*Clinica Chimica Acta*, 194 (1990) 73–90
Elsevier



CCA 04889

# Chemiluminescent DNA probes: a comparison of the acridinium ester and dioxetane detection systems and their use in clinical diagnostic assays

Norman C. Nelson and Daniel L. Kacian

*Gen-Probe, Inc., San Diego, CA (USA)*

(Received 11 June 1990; revision received 10 September 1990; accepted 14 September 1990)

*Key words:* Acridinium esters; Chemiluminescence; Dioxetane; DNA Probe; Hybridization

## Summary

Nucleic acid hybridization has the potential to markedly improve the diagnosis of infectious and genetic diseases. Recently, chemiluminescent hybridization assays using acridinium esters and stabilized dioxetanes have been described with sensitivities comparable to those obtained with radioactive labels.

Acridinium esters are used as direct labels that are attached to the probe throughout the hybridization reaction. Methods have been developed for labeling DNA probes with acridinium esters at high specific activity and for stabilizing the label under the relatively harsh conditions of hybridization reactions. The label does not affect the kinetics of the hybridization reaction or the stability of the resulting hybrid. The label emits light upon exposure to alkaline peroxide; thus, the assay format can be an extremely simple one. The acridinium ester labels are stable in storage and exhibit extremely rapid light-off kinetics which permit reading large numbers of samples within a brief period as well as limiting the contribution of background signal. A special property of acridinium esters allows chemical destruction of the label when it is present on unhybridized probe, whereas the label is stable to this process when the probe is hybridized. This behavior forms the basis of techniques to minimize assay background signals and allows a homogeneous assay format which does not require physical separation of hybridized and unhybridized probe.

The adamantyl-stabilized 1,2-dioxetanes have been used to produce high-sensitivity detection systems for clinical assays. The probe is labeled with enzymes such as alkaline phosphatase or $\beta$-D-galactosidase that hydrolyze the dioxetane derivative to

Correspondence to: Dr. N.C. Nelson, Gen-Probe, 9880 Campus Point Drive, San Diego, CA 92121, USA.

produce a chemiluminescent molecule. As with other enzyme-based labeling systems, the signal increases with time, allowing greater sensitivity to be achieved with longer incubations. The amount of light generated is sufficient to expose sensitive photographic film with extended incubation; therefore, convenient assay formats not requiring instrumentation can be used. Excellent analytical sensitivities have been reported, and by using labels with different light emissions and/or different enzymes on the probes, it is possible to distinguish multiple target sites within a single assay. Because the label is suited for use with solid supports such as polyacrylamide gels, membrane filters, or microscope slides, applications include DNA sequencing, dot and Southern blot hybridizations, and in situ hybridization.

## Introduction

In spite of recent advances in therapy, infectious diseases remain a major cause of morbidity and mortality. One of the main reasons is that methods of diagnosis have not kept pace with advances in treatment. In an unacceptable percentage of cases, the causative agent is either never identified or identified too late for the information to be of use in determining therapy.

Nucleic acid hybridization is one method that has the potential to markedly improve infectious disease diagnosis [1–3]. In recent years, considerable progress has been made in moving these techniques from the research to the clinical laboratory, and several DNA probe-based tests are now available commercially and are achieving widespread use. However, full exploitation of hybridization methods has been limited by the need to use radioactive labels, with their limited shelf-life and their safety and disposal problems. For this reason, effort has been devoted to developing suitable non-isotopic detection systems. The challenge has been to develop systems that are as sensitive as the radioisotopic labels, while still maintaining a rapid, convenient format suitable for routine clinical use.

Detection systems using chemiluminescent labels have been developed recently that are a viable alternative to radioisotopic methods [4–6]. Chemiluminescence provides an extremely sensitive mode of detection; chemiluminescent labels are easy to control (the reaction chemistry is typically very simple, yet very specific), require no special handling or disposal, and have excellent shelf-lives; and chemiluminescence can be detected using a wide variety of methods from simple photographic film to highly sophisticated photon counting instrumentation.

Recently, a number of hybridization assays using chemiluminescent labels have been described that achieve analytical and diagnostic sensitivities that meet or exceed those obtained with $^{32}P$ or $^{125}I$-labeled probes [3,7–13]. Among the most promising of these labels are acridinium esters and stabilized dioxetanes.

Methods have been developed to attach acridinium ester directly to a DNA probe without any loss of specific activity of the acridinium ester and without affecting hybridization characteristics of the DNA probe. The label is simple to detect, very sensitive, and gives signal proportional to concentration over a wide range. Furthermore, assay formats have been developed which are very rapid and

simple to perform, including a novel homogeneous format which does not require physical separation of hybridized and unhybridized probe [7].

The adamantyl-stabilized 1,2-dioxetanes are employed as substrates for enzymes such as alkaline phosphatase and $\beta$-D-galactosidase. Reaction with the enzyme yields a chemiluminescent compound. For use in a nucleic acid hybridization assay, the DNA probe is labeled (either directly or indirectly) with the appropriate enzyme, and after physical separation of the hybridized and unhybridized probe, the dioxetane substrate is added and chemiluminescence is generated over time. Excellent analytical sensitivities have been reported using this system [9,10,14,15].

In what follows, these two labels will be described in greater detail, and the advantages and disadvantages of each for specific purposes will be discussed.

## Materials and methods

Acridinium ester (AE) was synthesized as described previously [22]. Oligonucleotide probes were labeled by reacting the *N*-hydroxysuccinimide derivative of AE with a primary alkyl amine on a non-nucleotide-based phosphoramidite linker-arm [24] introduced into the oligomer during DNA synthesis. The acridinium ester-labeled probe (AE-probe) was then purified using high performance liquid chromatography. Chemiluminescence was detected in a Leader I luminometer (Gen-Probe, San Diego, CA) by the automatic injection of 200 $\mu$l of 0.1% $H_2O_2$ in $HNO_3$ (5 to 400 mmol/l), then 200 $\mu$l of 1 N NaOH (with or without surfactant). The measurement period was 2 to 5 seconds.

Hybridization of AE-probes was performed at 60 °C in 0.1 mol/l lithium succinate buffer, pH 5.2, containing 5–10% lithium laurel sulfate, 2 mmol/l EDTA and 2 mmol/l EGTA. Hybridization volumes ranged from 50 to 200 $\mu$l and incubation times ranged from 10 to 60 min. Differential hydrolysis was performed at 60 °C in 0.15–0.20 mol/l sodium tetraborate, 1–5% Triton X-100, pH 7.5–8.5. In a typical homogeneous format, hybridization was performed in a volume of 100 $\mu$l at 60 °C, followed by the addition of 300 $\mu$l of the tetraborate buffer and another 5–10 min incubation at 60 °C. Chemiluminescence was then measured as described above. In a differential hydrolysis with magnetic separation assay, amine-modified magnetic particles (Gen-Probe, San Diego, CA) were added either after or during the differential hydrolysis step to capture the hybridized AE-probe. The particles were separated with a magnet and the supernatant was discarded. The particles were washed once, and chemiluminescence was measured as described above.

## Results and discussion

### *Acridinium esters*

The basic acridine molecule was discovered in 1870, and since that time it has been studied and used extensively [16,17]. The *N*-methyl acridinium esters react with hydrogen peroxide in base to yield *N*-methyl acridone in the excited state

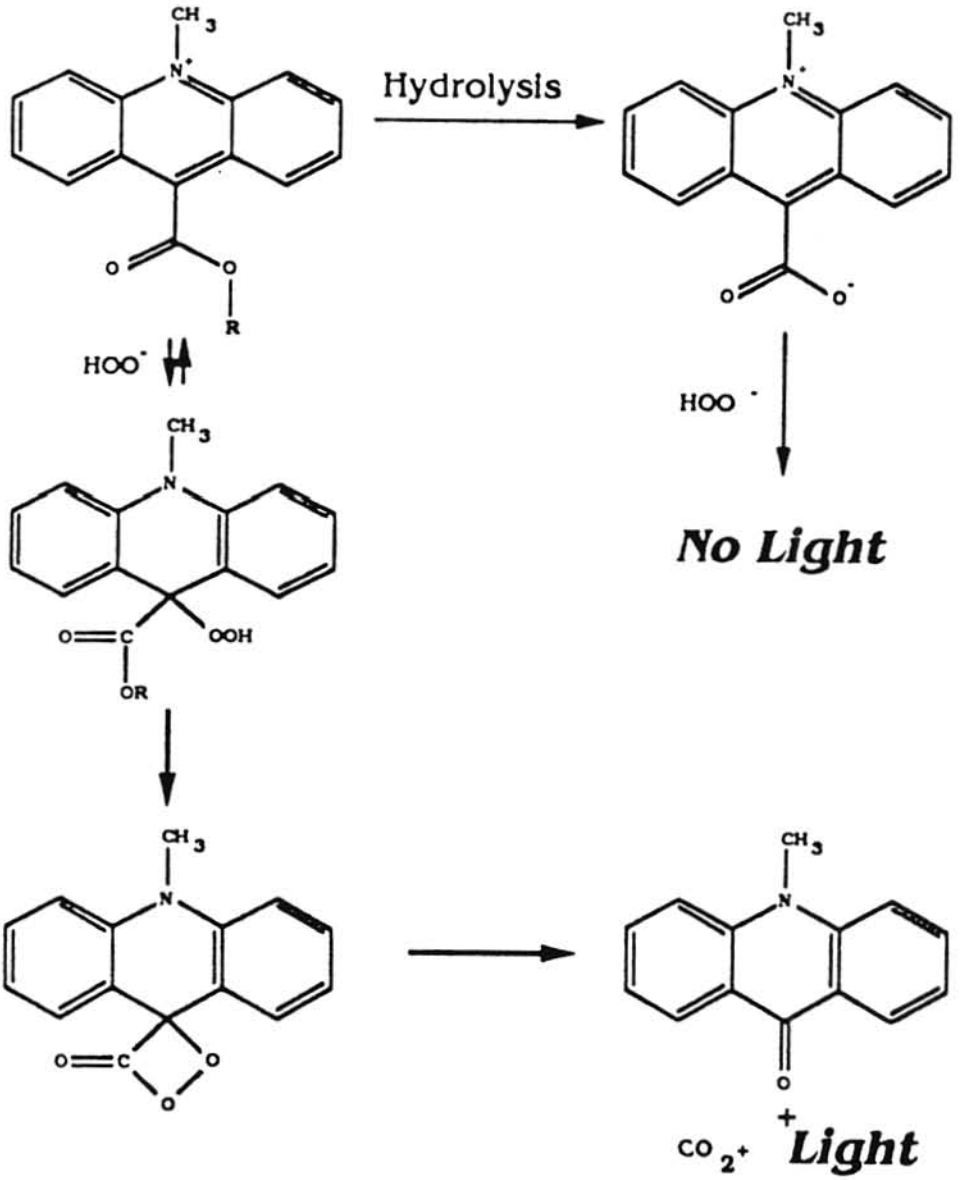


Fig. 1. Acridinium ester reaction pathways. In the pathway leading to chemiluminescence, hydroperoxy anion adds to the C9 position of the acridinium ring, followed by production of the electronically excited *N*-methyl acridone molecule through a cyclodioxetane-like intermediate. The excited *N*-methyl acridone then rapidly collapses to the ground state, resulting in the emission of light. In an alternate pathway, hydroxide ion catalyzes the hydrolysis of the ester bond, resulting in the non-chemiluminescent acridinium carboxylic acid molecule.

which then emits light at 430 nm (Fig. 1) [18–20]. The reaction characteristics and chemiluminescent properties of the acridinium esters are dependent upon the nature of the R group [21].

Recently, the phenyl ester shown in Fig. 2A was synthesized for use as a chemiluminescent label in immunoassays and other bioassays [22,23]. This compound contains an *N*-hydroxysuccinimide (NHS) group which reacts specifically

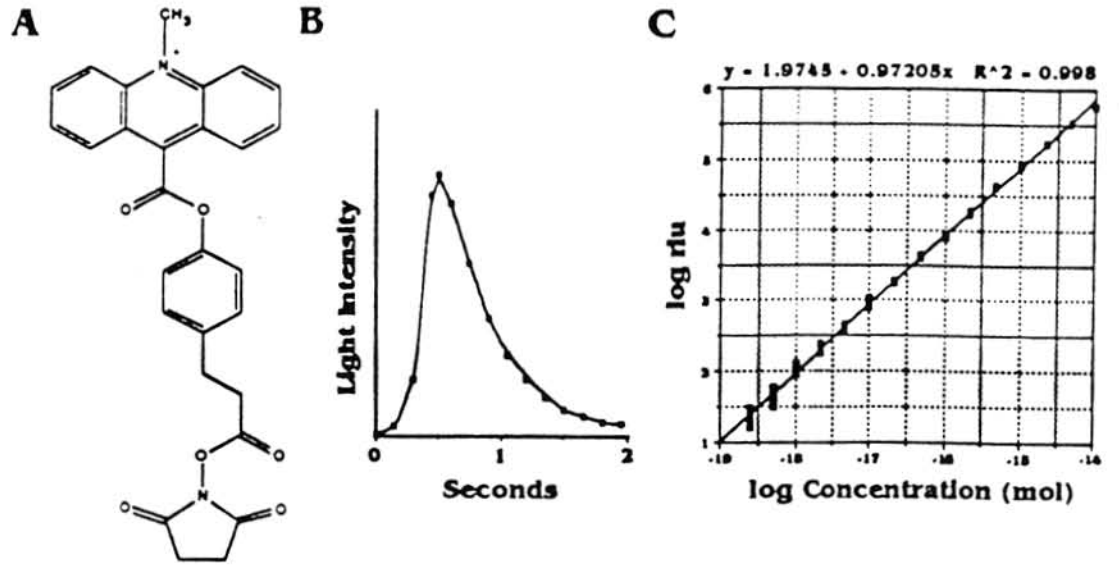


Fig. 2. *N*-Hydroxysuccinimide phenyl acridinium ester. Panel A: *N*-hydroxysuccinimide (NHS) phenyl acridinium ester used to label DNA probes containing primary alkyl amine groups. Panel B: Very rapid kinetics of light emission seen with acridinium ester-labeled probes. Panel C: AE-labeled probe was serially diluted, and chemiluminescence was measured as described in Materials and Methods. Four replicates were measured at each concentration; the average coefficient of variation for all determinations was 1.66%. Reagent and machine background combined were 49 RLU (subtracted from data before plotting). The limit of detection was about $5 \times 10^{-19}$ moles.

and covalently with primarily amines, providing an easy way to directly label amine-containing compounds. This phenyl acridinium ester (AE) reacts rapidly with alkaline peroxide to produce light (Fig. 2B), and detection is linear with concentration over more than 4 orders of magnitude. The detection limit is approximately $5 \times 10^{-19}$ mol (measured in a standard, commercially available luminometer sold for clinical laboratory use). The rapid reaction kinetics of acridinium esters improve sensitivity since the short read time limits the contribution of background noise and allows reading a large number of samples within a short time.

To provide a site of attachment for the AE within a deoxyoligonucleotide probe, non-nucleotide-based alkylamine linker-arms have been developed, which can be incorporated at any location in the probe during solid-phase phosphoramidite synthesis [24]. The amine is reacted with the NHS-AE (Fig. 2A), yielding a DNA probe with the chemiluminescent AE directly attached through a covalent (amide) bond. Direct labels greatly simplify assay formats since the 'capping', binding and washing steps required for indirect labels (those attached through a biotin/avidin interaction, for example) are not necessary, and separate reagent additions for label coupling or substrate addition are unneeded.

An additional, very important advantage of the AE label is that the acridinium ring is also cleaved from the DNA probe before light emission occurs (see Fig. 1), thus minimizing intramolecular quenching [22]. An AE-labeled DNA probe (AE-probe) displays the same reaction kinetics and specific activity as the free label (Fig. 2C), demonstrating that performance is not compromised by attachment to the

probe. Furthermore, AE-probes display hybridization characteristics (thermal stability, rate and extent of hybridization, and specificity) essentially equivalent to their $^{32}$P-labeled counterparts, demonstrating that attachment of the AE label does not compromise hybridization performance. Additionally, hybridization and detection of AE-probes can also be performed in the presence of relatively large amounts of clinically specimen material, which allows detection of clinically relevant microorganisms directly in patient specimens.

Several rapid and simple formats have been developed that use AE-probes for detecting target DNA or RNA sequences, and a number of these have been incorporated into commercially available assays [8,25–30; see also Table II]. In these formats, the hybridization and detection reactions are performed in solution, which offers significant advantages for routine clinical laboratory use compared to standard target immobilization techniques [3], including faster hybridization kinetics, availability of all the target molecules for hybridization, much better quantitation, fewer steps and less complexity, and much shorter time to result. The solution hybridization format is made possible by targeting single-stranded ribosomal RNA sequences, which also provide the additional advantages of increased sensitivity due to natural target amplification (each bacterial cell contains up to 10000 copies of the ribosomal RNAs) and increased specificity since rRNA sequences are excellent phylogenetic markers [1,2,31].

Separation of hybridized and unhybridized AE-probe is achieved through the use of polycationic sub-micron sized microspheres which selectively bind hybridized AE-probe. After a brief binding period (approximately 10 minutes), the microspheres are magnetically separated from the solution, the unhybridized AE-probe is washed away, and hybridized AE-probe associated with the microspheres is detected. This format is amenable to batch procedures and to automation.

TABLE I

Comparison of the acridinium ester (AE) and dioxetane chemiluminescent detection systems

| Parameter | AE | Dioxetane |
|---|---|---|
| Detection system sensitivity limit | $5 \times 10^{-19}$ mol | $10^{-21}$–$10^{-20}$ mol |
| Specificity | Superior | Good–excellent |
| Time to result [1] | 30–120 min | Hours-1 to 2 days |
| Ease of use [1] | Simple | More complex |
| Quantitation | Objective | Objective/subjective [2] |
| Linear dynamic range | | |
| Solution/luminometer | $>10^4$ | $>10^3$ |
| Full assay format | $>10^4$ | $10^1$–$10^2$ |
| Reliability and reproducibility | Excellent | Excellent |
| Shelf-life | Excellent | Excellent |
| Compatibility with clinical specimen | Excellent | Potention Problems [3] |
| Applicability to clinical diagnostics | Excellent | Moderate-good |

[1] Refers to the entire assay procedure, not just the detection step.
[2] Dependent on assay format and mode of detection; see text.
[3] Dependent on assay format and type of clinical specimen; see text.

TABLE II

Commercial acridinium ester DNA probe-based tests

| Test Kit [1] | FDA status | Organism(s) detected | Mode [2] | Format [3] |
|---|---|---|---|---|
| PACE 2 | Approved | *Chlamydia trachomatis* | D | DH/MS |
| | Approved | *Neisseria gonorrhoeae* | D | DH/MS |
| Urine Screen | Approved | All bacterial/All yeast | D | H |
| ACCUPROBE | Approved | *Campylobacter jejuni* | CC | H |
| | Approved | *Haemophilus influenzae* | CC | H |
| | Approved | *Neisseria gonorrhoeae* | CC | H |
| | Approved | *Streptococcus agalactiae* | CC | H |
| | Approved | *Enterococcus* species | CC | H |
| | Approved | *Mycobacterium tuberculosis* | CC | H |
| | Approved | *Mycobacterium avium* complex | CC | H |
| | Approved | *Mycobacterium gordonae* | CC | H |
| | Approved | *Histoplasma capsulatum* | CC | H |
| | Pending | *Mycobacterium kansasii* | CC | H |
| | Pending | *Mycobacterium avium* | CC | H |
| | Pending | *Mycobacterium intracellulare* | CC | H |
| | Pending | *Listeria monocytogenes* | CC | H |
| | Pending | *Staphylococcus aureus* | CC | H |
| | Pending | *Streptococcus pneumoniae* | CC | H |
| | Pending | *Streptococcus pyogenes* | CC | H |
| | Pending | *Neisseria meningitidis* | CC | H |
| | Pending | *Blastomyces dermatitidis* | CC | H |
| | Pending | *Coccidiodes immitis* | CC | H |
| | Pending | *Cryptococcus neoformans* | CC | H |
| ACCUSEARCH | N/A | HIV-1 gag-1 and gag-2 | R | H |
| | N/A | HIV [4] env-1 and env-2 | R | H |
| | N/A | HIV-2 vpx-1 and vpx-2 | R | H |
| | N/A | All bacteria | R | H |
| | N/A | SRB [5] 1 and 2 | R | H |
| | N/A | HIV-1 env-3 and env-4 | R(ID) | H |
| | N/A | HIV-2 LTR [6] | R(ID) | |
| | N/A | Philadelphia chromosome | R(ID) | |
| | N/A | Mycoplasma tissue culture | R(ID) | |

[1] Registered Trademarks of Gen-Probe, Inc., 9880 Campus Point Drive, San Diego CA, USA (Urine Screen Trademark pending); [2] Mode, D-direct specimen testing; CC-culture confirmation; R-research; ID-in development; [3] Format, DH/MS-differential hydrolysis + magnetic separation; H-homogeneous; [4] HIV, human immunodeficiency syndrome; [5] SRB, sulfate reducing bacteria; [6] LTR, long terminal repeat.

A completely homogeneous format requiring no physical separation step has also been developed using the acridinium ester label [7]. This format is based on differential chemical hydrolysis of the ester bond of the AE molecule (hydrolysis of this bond renders the AE permanently non-chemiluminescent, as shown in Fig. 1). The system is designed such that the rate of hydrolysis of the AE attached to unhybridized probe is rapid, whereas the rate of hydrolysis of AE attached to probe which is hybridized with its target nucleic acid is slow. By adjusting the reaction

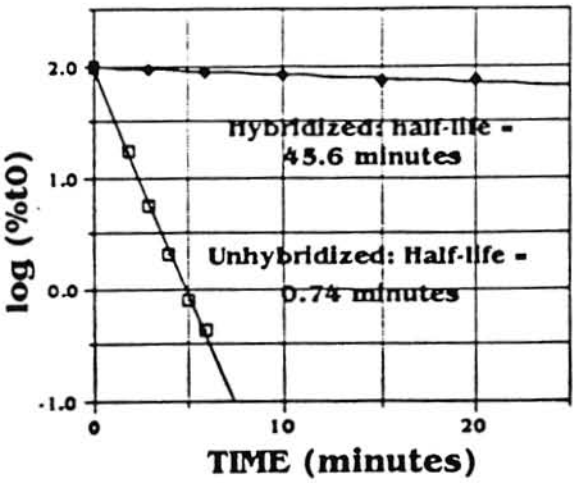


Fig. 3. Differential hydrolysis of hybridized and unhybridized acridinium ester-labeled DNA probe. AE-labelled probe was hybridized in the presence of excess target as described in Materials and Methods. Individual aliquots were then hydrolyzed for various times and chemiluminescence was measured. Results were plotted as the log of percent initial chemiluminescence versus time, and half-lives of hydrolysis were determined using standard linear regression analysis.

chemistry, the chemiluminescence associated with unhybridized probe is rapidly reduced to low levels, whereas chemiluminescence associated with hybridized probe is minimally affected. Thus, after this differential hydrolysis process, the remaining chemiluminescence is a direct measure of the amount of target present.

An example of differential hydolysis of hybridized and unhybridized probe is shown in Fig. 3, which illustrates loss of chemiluminescence with time due to ester hydrolysis. From linear regression analysis, the half-lives of hydrolysis were determined to be 45.6 and 0.74 min for hybridized and unhybridized probe, respectively. The theoretical percent remaining chemiluminescent label after a given hydrolysis time can be calculated using the equation

$$(0.5)^n \times 100 = \text{percent remaining chemiluminescence}$$

where $n$ is the elapsed time of differential hydrolysis expressed as multiples of the half-life of loss of the chemiluminescent label. Using the half-life values given above, the calculated values for percent remaining chemiluminescence after a 15 min differential hydrolysis step would be 80% for hybridized probe and 0.00008% for unhybridized probe. These provide a one million-fold discrimination between hybridized and unhybridized AE-probe, in 15 min, with the addition of a single reagent and without any physical separation.

An example of the use of this format in a clinical assay is shown in Fig. 4A, which shows the detection of various amounts of rRNA from *Chlamydia trachomatis* using the homogeneous format. The assay was quantitative, linear over a wide dynamic range, rapid and easy to perform. Results were available within 25 minutes, of which only 5 min were actual hands-on time. As little as $10^{-4}$ μg of rRNA ($6 \times 10^{-17}$ mol) were detected.

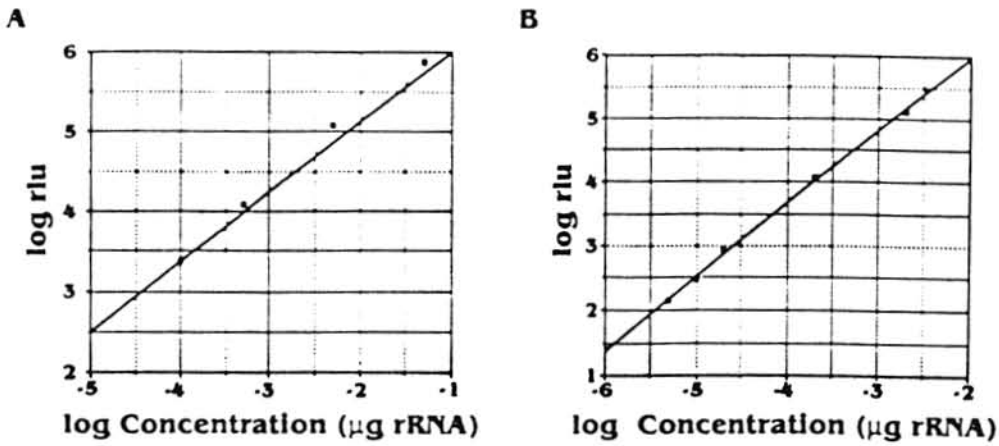


Fig. 4. Detection of decreasing amounts of target rRNA using homogeneous and differential hydrolysis with magnetic separation formats. A series of dilutions of purified rRNA from *Chlamydia trachomatis* was assayed using either a homogeneous (panel A) or differential hydrolysis with magnetic separation (panel B) format as described in 'Materials and Methods'. The rRNA was 'seeded' into a cervical swab specimen in the differential hydrolysis with magnetic separation format.

Another useful format combines differential hydrolysis with separation using cationic magnetic beads, thereby achieving even greater discrimination between hybridized and unhybridized AE-probe (Fig. 4B). In the commercially available clinical assays, there is only one more reagent addition step than in the homogeneous format, providing a method that is also very rapid and easy to perform.

*Dioxetanes*

Peroxides have been shown to thermally decompose to produce excited state carbonyl compounds which are capable of chemiluminescence [20]. The class of peroxides known as the 1,2-cyclodioxetanes have been studied extensively, and several hundred have been described in the literature [32,33]. The stability of the 1,2-dioxetanes varies according to the nature of the substituent groups [34]. Recently, a group of substituted 1,2-dioxetanes have been described that are substrates for various enzymes [35–39], and these have been used to develop detection systems for hybridization assays.

In these systems, the dioxetane is very stable until it reacts with the enzyme. The resulting compound spontaneously decomposes, producing an excited state fragment which subsequently chemiluminesces. An example of this type of compound is 3-(2′-spiroadamantane)-4-methoxy-4-(3″-phosphoryloxy)-1,2 dioxetane, commonly referred to as AMPPD (see Fig. 5). The reaction pathway leading to chemiluminescence begins with the cleavage of the phosphate group from AMPPD by the enzyme alkaline phosphatase, yielding the anion AMP-D. This anion is weakly to moderately stable and decomposes with a half-life of from approximately 2 minutes to several hours, depending on several factors such as pH, temperature and the nature of the local environment. Fragmentation of the AMP–D molecule yields adamantone in the ground state and the electronically excited methyl meta-oxybenzoate

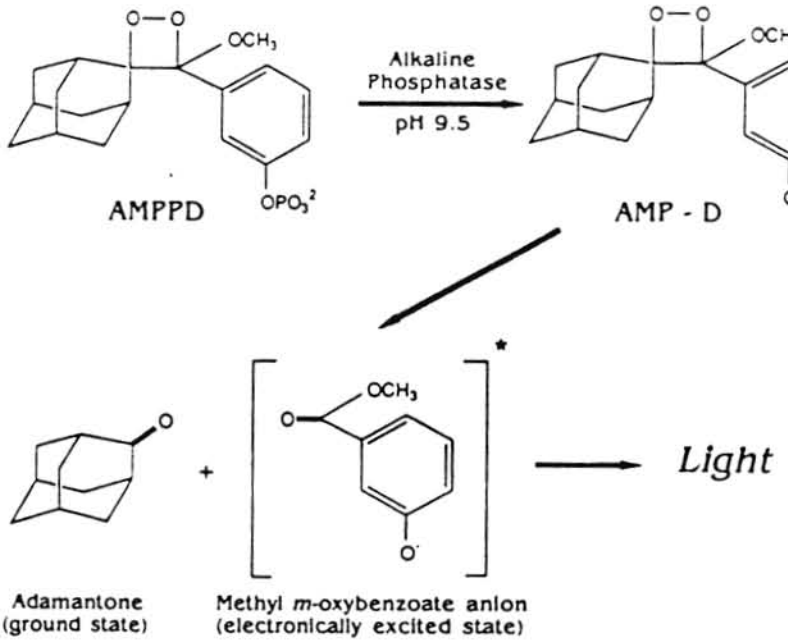


Fig. 5. Chemiluminescence reaction mechanism of AMPPD. Alkaline phosphatase cleaves the phosphate group from AMPPD, yielding the AMP-D anion which spontaneously decomposes, producing light.

anion which subsequently emits light at 470 nm. In this system, dephosphorylation proceeds at a rate dependent upon enzyme concentration, and the AMP-D anion accumulates. As the AMP-D slowly decomposes, chemiluminescence increases until a steady state 'glow' is reached, which can continue for up to several hours (depending on reaction conditions). The increase of the unstable intermediate species with time allows greater sensitivity to be achieved with longer incubation times. Chemiluminescence can be detected using a luminometer or photographic film. Using a luminometer, detection of alkaline phosphatase in solution at concentrations between $10^{-15}$ mol/l and $10^{-14}$ mol/l has been reported [38]. One limit to ultimate sensitivity is background chemiluminescence due to non-enzymatic dephosphorylation of AMPPD; however, signal to noise ratios are very good at pH 9.5.

The 1,2-dioxetane enzyme substrate detection system is most commonly used in a solid phase membrane hybridization format. Target nucleic acids are purified and then bound to a filter membrane. The membrane is then treated to block further non-specific binding of nucleic acids. The probe is hybridized with the target, and unhybridized probe is removed by careful washing. If the enzyme was not directly attached to the probe, alkaline phosphatase is attached to the hybridized probe through an indirect linkage (e.g. biotin-avidin coupling) and excess is washed away. The 1,2-dioxetane substrate is added, and the chemiluminescent species is generated during the subsequent incubation period. Chemiluminescence is typically detected with photographic film using various exposure times, but a luminometer can also be used.

The use of the AMPPD substrate in this format has been reported for a few model systems [14,40]. In one report [14], a nick-translated biotinylated probe was hybridized with plasmid pBR322 DNA digested with restriction endonuclease Rsa I using a Southern blot procedure and detected through a series of steps with streptavidin-alkaline phosphatase and AMPPD. The limit of detection was in the $10^{-20}$ to $10^{-19}$ mol range, which was reported as being more sensitive than 2 other commonly used alkaline phosphatase detection systems [12,41]. In another report [40], a DNA probe specific for sequences in the hepatitis B virus core antigen gene was directly labeled with alkaline phosphatase and hybridized with plasmid DNA containing the target sequences using a standard dot blot procedure. After washing the membrane to remove unhybridized probe, the bound alkaline phophatase was detected using AMPPD. The limit of sensitivity was around $10^{-19}$ mol, and direct comparison showed the AMPPD system to be more sensitive and/or more rapid than another sensitive alkaline phosphatase detection system.

*Comparison of assay formats using acridinium ester and dioxetane detection systems*

The probe labeling and detection method is one of the most important parts of a DNA probe assay. The overall sensitivity, specificity, convenience, and cost-effectiveness of the assay are all directly dependent upon this component. The performance characteristics of the acridinium ester-based and dioxetane-based formats in each of these categories are listed in Table I, as well as discussed in the following text.

*Sensitivity*

The AE and dioxetane detection systems are both very sensitive, with the dioxetane system offering higher inherent sensitivity due to the signal amplification achieved through enzyme catalysis. In routine use, the full sensitivity of these systems will not be realized if background signals are not sufficiently low. In the acridinium ester assay, low background signals are relatively easy to achieve since the light-producing reaction is very rapid and accumulation of background noise is thereby minimized. More importantly, the differential hydrolysis procedure described above, when used either in a homogeneous assay format or combined with traditional separation methods, is able to reduce backgrounds due to unhybridized label to as low as 1–2 ppm of the input AE. Sample chemiluminescence may still be a source of background in AE assays, and sample purification procedures or traditional separation methods may be needed to reduce interferences from this source to acceptable levels.

The solid phase membrane formats that have been used with the dioxetane system are traditionally associated with non-specific binding problems regardless of the label employed, and these can limit the actual sensitivity achieved in the assay [40]. Also limiting sensitivity with dioxetane substrates is the need for extended incubation since during this time background signal also accumulates. The point of greatest signal:noise is difficult to determine with accuracy in advance; therefore,

the full sensitivity of the system is less easily achieved in routine use. Specimen components may also decrease assay sensitivity with dioxetane assays. These may be chemiluminescent substances, materials promoting non-specific binding to the solid supports, or interferring enzyme activities that either reduce signal (e.g., proteases that attack the alkaline phosphatase enzyme) or increase background (e.g., specimen alkaline phosphatase).

*Quantitation*

Quantitation is relatively easy and straightforward with the AE label since it is directly attached to the probe, and the signal produced in the light-producing reaction is linear with respect to concentration over at least a 10000-fold range. The range can be extended by several orders of magnitude with appropriate instrumentation. The result produced by the luminometer is numerical; therefore, subjective interpretation of the magnitude of the signal is not required.

The dioxetane system provides similar performance when detecting enzyme in solution using a luminometer [38]. In the standard solid phase membrane assay format, results are usually obtained by exposing photographic film, and the technologist is required to judge the intensity of spots in comparison with a set of standards run under the same conditions. Under these conditions, the linear range is typically only one to two orders of magnitude [14,38,40]. In addition, variations in enzyme reaction conditions (pH, temperature, substrate concentration, etc.) must be carefully controlled across the membrane support for accurate results. Preferably, standards are included on each membrane processed. In spite of these drawbacks, the lower cost of film based detection systems compared to luminometers can be an advantage in some applications where highly accurate quantitation is not necessary.

*Specificity*

DNA probe assays are inherently highly specific if probes are properly chosen, and reaction conditions controlled to provide the necessary degree of stringency. In general, the label employed in hybridization assays does not affect specificity. Instead, other aspects of the format, such as use and type of solid support, reaction parameters (temperature, ionic strength, etc.), washing conditions, and method of separating hybridized and unhybridized probe are more important. In the dioxetane systems, all of these factors play essentially the same role as in traditional solid support membrane hybridization systems. The choice of label affords no new advantages with respect to assay specificity.

On the other hand, the differential hydrolysis assay formats made possible by the AE label are inherently more specific than those using other labels. This is because the AE label in these formats is sensitive to the structure of the hybrid that is formed and therefore offers an additional level of stringency not available with other labels. For example, an AE-labeled probe did not distinguish between 2 targets which differed by a single base in a standard assay format using physical methods to separate hybridized and unhybridized probe, whereas these targets were

clearly distinguished using the homogeneous format using differential hydrolysis to chemically destroy unhybridized label [42]. In practice, even such closely related organisms as *Neisseria gonorrhoeae* and *Neisseria meningitidis*, which are difficult to distinguish by ordinary hybridization methods, can be differentiated easily using the AE label and differential hydrolysis [7].

*Speed and convenience*

Both the dioxetane and acridinium ester labels avoid the hazards and inconvenience of dealing with radioisotopes while affording equal or greater sensitivity and shelf life. Assays using an indirect label such as the enzymes used with the dioxetane system are inherently neither as rapid nor as convenient as those using a direct label such as a radioisotope or the acridinium ester. Indirect labels require reagent addition and incubation steps for attachment of the label and/or development of the signal that are not needed with direct labels. Thus, in most cases, the formats using the acridinium ester label will be faster and simpler than those using the dioxetane label.

Other steps may be required as well depending upon the assay format. For example, assays using the dioxetane label can employ photographic film for detecting light output, whereas photographic detection cannot be used with acridinium ester labels due to the chemistry of the system, the rapid reaction kinetics, and the lower overall light emission. Photographic detection does, however, introduce additional steps, including immobilization of target nucleic acids; blocking, washing and drying of membrane filters; loading of film cassettes; and film development. Detection with film is, however, a convenient way of dealing with large numbers of samples and is applicable to work in the field or if the cost of a luminometer is prohibitive. Exposure times are typically lower with the dioxetanes than with radioisotopes, although relatively long exposure times are still required to achieve highest sensitivity. Long delay periods prior to maximum light output have also been reported [14]. Detection of dioxetanes with a luminometer is simple, but applicable only in certain formats.

In the AE-based system, the entire assay is rapid with time-to-result (start to finish, including the assay procedure and detection) as low as 30 min. The tests are also very easy to perform: the homogeneous format requires no physical separation at any point in the assay and less than 5 min of 'hands-on' time. The entire assay can be performed in a single test tube, and detection simply requires placing the tube into the luminometer, which automatically injects the detection reagents, reads the chemiluminescence and prints out the results.

*Reliability and reproducibility*

Both detection systems are reliable and reproducible, if all variables are carefully controlled; however, the AE-based systems have an inherent advantage since they use direct labeling and, as a consequence, have fewer steps. For example, relatively small variations in pH, ionic strength, and temperature or interferring sample

components might alter the activity of the enzyme labels in the dioxetane systems. Since the AE systems do not develop their signals using enzymes, they are not as susceptible to changes of similar magnitude in these variables.

*Susceptibility to interferences from clinical specimens*

No interference with the production of light, and no premature light emission has been observed in any of the clinical specimens tested with the AE assays. Some types of clinical specimens contain components that produce alkaline peroxide-activated chemiluminescence, which can raise background levels. In assay formats which include physical separation and washing steps, this chemiluminescence is simply washed away. In homogeneous formats, it is necessary to use chemical procedures for eliminating background signal from these sources.

With the dioxetane system, components present in clinical samples, such as hemoglobin and high concentrations of protein have been shown to interfere with detection [43]. Interferring enzyme activities could also present problems, either by activating the chemiluminescent intermediate (e.g., sample alkaline phosphatases) or by damaging the enzyme label (e.g., proteases). These can, however, be removed by thorough washing of membrane filters or other solid supports prior to adding the labeled probe.

*Present and future applications*

Acridinium esters have been used as labels in immunoassays, and several clinical diagnostic products are commercially available [44]. Several AE-based DNA probe tests have also been developed for use in the clinical laboratory (Table II). These have received FDA clearance and are currently being marketed. These tests are rapid and easy to perform and have been found to have excellent sensitivity and specificity when compared to standard culture techniques [8,26,27]. For example, direct specimen tests for *Chlamydia trachomatis* and *Neisseria gonorrhoeae* showed 97.2 and 98.2% agreement with culture, respectively (Table III), but provide results in a few hours compared to one or more days for conventional methods. Other tests now available use a homogeneous AE format to identify culture isolates in less than 30 min. Most of the tests to date exhibit 100% sensitivity and 100% specificity [25,28–30].

Several AE-based assays have also been developed for use in the research applications (Table II). These use the homogeneous format to specifically detect target nucleic acid from a variety of sources in under 30 minutes. One application to which this assay is particularly well suited is the detection of products from nucleic acid target amplification reactions, such as the polymerase chain reaction [45] and the transcription amplification system [46]. These reactions produce large numbers of copies of target nucleic acid sequences. When used with specific detection systems, these amplification systems can be used to develop clinical assays that uncouple the usual inverse relationship between sensitivity and specificity. Detection systems using ethidium bromide-stained agarose gels [47] lack specificity unless

Note: See final appended page for amendments to Table III.

TABLE III

Comparison of PACE 2 and culture assays for *Chlamydia trachomatis* and *Neisseria gonorrhoeae* in urogenital clinical specimens [1]

*Chlamydia trachomatis*, combined female and male specimens ($n = 1201$)

| | | Culture + | Culture − | |
|---|---|---|---|---|
| PACE 2 | + | 127 | 24 | Sensitivity = 92.0% |
| | | | | Specificity = 97.8% |
| | − | 11 | 1040 | Agreement = 97.2% |

*Neisseria gonorrhoeae*, combined female and male specimens ($n = 1762$)

| | | Culture − | Culture − | |
|---|---|---|---|---|
| PACE 2 | + | 233 | 19 | Sensitivity = 95.1% |
| | | | | Specificity = 98.7% |
| | − | 12 | 1498 | Agreement = 98.2% |

[1] Positivity rate for *C. trachomatis* (sample set = 1201 specimens) was 11%; positivity rate for *N. gonorrhoeae* (sample set = 1762 specimens) was 14%.

restriction enzyme mapping or specific hybridization is used to verify the identity of the amplified species. Reliance upon size of the amplified product alone is hazardous since non-target species of similar size can be produced in PCR reactions with some frequency [3,48].

Standard methods using probe hybridization (e.g., Southern blotting; see 47 also) are better if stringency is carefully controlled; however, these techniques are typically tedious and time consuming and subject to errors if not carefully done. Techniques which combine blot hybridization with restriction enzyme analysis are preferable to minimize false positive results but add additional complexity and time [3]. The homogeneous AE format is ideally suited for analyzing amplification products because it retains all the specificity of stringent probe hybridization, yet is very rapid and easy to perform. For example, the Philadelphia chromosome [49] was amplified from K562 cells and detected using either standard Southern analysis or the homogeneous AE format [7]. The assay not only gave more sensitive detection than the Southern blot, but it was complete in 30 minutes as compared to the 2 days required to perform the Southern analysis. Amplified HIV sequences have also been rapidly and specifically detected using the homogeneous AE format [50]. The ability of the homogeneous AE format to detect single base mismatches [42] should be particularly useful for use in target-amplified assays for genetic screening.

Stabilized dioxetane substrates for alkaline phosphatase and $\beta$-galactosidase are commercially available, and have been utilized in the research sector in a variety of DNA probe-based assays employing enzyme-mediated detection. Applications include detection of plasmid pBR322 DNA and the globin gene of human DNA in a

Southern blot hybridization procedure [14], cloned hepatitis B core antigen sequences [40] and cloned herpes simplex virus I DNA [51] in dot blot procedures; imaging of sequencing ladders; in situ detection of HSV I-infected Vero cells [49]; and detection of a *Chlamydia trachomatis* plasmid [15]. To date, no DNA probe-based clinical assays utilizing the dioxetane substrates are commercially available.

DNA probe-based assays will be used for a rapidly growing number of applications in the future, including detection and identification of infectious agents, screening for genetic susceptibility to disease, genetic screening, food testing, genetic typing, forensic science, therapeutic drug monitoring, environmental testing, and basic research. Both the dioxetane and the AE systems have the potential to be utilized in the applications listed above. At present, the dioxetane system is more particularly suited to applications where very high sensitivity is required and is more important than speed and ease of use or where detection of target immobilized on or within a solid support is required, as for example in DNA sequencing. The AE-based systems have broad applicability due to their unique combination of speed and ease of use, sensitivity, specificity, compatability with clinical specimens, and quantifiability. It has already been shown to be of great value in assay systems suitable for routine use in the clinical laboratory, even at a relatively early stage of development.

## References


1 Kohne DE. Application of DNA probe tests to the diagnosis of infectious disease. Am Clin Prod Rev 1986; November:20–29.

2 Enns RK. DNA Probes: An overview and comparison with current methods. Lab Medicine 1988;19:295–300.

3 Keller GH, Manak MM. DNA Probes. New York: Stockton Press, 1989.

4 Deluca MA, McElroy WD, eds. Methods Enzymol, Vol 133, Bioluminescence and Chemiluminescence Florida: Academic Press, Inc., 1986.

5 van Dyke K, ed. Bioluminescence and chemiluminescence: instruments and applications, Vols. I and II Florida: CRC Press, 1985.

6 Campbell AK. Chemiluminescence: Principles and applications in biology and medicine UK: Ellis Horwood Ltd and FRG: VCH Verlagsgesellschaft GmbH, 1988.

7 Arnold LJ Jr, Hammond PW, Wiese WA, Nelson NC. Assay formats involving acridinium-ester-labeled DNA probes. Clin Chem 1989;35:1588–1594.

8 Granato PA, Franz MR. Evaluation of a prototype DNA probe test for the nonculture diagnosis of gonorrhea. J Clin Micro 1989;27:632–635.

9 Bronstein I, Voyta JC, Lazzari KG, Murphy O, Edwards B, Kricka LJ. Rapid and sensitive detection of DNA in southern blots with chemiluminescence. Bio Techniques 1990;8:310–314.

10 Schaap AP, Akhavan H, Romano LJ. Chemiluminescent substrates for alkaline phosphatase: application to ultrasensitive enzyme-linked immunoassays and DNA probes. Clin Chem 1989;35:1863–1864.

11 Sanchez-Pescador R, Stempien MD, Urdea MS. Rapid chemiluminescent nucleic acid assays for detection of TEM-1 $\beta$-lactamase-mediated penicillin resistance in *Neisseria gonorrhoeae* and other bacteria. J Clin Micro 1988;26:1934–1938.

12 Urdea MS, Warner BD, Running JA, Stempien M, Clyne J, Horn T. A comparison of non-radioisotopic hybridization assay methods using fluorescent, chemiluminescent and enzyme labeled synthetic oligodeoxyribonucleotide probes. Nuc Acids Res 1988;16:4937–4956.

13 Durrant I. Light-based detection of biomolecules. Nature (London) 1990;346:297–298.

14 Carlson DP, Superko C, Mackey J, Gaskill ME, Hansen, P. Chemiluminescent detection of nucleic acid hybridization. Focus 1990;12:9–12.

15 Clyne JM, Running JA, Stempien M, et al. A rapid chemiluminescent DNA hybridization assay for the detection of *Chlamydia trachomatis*. J Biolum Chemilum 1989;4:357–366.
16 Acheson RM. Acridines. In: Weissberger A, Taylor EC, eds. The Chemistry of Heterocyclic Amines. New York: Interscience Publishers, 1973.
17 Albert A. The Acridines. London: Edward Arnold Ltd., 1966.
18 McCapra F. Chemiluminescence of organic compounds. Pure Appl Chem 1970;24:611–629.
19 McCapra F. Chemiluminescent reactions of acridines. Chem Heterocycl Compounds. 1973;9:615–630.
20 McCapra F, Perring KD. General Organic Chemiluminescence. In: Burr JG, ed. Chemi and Bioluminescence. New York: Marcel Dekker, Inc, 1985:259–320.
21 McCapra F. Chemical mechanisms in bioluminescence. Acc Chem Res 1976;9:201–108.
22 Weeks I, Beheshti I, McCapra F, Campbell AK, Woodhead JS. Acridinium esters as high-specific-activity labels in immunoassay. Clin Chem 1983;29:1474–1479.
23 Weeks I, Campbell AK, Woodhead JS. Two-site immunochemical assay for human å-fetoprotein. Clin Chem 1983;29:1480–1483.
24 Arnold LJ Jr, Bhatt RS, Reynolds MA. Non-nucleotide linking reagents for nucleotide probes. PCT/US88/03173, 1988.
25 Tenover FC, Carlson, L, Barbagallo S, Nachamkin I. DNA probe culture confirmation assay for identification of thermophilic *Campylobacter* Species. J Clin Micro 1990;28:1284–1287.
26 Gegg C, Kranig-Brown D, Hussain J, McDonough S, Kohne D, Shaw S. A clinical evaluation of a new DNA probe test for *Neisseria gonorrhoeae* that included analysis of specimens producing results discrepant with culture. Abstracts of the 90th Annual Meeting of the American Society for Microbiology 1990;356.
27 Kranig-Brown D, Gegg C, Hussain J, Johnson R, Shaw S. Use of PCR Amplification and unlabeled probe competition for analysis of specimens producing results discrepant with culture in an evaluation of a DNA probe test for *Chlamydia trachomatis*. Abstracts of the 90th Annual Meeting of the American Society for Microbiology 1990;356.
28 Watson M, Zepeda-Bakan M, Smith K, Alden M, Stolzenbach F, Johnson R. Evaluation of a DNA probe assay for screening urinary tract infections. Abstracts of the 90th Annual Meeting of the American Society for Microbiology 1990;392.
29 Snider E, Gordon P, Dean E, Trainor D. Development of a DNa probe culture confirmation test of *Haemophilus influenzae*. Abstracts of the 90th Annual Meeting of the American Society for Microbiology 1990;398.
30 Rubin SM, Murphy-Clark KA, Bee GG, Gordon PC, Roberts SS, Johnson R. Development of rapid non-isotopic DNA probe assays for fungi: *Blastomyces dermatitidis*. Abstracts of the 90th Annual Meeting of the American Society for Microbiology 1990;409.
31 Woese CR. Bacterial Evolution. Microbiol Rev 1987;51:221–271.
32 Adam W. The Chemistry of 1,2-dioxetans: Adv Heterocycl Chem 1977;21:438–481.
33 Wilson T. Chemiluminescence in the liquid phase: Thermal cleavage of dioxetans. Int Rev Sci Phys Chem 1976;2:265–322.
34 McCapra F, Beheshti, I. Selected chemical reactions that produce light. In: van Dyke K, ed. Bioluminescence and Chemiluminescence: Instruments and Applications Vol I. Florida: CRC Press 1985;9–42.
35 Schaap AP, Handley RS, Giri BP. Chemical and enzymatic triggering of 1,2-Dioxetanes. 1: Aryl Esterase-catalyzed chemiluminescence from a naphthyl acetate-substituted dioxetane. Tetrahedron Lett 1987;28:935–938.
36 Schaap AP, Chen TS, Handley RS, DeSilva R, Giri BP. Chemical and enzymatic triggering of 1,2-dioxetanes. 2: fluoride-induced chemiluminescence from tert-butyldimethylsilyloxy-substituted dioxetanes. Tetrahedron Lett 1987;28:1155–1158.
37 Schaap AP, Sandison MD, Handley RS. Chemical and enzymatic triggering of 1,2-dioxetanes. 3: Alkaline phosphatase-catalyzed chemiluminescence from an aryl phosphate-substituted dioxetane. Tetrahedron Lett 1987;28:1159–1162.
38 Bronstein I, Edwards B, Voyta JC. 1,2-Dioxetanes: Novel chemiluminescent enzyme substrates. Applications to immunoassays. J Biolum Chemilum 1989;4:99–111.
39 Bronstein I, McGrath P. Chemiluminescence lights up. Nature 1989;338:599–600.

40 Bronstein I, Voyta JC, Edwards B. A comparison of chemiluminescent and colorimetric substrates in a hepatitis B virus DNA Hybridization Assay. Anal. Biochem 1989;180:95-98.
41 Kricka LJ, Thorpe GHG. Photographic detection of chemiluminescent and bioluminescent reactions. In: Deluca MA, McElroy WD, eds. Methods in Enzymol Vol 133, Bioluminescence and Chemiluminescence. Florida: Academic Press, Inc, 1986;404-420.
42 Nelson NC, Hammond PW, Wiese WA, Arnold LJ, Jr Homogeneous and heterogeneous chemiluminescent DNA probe-based assay formats for the rapid and sensitive detection of target nucleic acids. In: van Dyke K, ed. Luminescence Immunoassay and Molecular Applications. Florida: CRC Press, Inc 1990;293-309.
43 Bronstein I, Boyta JC, Thorpe GHG, Kricka LJ, Armstrong G. Chemiluminescent Assay for Alkaline Phosphotase: Application in an Ultrasensitive Enzyme Immunoassay for thyrotropin. Clin Chem 1989;35:1441-1446.
44 Berry DJ, Clark PMS, Price CP. A laboratory and clinical evaluation of an immunochemiluminometric assay of thyrotropin in serum. Clin Chem 1988;34:2087-2090.
45 Mullis KB, Faloona FA. Specific synthesis of DNA in vitro via a polymerase catalyzed chain reaction. In: Wu R, ed. Methods in Enzymol Vol 155. Florida: Academic Press, Inc 1987;335-350.
46 Kwoh DY, Davis GR, Whitfield KM, Chappelle HL, DiMichele LJ, Gingeras TR. Transcription-based amplification system and detection of amplified human immunodeficiency virus type 1 with a bead-based sandwich hybridization format. Proc Natl Acad Sci USA 1989;86:1173-1177.
47 Gyllensten UB, Erlich HA. Generation of single-stranded DNA by the polymerase chain reaction and its application to the direct sequencing of the HLA-DQA locus. Proc Natl Acad Sci USA 1988;85:7652-7656.
48 Innis MA, Gelfand DH. Optimization of PCRs. In: Innes MA, Gelfand DH, Sninsky JJ, White TJ, eds. PCR Protocols. California: Academic Press, Inc. 1990;3-12.
49 Adams JM. Oncogene activation by fusion of chromosomes in leukemia. Nature (London) 1985;315:542-543.
50 McDonough SH, Ryder TB, Cabanas DK, et al. Rapid and sensitive detection of amplified DNA by a chemiluminescent DNA probe system (HPA). Abstracts of the Twenty-Ninth Interscience Conference on antimicrobial Agents and Chemotherapy, 1989:301.
51 Bronstein I, Voyta JC. Chemiluminescent detection of herpes simplex virus I DNA in blot and in situ hybridization assays. Clin Chem 1989;35:1856-1860.

After discrepant analysis and further testing, the values which appear in Table III have been updated and appear below.

**Table III**

Comparison of PACE 2 and Culture Assays for Chlamydia trachomatis and Neisseria gonorrhoeae in Urogenital Clinical Specimens[1]

Chlamydia trachomatis, combined female and male specimens (n=1590)

| | | Culture + | Culture - | |
|---|---|---|---|---|
| PACE 2 | + | 161 | 6 | Sensitivity = 92.5% |
| | - | 13 | 1410 | Specificity = 99.6%<br>Agreement = 99% |

Neisseria gonorrhoeae, combined female and male specimens (n=1762)

| | | Culture + | Culture - | |
|---|---|---|---|---|
| PACE 2 | + | 248 | 4 | Sensitivity = 95.4% |
| | - | 12 | 1498 | Specificity = 99.8%<br>Agreement = 99% |

[1]Positivity rate for C. trachomatis (sample set = 1590 specimens) was 11%; positivity rate for N. gonorrhoeae (sample set = 1762 specimens) was 15%