures, including nucleic acid probe-based systems [Nelson et al. (1995) in *Nonisotopic Probing, Blotting and Sequencing* (Kricka, L. J., Ed.) pp 391−428, Academic Press, Inc., San Diego, CA]. We have synthesized a number of derivatives of this AE and characterized their chemiluminescent properties. These derivatives display significant differences in the kinetics of the chemiluminescence reaction as well as optimal pH for light production. These differences allow two or more derivatives to be simultaneously detected and quantitated in a single reaction vessel. Several of these derivatives have been covalently linked to nucleic acid probe molecules and have been further characterized in regard to chemiluminescence properties as well as hydrolysis of the ester bond in both single- and double-stranded conformations. On the basis of these properties, homogeneous assay formats utilizing DNA probes labeled with various AE derivatives were developed. Simultaneous detection and quantitation of *Chlamydia trachomatis* and *Neisseria gonorrhoeae*, the *gag* and *pol* regions of HIV, and wild-type and mutant HIV sequences was achieved with high sensitivity and discrimination.

The use of nucleic acid probes for the detection of specific DNA or RNA sequences has expanded dramatically during the last decade. The extremely high affinity and specificity afforded by hybridization, including the ability to detect single mismatches, coupled with the high sensitivity of current systems, including nucleic acid amplification techniques, render probe-based assays a powerful tool in a wide variety of applications. Medicine is one field which has been profoundly affected, with probe technology allowing rapid characterization and detection of genetic lesions responsible for disease as well as ultrasensitive detection of a wide variety of infectious agents.

In many applications, the ability to detect two or more targets in a single reaction vessel provides distinct advantages. For example, clinical diagnostic assays can be performed in which two or more organisms, such as *Chlamydia trachomatis* and *Neisseria gonorrhoeae,* can be detected in a single specimen. This simplifies the assay, increases through-put, and minimizes the consumption of what can often be a limited amount of clinical specimen. In amplification reactions, detection of both the target sequence and an internal amplification standard in the same reaction vessel provides a positive control of the amplification process in each assay tube. Since target and positive control can be detected without splitting the sample, contamination is minimized. Similarly, simultaneous amplification and detection of two different regions of a given target, such as the *gag* and *pol* regions of the HIV,[1] allows one to internally confirm the presence of a positive signal. In mutational analysis, the ability to detect two or more variants in a single tube allows one to monitor, quantitatively, the appearance of mutant populations.

There have been a number of dual analyte assays described in the literature, the majority of which have had application in immunoassays. Two radioisotopes of different energies have been used to discriminate dual analytes in a single assay (Beinlich et al., 1985; Desai et al., 1988; Dahl et al., 1988). However, due to the health hazards, disposal issues, general inconvenience, and short half-lives of some of the most commonly used radioisotopes, nonisotopic labels have recently gained widespread popularity. Fluorescent dyes with different emission wavelengths have been used for dual analyte detection in flow cytometry (Traganos et al., 1977; Hämmerle & Löffler, 1989; Szejda et al., 1984), immunoassays (Sidki et al., 1985; Siitari, 1990; Vuori et al., 1991), and nucleic acid probe assays (Iitiä et al., 1992; Sjöroos et al., 1995). Yet another method for detecting two analytes uses two different enzymes which catalyze the hydrolysis of two different substrates which then absorb light at different wavelengths (Philo & Allen, 1992). In a chemiluminescence-based system, different dioxetanes are cleaved, typically enzymatically, to yield compounds that emit light at different wavelengths (Bronstein & Voyta, 1990). A different chemiluminescent system employs an acridinium or phenanthridinium derivative which yields a short-lived signal upon chemical triggering and a dioxetane derivative which yields a longer-lived signal upon enzymatic triggering (Khalil et al., 1992).

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[1] Abbreviations: 1-Me-AE, 1- or 3-methyl acridinium ester; 1-Me-di-m-F-AE, 1- or 3-methyl *m*-difluoroacridinium ester; di-m-F-AE, *m*-difluoroacridinium ester; di-*o*-Br-AE, *o*-dibromoacridinium ester; di-o-Cl-AE, *o*-dichloroacridinium ester; di-o-F-AE, *o*-difluoroacridinium ester; di-o-CH_3-AE, *o*-dimethylacridinium ester; di-o-OCH_3-AE, *o*-dimethoxyacridinium ester; HIV, human immunodeficiency virus; HPA, hybridization protection assay; o-CH_3-AE, *o*-methylacridinium ester; o-F-AE, *o*-fluoroacridinium ester; o-OCH_3-AE, *o*-methoxyacridinium ester; o-OCH_3(c)-AE, *o*-methoxyacridinium ester (cinnamate derivative); rRNA, ribosomal RNA; rlu, relative light units.

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Common problems associated with multiple analyte assays, which are almost exclusively heterogeneous by necessity, include complex assay formats, the need to split the sample before measurement of signals, low sensitivity and dynamic range, poor discrimination of two (or more) analytes, and the need for sophisticated instrumentation. In the system reported here, various acridinium ester derivatives are utilized as detection labels in a simple, homogeneous DNA probe-based assay format.

Acridinium ester (AE) is a highly chemiluminescent molecule which has been used as a detection label in a variety of applications (Nelson & Kacian, 1990; Nelson & McDonough, 1994; Weeks et al., 1983; Woodhead, 1995). Advantages of AE include high sensitivity (detection limit $= 5 \times 10^{-19}$ mol), a wide dynamic range (at least 4 orders of magnitude), simple reaction chemistry (alkaline hydrogen peroxide triggers the chemiluminescence reaction), rapid reaction kinetics, low backgrounds, low quenching, long shelf-life, ease of use, and safety of handling and disposal.

Acridinium ester has been used in a number of DNA probe-based assay formats. One format, referred to as the hybridization protection assay (HPA), can be used in a completely homogeneous mode, requiring no physical separation to discriminate between hybridized and unhybridized probe (Arnold et al., 1989). Hydrolysis of the ester bond of the AE molecule renders the AE permanently nonchemiluminescent. When present in a single-stranded probe, AE hydrolysis is rapid. However, when the AE probe hybridizes to its target nucleic acid, the label binds to the duplex and the ester bond is protected from hydrolysis. Therefore, after hybridization, reaction conditions are adjusted such that chemiluminescence associated with single-stranded probe is rapidly reduced to low levels, whereas chemiluminescence associated with hybridized probe is minimally affected. After differential hydrolysis, the remaining signal is a direct measure of the amount of target present.

The work presented here describes the use of acridinium ester as the label in a homogeneous DNA probe-based multiple analyte assay system. This required development of acridinium ester derivatives whose chemiluminescence was distinguishable, yet could be used in the same hybridization protection assay by virtue of the similarity of their ester hydrolysis characteristics. Advantages of this multi-analyte assay system include high sensitivity in both amplified and nonamplified formats; simplicity, which allows an entire assay to be carried out in one tube without separation steps; and exquisite specificity, which allows genetic differences as small as a single mismatch to be readily discriminated from one another. Examples are given for the simultaneous assay and detection of *C. trachomatis* and *N. gonorrhoeae*, the *gag* and *pol* regions of HIV, and wild-type and mutant HIV sequences.

MATERIALS AND METHODS

Acridinium Ester Synthesis, Purification, and Characterization

Oxalyl chloride, 3-methyldiphenylamine, benzyl bromide, acrylonitrile, acridine-9-carboxylic acid, and 3,5-difluorophenol were purchased from Aldrich. 4-Hydroxy-3-methoxycinnamic acid and 3-(4-hydroxyphenyl)propionitrile were purchased from Lancaster. TLC plates (Whatman 250μ AL-Sil/UV) were purchased from Fisher. Silica gel was Merck grade 60 (200−400 mesh). TLC analysis was performed with the following systems: 9:1 toluene−tetrahydrofuran (S_1), 9:1 methanol−5% ammonia (S_2), 7:3 hexane−ethyl acetate (S_3), 8:2 hexane−ethyl acetate (S_4), 7:3:0.1 methylene chloride−acetonitrile−acetic acid (S_5), and 8:2:0.1 methylene chloride−acetonitrile−acetic acid (S_6). Samples for UV analysis were dissolved in acetonitrile containing 10 mM hydrochloric acid. HPLC analysis was conducted on a reversed-phase C18-5μ column (Beckman) and run with isocratic elution using 20% water, 20% 100 mM triethylammonium phosphate (pH 2.2), and 60% acetonitrile at 1 mL/min.

(A) Preparation of 1- or 3-Methylacridine-9-carboxylic Acid. Oxalyl chloride (1.04 g, 8.17 mmol) was dissolved in 6 mL of anhydrous carbon disulfide and gently refluxed under magnetic stirring and 3-methyldiphenylamine (1.0 g, 5.4 mmol) added dropwise as a 1 mL solution in carbon disulfide. The resulting mixture was refluxed for 2 h, after which solvent and excess reagent were evaporated. The yellow residue was redissolved in 20 mL of anhydrous carbon disulfide and refluxed again. Anhydrous aluminum chloride (1.5 g, 11.23 mmol) was then added in portions over a period of 30 min and the resulting black tar refluxed for an additional 2 h. TLC analysis (S_1) showed complete conversion to the expected isatin. The black mixture was then evaporated to dryness and the residue treated with 50 mL of a mixture of 1:1 concentrated hydrochloric acid−ice and gently stirred. The product was then extracted with 3 $\times$ 50 mL of a 3:2 mixture of ether and toluene. The organic phase was dried over magnesium sulfate and evaporated to yield 1.66 g of an orange fluffy solid. The solid was remixed with 50 mL of 1 M KOH and refluxed for 12 h. The clear orange solution was then cooled to room temperature, filtered, and poured into a 300 mL mixture of 1:1 concentrated HCl−ice. The resultant orange solid was filtered off, washed with 10 mL of cold distilled water, and dried over phosphorus pentoxide under vacuum to yield 0.8 g (62%) of 1- or 3-methylacridine-9-carboxylic acid which ran as a single spot on TLC (S_2). IR (KBr): 3412.8, 2907.5, 1642.

(B) Preparation of 3-(4-Hydroxy-3-methoxybenzyl) Cinnamate. 4-Hydroxy-3-methoxycinnamic acid (200 mg, 1.03 mmol) was dissolved at 60 °C in 4 mL of water containing KOH (57.79 mg, 1.03 mmol). The mixture was then evaporated to dryness, yielding the potassium salt of 4-hydroxy-3-methoxycinnamic acid. The salt was redissolved in 6 mL of a mixture of 90:5:5 ethanol−isopropyl alcohol−water and treated dropwise with benzyl bromide (176.2 mg, 1.03 mmol) under reflux. After 2 h, potassium bromide, which forms as a byproduct, was filtered off and the filtrate evaporated to dryness. The residue was dissolved in 15 mL of ether, washed with 15 mL of saturated sodium bicarbonate followed by 15 mL of distilled water, and dried over magnesium sulfate. Evaporation to dryness yielded a colorless oil which was dried over phosphorus pentoxide under vacuum to yield 145 mg (50%) of pure product as judged by TLC (S_3). IR (KBr): 3442.1, 1756.4−1735.9.

(C) Preparation of 3-(4-Hydroxy-2,6-difluorophenyl)-propionitrile. 3,5-Difluorophenol (1.0 g, 7.68 mmol) and acrylonitrile (0.55 g, 7.68 mmol) were mixed by magnetic stirring, and anhydrous aluminum chloride (0.51 g, 3.84 mmol) was then added in small portions over a 20 min period. Dry HCl gas was bubbled into the mixture for a

period of 90 min and the resulting red oil heated at 80 °C for 2 h. After the oil cooled to room temperature, 20 g of crushed ice was added slowly to the amorphous red glass, and the resulting slurry was dissolved by stirring for 2 h. The solution was adjusted to 40 mL with water and the product extracted with 3 × 60 mL of methylene chloride, dried over magnesium sulfate, filtered, and evaporated to give a pale yellow oil. Purification by silica gel chromatography using S_4 yielded 0.4 g (31%) of a colorless oil which was judged pure by TLC (S_3). IR (KBr): 3340.6, 2260.1, 999.1. ^{1}H-NMR (DMSO-d_6): 2.75 (t, 4H, CH_2CH_2), 7.10 (s, 2H aromatic), 10.04 (br s, 1H, OH).

(D) Preparation of 3-(4-Hydroxy-3,5-dibromophenyl)-propionitrile. Bromine (300 μL, 6.0 mmol) was added dropwise to a stirred solution of 3-(4-hydroxyphenyl)-propionitrile (150 mg, 1.02 mmol) in 10 mL of glacial acetic acid. The resulting red solution was heated at 100 °C for 4 h. TLC analysis (S_3) showed nearly complete conversion to the desired product. The solvent was evaporated, and the residue was dissolved in 10 mL of methylene chloride and washed with 10 mL of water. The organic phase was dried over magnesium sulfate, filtered, and evaporated to yield a light brown residue which was a mixture of the 3,5-dibromophenyl and 3-bromophenyl isomers as judged by TLC (S_3). Purification by silica gel chromatography (S_4) yielded 93.3 mg (30%) of pure product as judged by TLC (S_3). IR (KBr): 3340, 2260.0, 662.3. ^{1}H-NMR (DMSO-d_6): 3.0−3.30 (2t, CH_2CH_2), 7.88 (s, 2H aromatic), 10.16 (br s, 1H, OH).

(E) Preparation of 4-(2-Succinimidyloxycarboxyethyl)-phenyl-10-methylacridinium 9-Carboxylate Trifluoromethane Sulfonate. Acridine-9-carbonyl chloride and 3-(4-hydroxyphenyl)benzyl propionate were condensed and converted to the desired product as previously described (Weeks et al., 1983). TLC (S_5): R_f 0.2. UV: 365.5 (3.60), 260.5 (4.35). IR (KBr): 1810, 1783.8, 1745.7. ^{1}H-NMR (DMSO-d_6): 3.05 (t, 4H, CH_2 succinyl), 3.29 (2t, 4H, CH_2CH_2), 4.99 (s, 3H, NCH_3), 7.52−7.74 (2d, 4H phenyl), 7.83−9.00 (2d, 2t, 8H aromatic).

(F) Preparation of 4-(2-Succinimidyloxycarboxyethylenyl)-2-methoxyphenyl-10-methylacridinium 9-Carboxylate Trifluoromethane Sulfonate. Acridine-9-carbonyl chloride and 3-(4-hydroxy-3-methoxy)benzyl cinnamate were condensed and converted to the desired product as previously described for unsubstituted AE (Weeks et al., 1983). UV: 365.3 (3.37), 260.8 (4.40). TLC (S_6): R_f 0.1. IR (KBr): 1810, 1784.3, 1739.1. ^{1}H-NMR (DMSO-d_6): 3.01 (s, 4H, CH_2 succinyl), 3.60 (s, 3H, OCH_3), 4.99 (s, 3H, NCH_3), 5.60−6.20 (2s, CH═CH), 7.24−7.81 (1s + 2d, 3H, phenyl), 7.89−9.00 (2d + 2t, 8H aromatic). MS-FAB+: 513 (M − CF_3SO_3)$^+$.

(G) Preparation of 4-(2-Succinimidyloxycarboxyethyl)-2,6-dibromophenyl-10-methylacridinium 9-Carboxylate Trifluoromethane Sulfonate. Acridine-9-carbonyl chloride was condensed with 3-(4-hydroxy-3,5-dibromophenyl)propionitrile to yield acridine-9-carboxyl(2,6-dibromophenyl)propionitrile ester. The nitrile group was hydrolyzed to the carboxylic acid using concentrated HCl at 80 °C for 2 h. The product was isolated in 63% yield by precipitation in cold water and subsequently converted to the desired product as previously described for unsubstituted AE (Weeks et al., 1983). TLC (S_5): R_f 0.3. UV: 369.4 (3.52), 262.5 (4.46). IR (KBr): 1813.7, 1783.6, 1746.9. ^{1}H-NMR (DMSO-d_6): 2.78 (s, 4H, CH_2 succinyl), 3.00−3.20 (2t, CH_2CH_2), 4.98 (s, 3H, NCH_3), 7.68 (s, 2H phenyl), 7.96−9.08 (2t + 2d, 8H aromatic). MS-FAB+: 639 (M − CF_3SO_3)$^+$.

(H) Preparation of 4-(2-Succinimidyloxycarboxyethyl)-phenyl-1(3),10-dimethylacridinium 9-Carboxylate Trifloromethane Sulfonate. 1- or 3-methylacridine-9-carboxylic acid was converted to the carbonyl chloride, condensed with 3-(4-hydroxyphenyl)benzyl propionate, and converted to the desired product as previously described for unsubstituted AE (Weeks et al., 1983). TLC (S_6): R_f 0.4. UV: 370.6 (3.49), 263.2 (4.41). IR (KBr): 1814, 1784.2, 1740.0. ^{1}H-NMR (DMSO-d_6): 2.90 (s, 3H, CH_3), 3.10 (s, 4H, CH_2 succinyl), 3.30 (2t, 4H, CH_2CH_2), 4.99 (s, 3H, NCH_3), 7.50−7.65 (2d, 4H, phenyl), 7.92−8.95 (1s + 4d + 2m, 8H). MS-FAB+: 497 (M − CF_3SO_3)$^+$.

(I) Preparation of 4-(2-Succinimidyloxycarboxyethyl)-3,5-difluorophenyl-1(3),10-dimethylacridinium 9-Carboxylate Trifluoromethane Sulfonate. This compound was prepared by the same pathway used to prepare the corresponding (2,6-dibromophenyl)acridinium ester derivative. TLC (S_5): R_f 0.3. UV: 371.9 (3.41), 263.3 (4.46). IR (KBr): 1814, 1783.6, 1767.1. ^{1}H-NMR (DMSO-d_6): 2.90 (s, 3H, CH_3), 3.14 (s, 4H, CH_2 succinyl), 3.29 (2t, 4H, CH_2CH_2), 4.99 (s, 3H, NCH_3), 7.83 (s, 2H phenyl), 7.92−9.10 (1s + 3d + 2t, 7H aromatic). MS-FAB+: 533 (M − CF_3SO_3)$^+$.

Synthesis and Purification of Acridinium Ester-Labeled DNA Probes

Oligonucleotide probes were synthesized using standard solid-phase phosphoramidite chemistry on a Biosearch 8750 or ABI 380A DNA Synthesizer and purified using standard polyacrylamide gel electrophoresis. An amine-terminated linker arm was incorporated at a predetermined position in each oligonucleotide during synthesis utilizing a non-nucleotide-based, phosphoramidite linker arm reagent described elsewhere (Arnold & Bhatt, 1989). The oligomers were then labeled with various acridinium ester compounds and subsequently purified using high-performance liquid chromatography as described previously (Arnold & Nelson, 1993).

Measurement of Chemiluminescence

The chemiluminescent signal from each of the acridinium ester derivatives was measured in either a LEADER I or LEADER 50 single tube luminometer (Gen-Probe Inc., San Diego, CA). Data are expressed as relative light units, or rlu. The kinetics of each chemiluminescent reaction were measured in the "kinetic mode" of the luminometer, which collects and integrates the signal over time intervals as short as 20 ms.

Characterization of the Chemiluminescence Properties of Various Acridinium Ester-Labeled DNA Probes

Acridinium ester-labeled probes were diluted in 0.01 M lithium succinate (pH 5) and 0.1% (w/v) lithium lauryl sulfate to yield approximately 100000−200000 rlu per 10 μL. Aliquots of 10 μL were added to 12 × 75 mm polystyrene tubes (Sarstedt), and chemiluminescence was read for various times in the luminometer using a 0.1 s delay between automatic injections of 200 μL of 0.1% H_2O_2, 1 mM HNO_3 (detection reagent 1), and 1 N NaOH (detection reagent 2A). From these data, the time-to-peak and duration of the

chemiluminescence were determined for each labeled probe (duration was arbitrarily defined as the time required for the rlu to return within 10% of base line). The pH required for maximal rlu of each label (optimum pH) was determined using the same method described above, except that the second injection was changed to 0.24 M sodium borate buffer at various pH values. Time-to-peak as well as duration were also measured at the optimum pH.

Characterization of the Differential Hydrolysis Properties of Acridinium Ester-Labeled DNA Probes

The hydrolysis rates of the various acridinium esters attached to oligonucleotide probes were determined for both hybridized and single-stranded forms as described previously (Nelson et al., 1995). Briefly, approximately 0.1 pmol of labeled probe and 1 pmol of target nucleic acid were combined in 30 μL of 0.1 M lithium succinate (pH 5.2), 8.5% lithium lauryl sulfate (w/v), 1.5 mM EDTA, and 1.5 mM EGTA (succinate buffer 1) and incubated at 60 °C for 30 min. The reaction mixture was then diluted with 270 μL of succinate buffer 1, and 10 μL aliquots were placed in replicate 12 × 75 mm polystyrene tubes. To each tube was added 100 μL of 0.19 M $Na_2B_4O_7$ (pH 7.6) and 5% Triton X-100, and the tubes were placed at 60 °C. At various time points, 200 μL of 0.4 N HCl and 0.1% H_2O_2 was added to one of the replicates and the chemiluminescence was measured in the luminometer using the automatic injection of detection reagent 2A followed by a 5 s read time. The data were then plotted as the log of the percent of the remaining chemiluminescence (compared with time zero) versus time, and hydrolysis rates were calculated from the slope using standard regression analysis.

Simultaneous Detection of Two Acridinium Ester-Labeled DNA Probes

1-Me-di-m-F-AE- and 1-Me-AE-labeled single-stranded probes were diluted in succinate buffer 1 to yield a primary stock containing approximately 200000 rlu per 50 μL aliquot, and various dilutions of the primary stocks were also prepared in succinate buffer 1. Samples were prepared for detection in 12 × 75 mm tubes by adding various amounts of either probe to a final volume of 100 μL of succinate buffer 1. To each sample was then added 300 μL of 90 mM $Na_2B_4O_7$ buffer (pH 8.5) and 2% (v/v) Triton X-100, and the chemiluminescence was read every 0.04 s (2 s total) in the luminometer using a 0.1 s delay between automatic injections of detection reagent 1 and 1.5 N NaOH (detection reagent 2B). Data were collected directly into a standard personal computer, and the amount of each label was calculated using the resolving routine described below.

Resolution of Overlapping Chemiluminescence Profiles

Samples containing only one labeled probe were used as standards, while samples containing no probe were used as blanks. As described above, kinetic data were collected over multiple time intervals during the chemiluminescence reaction. The blank values at each interval were first subtracted from the standard values at each corresponding interval. Then a ratio between the sum of the rlu in one range of intervals and the sum of the rlu in another range of intervals was determined for each standard ($R = \sum_{\text{range 1}}/\sum_{\text{range 2}}$). Commonly used ranges were as follows: range 1 = intervals 41−50 and range 2 = intervals 1−6. The total rlu of a sample (T) was also determined for each standard and expressed as a ratio with a given range. Commonly used values were as follows: $T_{1\text{-Me-di-m-F-AE}} = \sum_{1-50}/\sum_{1-6}$ and $T_{1\text{-Me-AE}} = \sum_{1-50}/\sum_{41-50}$. To calculate the amount of each label in a mixture of the two, the blank values were first subtracted at each interval and then the rlu in range 1 were summed and divided by $R_{1\text{-Me-AE}}$, yielding the rlu contributed in range 2 by the 1-Me-AE. This assumes that all the rlu in range 1 were contributed by 1-Me-AE; this is obviously not correct in a mixture of the two labels, but this error is corrected as the calculation proceeds. This value was then subtracted from the total rlu in range 2, yielding the rlu contributed by the 1-Me-di-m-F-AE in these intervals. This value was then multiplied by $R_{1\text{-Me-di-m-F-AE}}$, yielding the rlu contributed in range 1 by 1-Me-di-m-F-AE. This value was then subtracted from the total rlu in range 1, yielding a *corrected* value for 1-Me-AE in this range. This new value for 1-Me-AE was then used to repeat the entire calculation, and this cycle of calculation and correction was continued until the values were constant. The total rlu for each label was then calculated from these values using T calculated for each standard: total rlu = $T_{1\text{-Me-di-m-F-AE}}$(final value for 1-Me-di-m-F-AE in range 2) and total rlu = $T_{1\text{-Me-AE}}$(final value for 1-Me-AE in range 1).

Simultaneous Detection of C. trachomatis and N. gonorrhoeae Nucleic Acid Targets in a Homogeneous Assay Format

The probes utilized for detection of *C. trachomatis* and *N. gonorrhoeae* were those used in commercially available assay kits for these two organisms (PACE 2 CTD and NGD assays, Gen-Probe Inc., San Diego, CA). The probes for *N. gonorrhoeae* were labeled with 1-Me-di-m-F-AE, and the probes for *C. trachomatis* were labeled with 1-Me-AE as described above. Target nucleic acid was ribosomal RNA (rRNA) from the two different organisms. Samples were prepared for assay by adding the desired amount of rRNA target to 30 mM sodium phosphate buffer (pH 6.8), 3% (w/v) lithium lauryl sulfate, 1 mM EDTA, and 1 mM EGTA. Probe reagent was prepared by combining the 1-Me-di-m-F-AE *N. gonorrhoeae* and 1-Me-AE *C. trachomatis* probes (approximately 0.2 pmol each per 50 μL) in 200 mM lithium succinate buffer (pH 5.2), 17% (w/v) lithium lauryl sulfate, 3 mM EDTA, and 3 mM EGTA (succinate buffer 2). Hybridization was achieved by mixing 50 μL of the target sample with 50 μL of the probe reagent and incubating at 55 °C for 60 min. Hybridized and nonhybridized probe were discriminated using the hybridization protection assay by adding 300 μL of 0.15 M $Na_2B_4O_7$ (pH 8.5) and 2% (v/v) Triton X-100 and incubating at 55 °C for 20 min. The chemiluminescence of each sample was then measured in a luminometer using a 2 s delay between automatic injections of detection reagent 1 and 1 N NaOH, 2% (w/v) Zwittergent 3-14 (CalBiochem, La Jolla, CA) (detection reagent 2C). Signal was collected in 0.04 s intervals for 2 s and resolved as described above.

Simultaneous Detection of the gag and pol Regions of HIV DNA in a Homogeneous Assay Format

Probes complementary to the *gag* and *pol* regions of HIV-1 were synthesized and labeled with 1-Me-AE and di-o-Br-

AE, respectively (*gag* sequence = 5′-GTC ATC CAT CCT ATT TGT TCC TGA AGG GTA C-3′; *pol* sequence = 5′-CTA CTA TTC TTT CCC CTG CAC TGT ACC CC-3′). A probe reagent containing 5.5 fmol of 1-Me-AE *gag* probe and 16 fmol of di-o-Br-AE *pol* probe per 100 μL of succinate buffer 2 was prepared. A cloned HIV-1 DNA fragment containing both the *gag* and *pol* regions was amplified (100 μL reaction volume) using TMA (transcription-mediated amplification) as described elsewhere (Kacian & Fultz, 1995). Following amplification, 100 μL of the probe reagent was added and the reaction mixture was incubated at 60 °C for 30 min. Three hundred microliters of 0.13 M $Na_2B_4O_7$ (pH 9.3), 2% (v/v) Triton X-100, and 13 mM iodoacetic acid was then added, and the mixture was incubated at 60 °C for 20 min. The chemiluminescence of each sample was then measured in a luminometer using a 2 s delay between the automatic injection of detection reagent 1 and detection reagent 2B. Signal was collected in 0.04 s intervals for 2 s and resolved as described above.

Simultaneous Detection of Wild-Type and Mutant HIV-1 Nucleic Acid Targets in a Homogeneous Assay Format

The target for this assay was the HIV-1 reverse transcriptase coding region, which contains a commonly occurring point mutation in codon 181 (Richman et al., 1991). The wild-type sequence of codon 181 is TAT, whereas the mutant sequence is TGT. Oligonucleotide probes were designed for this region, one exactly complementary to the wild-type sequence and one exactly complementary to the mutant sequence. Each probe was synthesized with an amine-terminated linker arm located immediately adjacent to the site of mutation: wild-type probe = 5′-···AT#A···-3′ and mutant probe = 5′-···AC#A···-3′ (# represents the site of linker arm attachment). The wild-type probe was labeled with 1-Me-di-m-F-AE, and the mutant probe was labeled with 1-Me-AE as described above.

Probe reagent was prepared by combining the 1-Me-di-m-F-AE wild-type and 1-Me-AE mutant probes (approximately 0.05 pmol each per 50 μL) in lithium succinate buffer 2. Hybridization was achieved by mixing 50 μL of the desired target sample in water with 50 μL of the probe reagent and incubating at 60 °C for 60 min. Hybridized and nonhybridized probe were discriminated using the hybridization protection assay by adding 300 μL of 0.09 M $Na_2B_4O_7$ (pH 8.5) and 5% (v/v) Triton X-100 and incubating at 62.5 °C for 12 min. The chemiluminescence of each sample was then measured in a luminometer using a 2 s delay between the automatic injection of detection reagent 1 and detection reagent 2B. Signal was collected in 0.04 s intervals for 2 s and resolved as described above.

RESULTS

The goal of this work was to develop a homogeneous, DNA probe assay for the simultaneous detection of multiple analytes. In order to achieve this goal, we had to identify AE-labeled probes with distinct chemiluminescence characteristics yet similar hydrolysis characteristics. Basic hydrogen peroxide reacts with the 9 position of AE to form a highly strained dioxetanone ring, decomposition of which results in the emission of light (Figure 1). The addition of substituents to AE that alter the electron density of the 9 position or that alter the basicity of the phenol leaving group

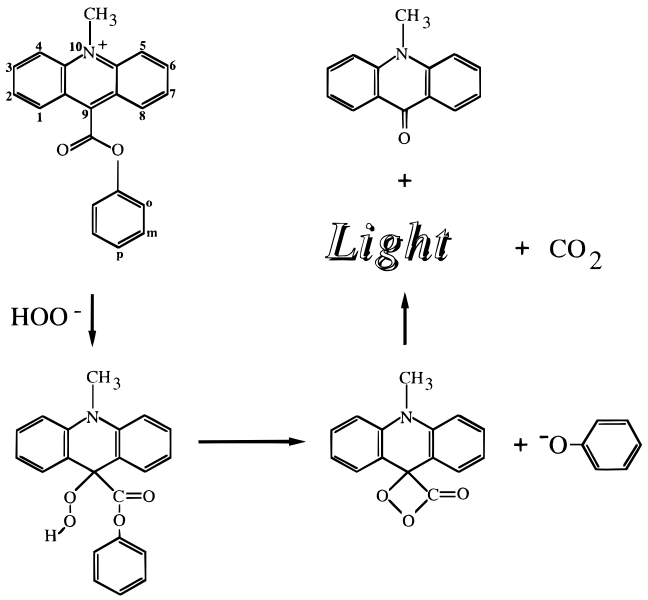

FIGURE 1: Reaction of peroxy anion (OOH^-) with acridinium ester to generate light. See text for details.

would be expected to alter the kinetics of chemiluminescence as well as the hydrolysis characteristics of AE. Therefore, a number of acridinium ester derivatives containing electron-donating and/or electron-withdrawing groups were synthesized, and DNA probes were labeled with these derivatives and characterized. Several AE derivatives were identified which possess distinct chemiluminescence characteristics and yet have similar hydrolysis characteristics (Table 1). For example, 1-Me-AE and 1-Me-di-m-F-AE have similar hydrolysis rates, but the kinetics of light emission for 1-Me-di-m-F-AE are much faster than those for 1-Me-AE. Similarly matched pairs of labels include 1-Me-AE and o-OCH_3(c)-AE, unmodified AE and o-OCH_3(c)-AE, and 1-Me-AE and di-o-Br-AE.

To demonstrate that two different probes could be detected simultaneously in one tube, we examined the individual and combined chemiluminescence characteristics of a 1-Me-di-m-F-AE-labeled probe and a 1-Me-AE-labeled probe. As shown in Figure 2A, the light emission profiles of these two labels exhibit distinct time-to-peak and signal duration and can therefore be clearly differentiated from one another. In Figure 2B, the light emission profile of an authentic mixture of both labels is compared to a computer generated emission profile obtained by summing the emission profiles of the individual labels. The experimental and computer-generated profiles are nearly identical, demonstrating that the two labels react independently of one another in the mixture.

We next investigated whether solutions containing differing amounts of these two labels could be accurately detected in a single tube. The chemiluminescence of various amounts of each labeled probe as well as various combinations of both labeled probes were detected as described in Materials and Methods. The amount of each label in various mixtures was determined by resolving the overlapping profiles of the labels using a reiterative method as described in Materials and Methods. As summarized in Table 2, the presence of as little as 0.5% of either label in a mixture could be accurately detected by this method.

We next investigated whether two different nucleic acid targets could be detected in the same tube by this method.

Table 1: Chemiluminescence and Hydrolysis Properties of Various Acridinium Ester Derivatives[a]

	chemiluminescence characteristics					hydrolysis characteristics				
	standard conditions		optimal conditions					$t_{1/2}$ (min)		
compound	peak (s)	duration (s)	pH	peak (s)	duration (s)	temp (°C)	pH	hyb.	unhyb.	ratio
unmodified AE	0.40	3.0	11.9	0.42	4.6	60	7.6	18.1	0.67	27.0
di-o-Br-AE	0.16	0.42	10.2	0.22	1.8	60	7.6	25.0	2.68	9.3
1-Me-AE	0.50	3.0	11.9	0.75	14	60	7.6	215	2.00	108
o-OCH_3(c)-AE	0.60	8.0	13.0	0.60	8.4	60	7.6	63.2	2.10	30.2
1-Me-di-m-F-AE	0.25	0.45	11.3	0.22	1.2	60	7.6	179	2.24	79.9

[a] Standard and optimum light-off conditions are described in Materials and Methods. The time to reach maximum light intensity is denoted as peak, while the time for chemiluminescence to return to within 10% of base line is denoted as duration. Half-lives ($t_{1/2}$) of hydrolysis for hybrids (hyb.) and single-stranded probes (unhyb.) were determined as described in Materials and Methods. Ratio = $t_{1/2}$ (hyb.)/$t_{1/2}$ (unhyb.). The label nomenclature is as follows. Unmodified AE has a propionate linking group in the para position of the phenyl ring. di-o-Br-AE is the same as unmodified AE, except it has a bromine in each of the ortho positions of the phenyl ring. 1-Me-AE is the same as unmodified AE, except it has a methyl group in the 1 or 3 position of the acridinium ring (regioisomers). o-OCH_3(c)-AE is the same as unmodified AE, except it has a methoxy group in the ortho position of the phenyl ring and a cinnamate linking group instead of a propionate linking group. 1-Me-di-m-F-AE is the same as unmodified AE, except it has a methyl group in the 1 or 3 position of the acridinium ring (regioisomers) and a fluorine in each of the meta positions of the phenyl ring.

Table 2: Simultaneous Detection of Two Acridinium Ester-labeled Probes[a]

[probe] (amol)		expected values		observed values	
probe 1	probe 2	probe 1	probe 2	probe 1	probe 2
2240	0	207000	0	209711	358
680	0	62000	0	58883	113
280	0	26000	0	27662	0
70	0	6300	0	5401	25
28	0	2600	0	2490	89
14	0	1300	0	1384	0
0	2000	0	213000	0	210560
0	500	0	54000	256	51242
0	200	0	22000	143	21005
0	50	0	5400	31	5191
0	20	0	2300	40	2141
0	10	0	1100	0	1002
2240	2000	207000	213000	208873	206861
2240	500	207000	54000	204797	52827
2240	200	207000	22000	198493	21723
2240	50	207000	5400	193049	5689
2240	20	207000	2300	207762	2326
2240	10	207000	1100	226934	1329
2240	2000	207000	213000	208873	206861
680	2000	62000	213000	60673	209060
280	2000	26000	213000	24952	205735
70	2000	6300	213000	4203	207142
28	2000	2600	213000	2125	204559

[a] Probe 1 was labeled with 1-Me-di-m-F-AE; probe 2 was labeled with 1-Me-AE. Observed values for each label were calculated using the method for resolution of overlapping chemiluminescence profiles described in Materials and Methods and represent the average of two or more replicates. Expected values were calculated from the known specific activities of each probe. Data were collected for 2 s in 0.04 s intervals, and calculation ranges were intervals 1−6 (range 2) and 31−50 (range 1) (see Figure 2A for the chemiluminescence profiles of these two labels and the location of these ranges within each profile).

Table 3: Simultaneous Detection of *C. trachomatis* and *N. gonorrhoeae*[a]

[*N. gon.*] (fmol)	[*C. trach.*] (fmol)	expected values		calculated values	
		N. gon.	*C. trach.*	*N. gon.*	*C. trach.*
10.0	0	102000	0	102857	243
2.5	0	29000	0	27769	948
0.5	0	7600	0	5533	2078
0	3.00	0	103000	0	110774
0	0.75	0	30000	640	30419
0	0.15	0	7400	266	7510
10.0	3.00	102000	103000	98729	116462
10.0	0.75	102000	30000	89825	36738
10.0	0.15	102000	7400	102238	4411
2.5	3.00	29000	103000	28441	115172
2.5	0.75	29000	30000	23564	37321
2.5	0.15	29000	7400	27282	8531
0.5	3.00	7600	103000	2317	118248
0.5	0.75	7600	30000	4223	33129
0.5	0.15	7600	7400	5959	7449
0	0	0	0	355	1811

[a] Various concentrations of *C. trachomatis* (*C. trach.*) and *N. gonorrhoeae* (*N. gon.*) rRNAs were assayed using the hybridization protection assay format as described in Materials and Methods. Observed values for each label were calculated from the experimental data using the resolving method described in Materials and Methods and represent the average of two or more replicates. Expected values were calculated from the known specific activities of each probe. Data were collected for 2 s in 0.04 s intervals, and calculation ranges were intervals 1−6 (range 2) and 41−50 (range 1).

In these and subsequent measurements, the homogeneous HPA format was used to discriminate between unhybridized and hybridized probes. Differing amounts of rRNA from *C. trachomatis* and *N. gonorrhoeae* were first mixed together. Two probes, one specific for *N. gonorrhoeae* and labeled with 1-Me-di-m-F-AE and one specific for *C. trachomatis* and labeled with 1-Me-AE, were hybridized to the mixture of rRNAs, and the AE attached to unhybridized probe was destroyed *in situ* by alkaline hydrolysis (pH 8.5) at 55 °C for 20 min. The amount of each hybridized target was then determined by resolving the emission profiles of each label. The presence of as little as 1.5% of either rRNA target in a mixture of the two could be accurately detected by this method (Table 3).

To determine whether two different genes from the same genome could be detected in the same tube by this method, DNA probes complementary to the *gag* and *pol* regions of HIV-1 were synthesized and labeled with 1-Me-AE and di-o-Br-AE, respectively. Decreasing amounts of a cloned HIV-1 DNA fragment containing both the *gag* and *pol* regions was amplified using TMA (transcription-mediated amplification) (Kacian & Fultz, 1995). Following amplification, a mixture of both probes was hybridized to the amplified DNA. AE associated with unhybridized probe was then destroyed *in situ* by alkaline hydrolysis at 60 °C, and the amounts of *gag* and *pol* targets were determined by resolving the emission profiles of each label. As summarized in Table 4, both the *gag* and *pol* genes could be detected simultaneously from as little as 1.25 copies of HIV-1 DNA. The data sets for 2.5 and 1.25 copies have what appear to be

Table 4: Simultaneous Detection of the *gag* and *pol* Regions of HIV-1[a]

		calculated values	
input template (copies)	total rlu	*pol*	*gag*
20	424149	163116	275832
20	474982	181555	288683
20	502688	175009	326109
20	487885	167060	343985
5	168892	72321	78129
5	275045	84425	166487
5	262052	102490	137456
5	290219	121739	140858
2.5	181562	31211	121724
2.5	221174	53702	146548
2.5	242543	115704	116987
2.5	12327	4205	8943
1.25	214078	86608	117385
1.25	7036	4738	1637
1.25	403548	112971	277915
1.25	3246	2265	497
0	4035	3019	508
0	4119	2634	2255
0	4396	3079	730
0	4340	2954	1542

[a] A cloned HIV-1 DNA fragment containing both the *gag* and *pol* regions was amplified, and the product was assayed using the hybridization protection assay format as described in Materials and Methods. Observed values for each label were calculated using the resolving method described in Materials and Methods (each of the four replicates shown for each target amount represents a separate amplification reaction). Data were collected for 2 s in 0.04 s intervals, and calculation ranges were intervals 1−4 (range 2) and 41−50 (range 1).

aberrantly low values for one or two of the replicates. These are not aberrant points, but rather samples which did not receive input target before amplification. This is always observed at low copy number since there is a statistically defined probability that no template will be delivered in a given pipetting operation. In fact, the percentage of samples which are predicted statistically to not receive template at low copy number must be taken into account when determining sensitivity of the amplification procedure.

A characteristic feature of HIV-1 replication is the appearance of mutant genomes. One point mutation occurs at codon 181 in the reverse transcriptase coding region (Richman et al., 1991). To determine whether a small amount of this mutant genome could be detected in the presence of excess wild-type genome, we prepared various mixtures of both genomes and hybridized wild-type and mutant probes labeled with 1-Me-AE and 1-Me-di-m-F-AE, respectively. Although under our hybridization conditions mutant probe hybridizes to wild-type target and wild-type probe hybridizes to mutant target, the resultant mismatched hybrids can be distinguished from the desired matched hybrids by selective alkaline hydrolysis of mismatched hybrids (Nelson et al., 1990). Following hybridization, AE associated with unhybridized probes and mismatched targets was destroyed *in situ* by alkaline hydrolysis, and the amounts of mutant and wild-type targets were determined by resolving the emission profiles of each label. As little as 2% of the mutant reverse transcriptase gene could be detected in the presence of the wild-type reverse transcriptase gene (Table 5).

Table 5: Simultaneous Detection of Wild-type and Mutant HIV-1[a]

concentration (fmol)		expected values		calculated values	
wild type	mutant	wild type	mutant	wild type	mutant
5.0	0	75000	0	86632	109
0.5	0	7700	0	5632	109
0.1	0	1600	0	2013	109
0	5.0	0	128000	0	112047
0	0.5	0	13200	20	10832
0	0.1	0	2000	95	1750
5.0	5.0	75000	128000	79812	116314
5.0	0.5	75000	13200	68507	10942
5.0	0.1	75000	2000	77682	765
0.5	5.0	7700	128000	6833	113469
0.5	0.5	7700	13200	8280	10395
0.5	0.1	7700	2000	7400	1422
0.1	5.0	1600	128000	1010	102308
0.1	0.5	1600	13200	1133	10504
0.1	0.1	1600	2000	1570	1750
0	0	0	0	273	0

[a] Wild-type and mutant sequences in the codon 181 region of the reverse transcriptase gene of HIV-1 were simultaneously detected using the hybridization protection assay format as described in Materials and Methods. Observed values for each label were calculated using the resolving method described in Materials and Methods and represent the average of two or more replicates. Expected values were calculated from the known specific activities of each probe. Data were collected for 2 s in 0.04 s intervals, and calculation ranges were intervals 1−4 (range 2) and 41−50 (range 1).

DISCUSSION

Acridinium ester reacts rapidly with hydrogen peroxide under alkaline conditions by the mechanism depicted in Figure 1 to produce light at 430 nm. In order to discriminate between two or more acridinium ester labels in the same solution, one or more of the properties of this chemiluminescent reaction must be altered. In the research presented here, alteration of the kinetics of the reaction was the primary approach used to accomplish this goal.

Chemical modification of the phenyl ring can affect the chemiluminescence reaction in a variety of ways. One reason that phenylacridinium esters exhibit relatively high chemiluminescence yields is that phenol is a good leaving group. Changes in the pK_a of this leaving group directly affect the quantum yield and rate of the chemiluminescent reaction (Batmanghelich et al., 1991). Addition of electron-withdrawing groups to the phenyl ring lowers the pK_a of the phenol, making it a better leaving group, which results in increased quantum yield and an increased rate of the chemiluminescent reaction. In contrast, addition of electron-donating groups raises the pK_a of the phenol, making it a worse leaving group, which results in decreased quantum yield and a decreased rate of the chemiluminescent reaction. Substitutions on the phenyl ring may also affect the chemiluminescence reaction by altering the electron density of the acridinium ring. Electron-withdrawing groups could polarize the electron density toward the phenyl ring, decreasing the charge density of the C9 position of the acridinium ring, resulting in a more rapid reaction with the nucleophilic hydroperoxide anion to initiate chemiluminescence (see Figure 1). Conversely, electron-donating groups could increase the charge density of the C9 position, thereby reducing the rate of reaction with peroxide. Leaving group pK_a and inductive effects work in the same direction for a given substituent to either increase or decrease the rate of the chemiluminescent reaction. Finally, substitutions on the phenyl ring, particularly those in the ortho position, may affect light emission by sterically hindering reaction with hydrogen peroxide.

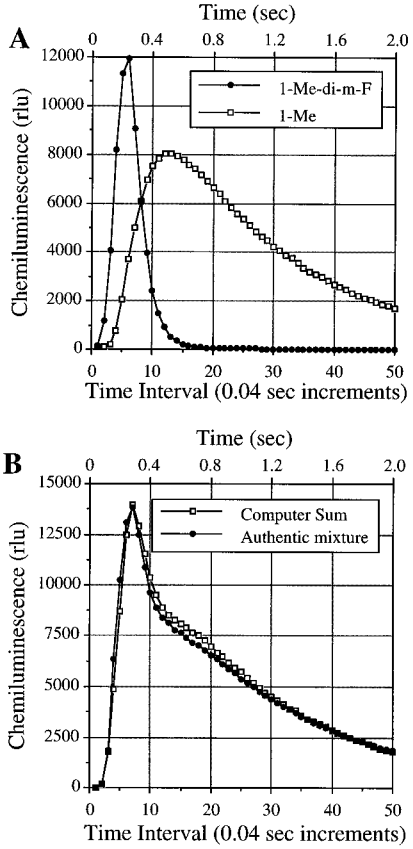

FIGURE 2: Plots of chemiluminescence versus time for 1-Me-di-m-F-AE and 1-Me-AE probes. Measurements were performed as described in Materials and Methods, with data collected at 0.04 s intervals for 2 s: (A) plots of the individual labels (●, 1-Me-di-m-F-AE; □, 1-Me-AE) and (B) comparison of the sum of the chemiluminescence emission profiles of the individual labels to the chemiluminescence emission profile of the simultaneous reaction of both labels in a single tube (□, 1-Me-di-m-F-AE + 1-Me-AE, sum of profiles from panel A; ●, 1-Me-di-m-F-AE + 1-Me-AE, chemiluminescent reaction of a mixture of both labels).

Examples of these effects in various AE derivatives are shown in Table 1 and elsewhere (Nelson et al., 1996). Addition of electron-withdrawing groups (di-o-Br-, o-F-, di-o-F-, di-o-Cl-, and di-m-F-AE) increased the rate of the chemiluminescence reaction, while addition of electron-donating groups (o-OCH_3-, di-o-OCH_3-, o-Me-, and di-o-Me-AE) decreased the rate of reaction. Steric effects appear to be rather small, since substitution with two relatively large bromine atoms still yielded a rapidly reacting AE.

Addition of substituents to the acridinium ring can also affect the chemiluminescence reaction by inductive as well as steric effects in a manner analogous to that described above for phenyl ring substitutions. A weakly electron-donating group, such as a methyl group, slowed the chemiluminescence reaction slightly (1-Me-AE, Table 1), while two methyl substituents (2,7-di-Me-AE) slowed the reaction even more. Substituents on both the acridinium and phenyl rings yielded molecules whose rate of reaction was a composite of the effects of each substituent (1-Me-di-m-F-AE).

Particular chemical substitutions, therefore, can yield acridinium esters which possess distinct chemiluminescence reaction kinetics. Figure 2 shows the kinetics of light emission for 1-Me-di-m-F-AE and 1-Me-AE. Although the profiles overlap, each compound could be clearly discriminated from the other. The signal from 1-Me-di-m-F-AE decayed to base line very rapidly, well before the signal from 1-Me-AE returned to base line. This key feature was the starting point for the resolving method described in Materials and Methods, which first assumes that the contribution from the fast acridinium ester label to the signal in the later time intervals was zero. On the basis of this assumption, the amount of each label was calculated, and these initial calculations were refined using a reiterative process (see Materials and Methods). With this method, two such labels in a mixture could be accurately quantitated up to an approximately 200-fold difference in the relative amounts of the labels. Other, more complex mathematical treatments of the data were explored, but none yielded significant improvement in label discrimination. The 200-fold limit is due to kinetic overlap, as the excess label obscures the signal from the other label. All labeling compounds we have evaluated, acridinium esters as well as other chromophores, begin emitting chemiluminescence rapidly upon contact with alkaline peroxide even though other kinetic characteristics of the reaction (time-to-peak, amplitude of peak, and duration) are variable. It is therefore likely that compounds which produce light via an oxidative decomposition mechanism as described here will overlap one another to some degree.

In addition to 1-Me-di-m-F-AE and 1-Me-AE, other pairs of AE derivatives could also be simultaneously detected. For example, 1-Me-di-m-F-AE could be paired with unmodified AE or o-OCH_3(c)-AE, and di-o-Br-AE could be paired with 1-Me-AE, unmodified AE, or o-OCH_3(c)-AE (see Table 1). It was also possible to simultaneously detect more than two labels. For example, di-o-Br-, 2,7-di-Me-, o-OCH_3(c)-, o-Me-, and di-o-Me-AE possess distinct light emission profiles and were clearly discriminated from one another in a single reaction (data not shown).

An alternative method to simultaneously detect two or more AE derivatives in a single tube exploits differences in the pH optima of the chemiluminescence reaction arising from chemical modification of AE (see Table 1). For example, when di-o-Br-AE and unmodified AE were mixed in a single tube, di-o-Br-AE was detected with an initial injection of a pH 10.2 peroxide solution and unmodified AE was detected with a subsequent injection of a pH 11.9 peroxide solution. The chemiluminescence of each label weakly overlapped the other, but discrimination was clear (data not shown). In another mode, discrimination using both pH optima and kinetic differences allowed the detection of four different AE derivatives (di-o-Br-, o-F-, unmodified-, and o-OCH_3-AE) in a single tube. Injection of a pH 10.2 solution activated di-o-Br and o-F, whose chemiluminescent profiles were resolved kinetically as described above. Subsequent injection of a pH 11.9 solution activated unmodified AE and o-OCH_3-AE, whose chemiluminescent profiles were also resolved kinetically (data not shown). Once again, a small amount of overlap was observed, but all four labels could be clearly discriminated from one another. Another possible method to simultaneously detect two or more AE derivatives uses differences in the wavelengths of light emitted from the different *N*-methylacridone molecules which result from chemical modification of the acridinium ring. For example, addition of two methyl groups to AE (2,7-di-Me-AE) shifted the wavelength of emission of AE by 15 nm (results not shown).

In addition to the chemiluminescence reaction, acridinium ester can also undergo hydrolysis, which renders AE permanently nonchemiluminescent. This property is utilized in the homogeneous HPA format where AE hydrolysis is rapid for unhybridized probe and slow for hybridized probe (Nelson et al., 1995). In order for AE labels to be useful for simultaneous detection of multiple analytes when hydrolysis is used to discriminate between hybridized and unhybridized probe, they must have distinct chemiluminescence characteristics yet similar hydrolysis characteristics. Otherwise, labels within the mixture would be hydrolyzing at different rates, leading to increased backgrounds and/or decreased signals from hybridized probes.

Chemical substitution of the AE molecule affects not only the chemiluminescence reaction but also the hydrolysis reaction. The chemiluminescence and hydrolysis reactions are similar in that both involve nucleophilic attack, the sites of which are only one carbon apart, and both result in cleavage of the ester bond and leaving of a phenol. One might therefore predict that a particular chemical modification would similarly affect the hydrolysis and chemiluminescence reactions. This prediction is true in some but not all cases [Table 1 and Nelson et al. (1996)]. For example, the addition of electron-donating groups to the phenyl ring (o-OCH_3-, o-Me-, and di-o-Me-AE) slowed the light emission and hydrolysis of AE to about the same degree. Addition of electron-donating groups to the acridinium ring (1-Me-, 2,7-di-Me-, and 4,5-di-Me-AE) also slowed both light emission and hydrolysis. However, the magnitude of the effect was unexpectedly much greater for hydrolysis than it was for light emission. For example, the duration of light emission was 33% longer for 2,7-di-Me-AE than for unmodified AE, whereas the hydrolysis rate was more than 8-fold slower for 2,7-di-Me-AE than for unmodified AE. Addition of an electron-withdrawing group to the phenyl ring (o-F-AE) greatly accelerated light emission but slowed hydrolysis. Addition of two bulky electron-withdrawing groups in the ortho position (di-o-Br-AE) again accelerated light emission but in this case slowed hydrolysis approximately 4-fold. Clearly, steric factors play a significant role in the hydrolysis of these AE derivatives. In support of this hypothesis, the addition of two methyl groups to the ortho position of the phenyl ring (di-o-Me-AE) slowed hydrolysis much more than the addition of two methyl groups to the 2 and 7 or 4 and 5 positions of the acridinium ring.

As noted earlier, steric effects do not appear to greatly affect the chemiluminescence characteristics of AE derivatives. Since steric effects do affect the hydrolysis characteristics of these derivatives, it is possible to identify pairs of AE derivatives which possess distinct chemiluminescence characteristics yet exhibit similar hydrolysis characteristics. Examples of these pairs include 1-Me-di-m-F- and 1-Me-AE, di-o-Br- and 2,7-di-Me-AE, and o-F- and unmodified AE.

Using these pairs of AE derivatives, homogeneous HPA assay formats for the simultaneous detection of various nucleic acid targets were developed, and the method was tested in a variety of applications. In one application, *C. trachomatis* and *N. gonorrhoeae*, common pathogens which infect the urogenital tract, were assayed. Ribosomal RNAs from both organisms, alone and in various combinations, were accurately detected (Table 3). In another application, wild-type and mutant sequences associated with the HIV-1 reverse transcriptase were simultaneously detected. A point mutation in the reverse transcriptase coding region at codon 181 causes broad resistance to non-nucleoside inhibitors such as nevirapine (Richman et al., 1991). As described elsewhere (Nelson et al., 1990), when an AE probe is hybridized to a target containing a single mismatch, protection of AE is disrupted and hydrolysis is much more rapid compared to that of an AE probe hybridized to a perfectly matched target. Combining this single mismatch discrimination by the HPA with the dual label detection described here allowed simultaneous detection and quantitation of the wild-type and mutant strains of the virus in the same assay tube (Table 5). In another application, the *gag* and *pol* regions of HIV were simultaneously detected (Table 4). In this assay, the HIV DNA was first amplified using TMA (transcription-mediated amplification) and the product nucleic acid detected using the HPA. Again, both targets were clearly detected. An advantage of simultaneously assaying two HIV targets is that detection of the second target confirms the presence of the first target due to the low likelihood of two false positive signals in the same reaction tube. This assay was also performed in blood spiked with HIV DNA with essentially identical results (data not shown).

In addition to the HPA format, other assay formats can be utilized with the modified acridinium esters described in this report. For example, one format combines the HPA format with physical separation of hybridized probe from remaining unhybridized probe and clinical specimen using magnetic amine microspheres (Arnold et al., 1989). This format was applied to the simultaneous detection of *C. trachomatis* and *N. gonorrhoeae*, resulting in accurate and sensitive quantitation of the two analytes in a single tube, even in the presence of a urogenital clinical specimen (data not shown).

Utilizing acridinium ester labels in the homogeneous HPA format for multi-analyte detection provides several advantages over other approaches. The assay is sensitive, owing to the high inherent specific activity and low background of acridinium esters and the ability to selectively hydrolyze and thus destroy the chemiluminescence of the large excesses of AE-labeled probes required to detect low levels of target sequences. The assay is specific due to the high specificity of nucleic acid hybridization, which is further enhanced by the exquisite sensitivity of AE hydrolysis to small perturbations in duplex structure, such as mismatches. The assay is very simple and fast and can be performed in a single tube without sample transfer, which is especially important in amplified assays to avoid contamination problems. The homogeneous assay possesses the further advantages of rapid hybridization kinetics and availability of all the target molecules afforded by solution hybridization as well as the use of direct rather than indirect labels, which greatly reduces the complexity of the assay. The instrumentation required for detection is simple, and detection is very rapid. Multiple labels can be detected in a very short time, and the data can be analyzed and the results reported within 1−2 s after signal measurement. The assays are quantitative with a linear response over a wide dynamic range, and pairs of labels are discernible at relative ratios up to 200-fold. Finally, the assays are robust, reproducible, and versatile and can be performed in the presence of a clinical specimen, making the system especially suited for clinical diagnostics.

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