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Detection Methods Using Chemiluminescence

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I. GENERAL INTRODUCTION

Chemiluminescence, the emission of light from a chemical reaction, has been studied extensively for many decades. Chemiluminescent processes constitute a very special class of chemical reactions in which products (or intermediates) are produced in electronically excited states that are very short-lived and rapidly decay with concomitant emission of light. Similar chemiluminescent reactions, called bioluminescence, occur in nature in species as diverse as the firefly (*Photinus pyralis*), marine bacteria (*Vibrio harveyi*), and others. Most chemiluminescence reactions involve oxidations of

a variety of organic compounds as well as naturally occurring materials, resulting in the generation of light-emitting excited states. This phenomenon was first described with synthetic organic compounds in 1877 (Radziszewski, 1877).

Chemiluminescent reactions do not produce very high intensity light signals because of many efficient quenching processes that compete with the radiative decay of the excited states. Nevertheless, chemiluminescence has been used effectively as a very sensitive detection system in many applications (Carter and Kricka, 1982; Harber, 1982; Kricka and Carter, 1982), largely because no background light signals are generated since the emitting excited state is created in a dark chemical reaction (compared to scattered excitation light in fluorescence.) Therefore, in theory, every photon detected is a true signal of the assay. This feature of chemiluminescent molecules—coupled with long shelf-life, elimination of hazards associated with the use of radioisotopes, and their detectability at 10^{-21} moles (detection of alkaline phosphatase with chemiluminescent dioxetane substrate; Kricka, 1992)—makes them ideal as a reporter system for immunoassays and DNA probe hybridization assays.

In this chapter we describe the use of various chemiluminescence methodologies for the detection of viruses in DNA hybridization assays. A short discussion of instrumentation used in chemiluminescence measurement is also included.

II. CHEMILUMINESCENCE METHODS

A. Dioxetanes

Dioxetanes are four-membered cyclic peroxides that have been implicated as short-lived unstable intermediates in oxidation reactions that result in chemiluminescence (McCapra, 1966). Thus, 1,2-dioxetanes differ from most other chemiluminescence systems because these compounds do not require oxidation to emit light. Recently developed 1,2-dioxetanes that can be activated to luminesce by enzymes have been used successfully for bio-analyte detection. Dephosphorylation of adamantyl- and derivatized adamantyl-1,2-dioxetane phosphate substrates, such as AMPPD[®] [disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2'-tricyclo[3.3.1.1^{3,7}]decan}-4-yl)phenyl phosphate] and CSPD[®] [disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2'-(5'-chloro)tricyclo[3.3.1.1^{3,7}]decan}-4-yl)phenyl phosphate] by alkaline phosphatase results in the formation of a destabilized anion that fragments further to form an excited state of methyl *meta*-oxybenzoate anion that emits light at

477 nm (Fig. 1; Bronstein *et al.*, 1989a,1991; Bronstein and Dimond, 1990; Bronstein and Sparks, 1992).

1,2-Dioxetane substrates for alkaline phosphatase are widely used in DNA hybridization assays (Bronstein, *et al.*, 1990; Pollard-Knight *et al.*, 1990b; Tumolo *et al.*, 1992). DNA probes are labeled with alkaline phosphatase either indirectly, with a biotin or hapten label followed by binding streptavidin- or antibody-alkaline phosphatase conjugates, or directly by covalent bonding to enzyme (oligonucleotide probes). Biotin has been the most popular ligand for indirect labeling, but hapten labels other than biotin have also been employed including digoxigenin, fluorescein, and 2,4-dinitrophenyl. Dioxetane-based chemiluminescent indirect labeling and detection systems for DNA hybridization assays, as well as for immunoassays and DNA sequencing, are widely available from many commercial suppliers. With a nick-translated biotinylated DNA probe, as little as 380 fg (7.9×10^4 copies) of target pBR322 DNA can be detected on a Southern blot (Bronstein *et al.*, 1990). Using digoxigenin-labeled random-primed DNA probes with a membrane-based assay and photographic film detection, a sensitivity level of 10–50 fg of target DNA was obtained for the detection of purified cytomegalovirus (CMV) or parvovirus B19 DNA (Musiani *et al.*, 1991a).

Direct labeling of oligonucleotide probes with alkaline phosphatase (Jablonski *et al.*, 1986) is possible with systems from Promega (Madison,

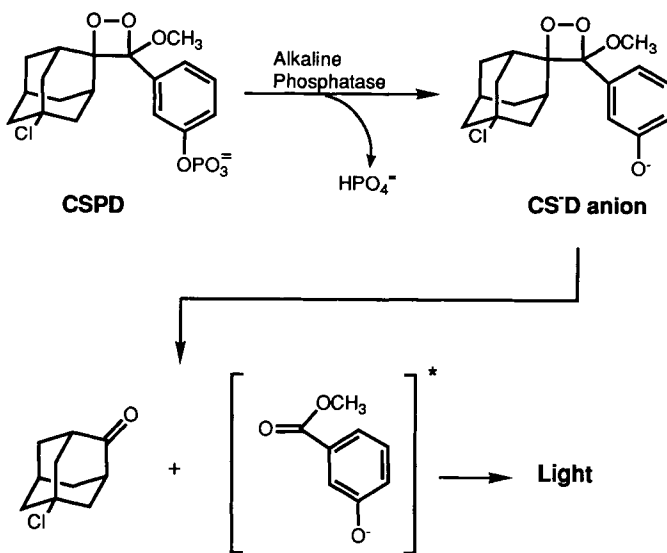


Figure 1 Chemiluminescent decomposition of CSPD® 1,2-dioxetane triggered by enzymatic dephosphorylation.

WI) and Cambridge Research Biochemicals (Wilmington, DE). The detection of a single copy gene in 0.25 μg human genomic DNA with Southern blot analysis has been achieved with an alkaline phosphatase-labeled oligonucleotide probe (Cate *et al.*, 1991).

Comparisons have shown that the sensitivity of alkaline phosphatase–dioxetane chemiluminescence detection is comparable to or better than that of ^{32}P -based detection. In a human genomic Southern blot analysis of the tissue plasminogen activator gene, the sensitivity achieved with an alkaline phosphatase-labeled oligonucleotide probe was 12-fold higher than that achieved with the same ^{32}P -5'-end-labeled probe, and the speed of detection was enhanced 40-fold with the alkaline phosphatase-labeled probe (Cate *et al.*, 1991). Slot blot hybridization of human serum samples with the alkaline phosphatase-labeled AmpliProbe® system (ImClone Systems, New York) showed a higher sensitivity in the detection of hepatitis B virus (HBV) relative to ^{32}P -labeled nick-translated probes (Yang *et al.*, 1991). Similar sensitivities were obtained with an indirect digoxigenin-labeled probe and a random-primed ^{32}P -labeled probe in a dot blot hybridization assay for amplified human immunodeficiency virus type 1 (HIV-1) DNA (Zachar *et al.*, 1991).

The sensitivity of alkaline phosphatase–dioxetane chemiluminescence detection has been shown to be superior to other nonisotopic systems based on colorimetric detection in membrane-based hybridization assays (Bronstein and Kricka, 1989; Bronstein and Voyta, 1989; Bronstein *et al.*, 1989c; Musiani *et al.*, 1991a, 1992). Finally, alkaline phosphatase–dioxetane detection has been demonstrated to be two to five times more sensitive than enhanced luminol chemiluminescent detection (described subsequently) in a solution hybridization assay system (Clyne *et al.*, 1989; Urdea *et al.*, 1990). Furthermore, the alkaline phosphatase–dioxetane detection system consists of fewer components, which are more stable than those required for an enhanced luminol chemiluminescent reaction (Beck and Köster, 1990). Although several other alkaline phosphatase-based chemiluminescent assays also exist, involving alternative substrates and coupled reactions, the most sensitive and widely used assays are those with 1,2-dioxetane substrates (for review of alternative systems, see Kricka, 1991).

B. Luminol

Luminol and other cyclic diacylhydrazide derivatives can be oxidized in the presence of peroxide and peroxidase to generate an unstable intermediate in the excited state that chemiluminesces. Luminols can be used as direct chemiluminescent labels or as the chemiluminescent detectors of a peroxidase enzyme label (Kricka, 1991). Activation of luminol chemiluminescence with horseradish peroxidase (HRP) using an enhanced luminol system (en-

hanced chemiluminescence, ECL) has been done in DNA hybridization assays (Matthews *et al.*, 1985; Durrant *et al.*, 1990; Durrant, 1992) and immunoassays (reviewed by Bronstein and Sparks, 1992; Whitehead *et al.*, 1983; Thorpe *et al.*, 1985; Kricka *et al.*, 1987).

DNA probes can be labeled indirectly with HRP by binding streptavidin–HRP or anti-hapten–HRP conjugates or covalently by direct enzyme conjugation with oligonucleotides and longer double-stranded DNAs. The detection of single copy genes in 0.5 μg human genomic DNA has been reported with indirectly labeled probes (Simmonds *et al.*, 1991). Direct HRP-labeled DNA probes have been used for both membrane-based DNA hybridization assays (Pollard-Knight *et al.*, 1990a; Simmonds *et al.*, 1991) and solution-phase hybridization assays (Urdea *et al.*, 1990). Detection of a single-copy gene on a Southern blot of <2 μg human genomic DNA, with a sensitivity of <1 amol target DNA, has been demonstrated (Pollard-Knight *et al.*, 1990a) using direct HRP-labeled probes 0.3–5.1 kb in length. Similar sensitivity (1 amol target DNA) was also reported by Durrant *et al.* (1990). Both indirect and direct HRP labeling systems for nucleic acids and detection systems for HRP-catalyzed chemiluminescent reactions (ECL gene detection system) are available from Amersham (Arlington Heights, IL).

C. Acridinium Esters

Acridinium esters (AE) are direct chemiluminescent labels for antibodies (Weeks *et al.*, 1983) and DNA probes (Septak, 1989; Nelson and Kacian, 1990; Nelson *et al.*, 1992), in contrast to dioxetane and luminol systems, in which an enzyme label catalyzes the chemiluminescent reaction. *N*-Methyl acridinium esters react with hydrogen peroxide under basic conditions to yield an excited state *N*-methylacridone which emits light at 430 nm (reviewed by Nelson and Kacian, 1990). Oligonucleotide DNA probes can be labeled covalently with AEs by reaction of modified *N*-hydroxysuccinimide-AE with a primary alkyl amine on a linker arm that was previously incorporated during oligonucleotide synthesis (Nelson and Kacian, 1990). Preparation of AE-labeled oligonucleotide probes has also been described by Septak (1989). The AE label does not affect probe hybridization characteristics; relatively large amounts of clinical specimen material may be used without interfering with hybridization and detection of AE-labeled probes.

Probe hybridization and detection reactions are performed in solution, using either separation or nonseparation formats. In a separation or heterogeneous assay, hybridized probe may be separated and detected by selective binding to microspheres, which can be separated from solution magnetically. In a nonseparation or homogeneous format, also termed a hybridization protection assay (HPA), the ester bond of the unhybridized probe can be hydrolyzed by differential chemical hydrolysis, thus rendering its AE label

nonchemiluminescent, whereas the AE label of the hybridized probe is minimally affected (Nelson and Kacian, 1990). This type of assay is possible because hybridization provides an intercalation site for the AE label, thereby protecting the AE molecule residing in the hybridized region from hydrolysis (Arnold *et al.*, 1989).

The sensitivity of this detection system is approximately 5×10^{-19} mol AE-labeled oligonucleotide, and the linear dynamic range is greater than four orders of magnitude (Nelson and Kacian, 1990). Similar sensitivities for the detection of an amplified *gag* sequence (4 HIV proviral copies per 150,000 cells) were achieved with colorimetric, chemiluminescence and ^{32}P -labeling methods (Ou *et al.*, 1990; Rapier *et al.*, 1993). Schmidt (1991) was able to detect 0.05 fmol target HIV-1 DNA with AE-labeled *gag* probes and obtained greater sensitivity with chemiluminescence than with the same ^{32}P -end-labeled probe in a dot blot hybridization assay.

D. Electrochemiluminescence

Electrochemiluminescence is a process in which the excited state products are generated via an electrochemical reaction (Faulkner and Glass, 1982). Electrochemiluminescence occurs when specific metal chelates such as ruthenium (II) tris(bipyridyl) [$\text{Ru}(\text{bpy})_3^{2+}$], utilized as labels, undergo a series of chemical reactions at an electrode surface. Electrochemiluminescent labels for DNA hybridization assays have been utilized in a highly sensitive, simple, and versatile assay system. Oligonucleotide probes, synthesized with a free 5'-amino group, are readily labeled with $\text{Ru}(\text{bpy})_3^{2+}$ -NHS ester (Blackburn *et al.*, 1991; Kenten *et al.*, 1991). Alternatively, oligonucleotide probes may be labeled during synthesis by incorporating labeled phosphoramidites (Kenten *et al.*, 1992; DiCesare *et al.*, 1993).

Electrochemiluminescent labels are relatively small molecules (~ 1000 dalton) that are extremely stable and may be coupled to nucleic acids, haptens, or proteins without affecting immunoreactivity or hybridization characteristics. The dynamic range for detection of these labels has been reported to be over six orders of magnitude (Blackburn *et al.*, 1991). These advantages, compared with other nonisotopic detection methods, provide potential wide utility in automated nonradioactive clinical diagnostic assays, including both DNA hybridization and immunoassay formats. A disadvantage of electrochemiluminescence, however, is a need for specialized instrumentation that can induce generation of electrochemically-excited states coupled with sensitive light detection.

Blackburn *et al.* (1991) used electrochemiluminescence detection with a DNA probe assay to quantify polymerase chain reaction (PCR)-amplified HIV-1 *gag* sequences. Double-stranded biotinylated PCR product was captured on streptavidin-coated microparticles and treated with alkali.

Ru(bpy)₃²⁺-labeled oligonucleotide probe was then hybridized to the particle-bound DNA, washed, and quantified. A linear response was generated over the range of 50 to 2000 gene copies, and the detection of less than 10 copies of the HIV-1 *gag* was attained. An automated system for electrochemiluminescence quantification of PCR products (QPCR System 5000; Perkin-Elmer Corporation, Norwalk, CT) has been developed (DiCesare *et al.*, 1993) and is used for detection of viral disease. This system provides detection limits of 10–200 amol and a linear dynamic range greater than three orders of magnitude. The system has been used for the detection of HIV-1 over a range of 3 to 10⁶ copies of target DNA (Wages *et al.*, 1993).

Because of the electrogeneration of the emitting species, which requires contact of the metal chelate label with an electrode, it is difficult to envision that simple membrane-based blotting assays that can be imaged on film could be designed using electrochemiluminescence.

E. Bioluminescence

Bioluminescent reactions, a special class of chemiluminescent reactions that occur in nature and are catalyzed by a luciferase or photoproteins, offer an alternative method for luminescence detection of protein and DNA (Kricka, 1991). Two bioluminescent reaction systems have been used for DNA hybridization assays, both of which are coupled enzymatic reactions. One system, used for membrane-based DNA hybridization, couples the production of D-luciferin from D-luciferin-*O*-phosphate, catalyzed by alkaline phosphatase (as a direct or indirect label) and the oxidation of D-luciferin, catalyzed by firefly luciferase, with concomitant light emission (Hauber and Geiger, 1987, 1988; Hauber *et al.*, 1988, 1989; Geiger, 1992). The other system, used with both membrane-based and solution hybridization assays, couples reactions catalyzed by glucose-6-phosphate dehydrogenase (G6PDH), NAD(P)H : FMN oxidoreductase, and marine bacterial luciferase to produce the light (Balaguer *et al.*, 1989a,b, 1991a,b; Nicolas *et al.*, 1990, 1992). Although bioluminescence-based DNA detection systems have not become as widely used as chemiluminescence systems for DNA hybridization assays, they do offer another alternative for sensitive nonradioactive biomolecule detection.

III. INSTRUMENTATION FOR CHEMILUMINESCENCE ASSAYS

A wide spectrum of instruments is currently available for recording and quantifying chemiluminescent signal intensities. These instruments, known as luminometers, use a light detector that consists of a photomultiplier tube

in photon counting mode, positioned close to the light source (microtiter plate or tube) to maximize photon collection efficiency. Among commercially available luminometers, semi-automated tube instruments such as the AutoClinilumat LB952T (Berthold/EG&G, Wallac, Inc., Gaithersburg, MD) and microtiter plate readers such as the ML 1000 (Dynatech Laboratories, Chantilly, VA) are most popular (reviewed by Bronstein and Kricka, 1990; Stanley, 1992a,b,1993b).

Chemiluminescence signals originating from blotting experiments performed on membranes can be detected by imaging on X-ray or instant photographic films. These films offer simple, convenient, and inexpensive detectors of chemiluminescence that can be used successfully for qualitative determinations and some signal quantification. Camera luminometers that house instant photographic film are suitable for the detection of light emission from blots and microtiter plate wells, and are available from Amersham, Analytical Luminescence Laboratory (San Diego, CA), Dynatech Laboratories, and Tropix, Inc. (Bedford, MA).

Finally, photon-counting cameras are available and are most suitable for the detection and accurate quantification of low-light signals. This instrumentation usually consists of a light detector such as a silicon target, silicon diode array, or charge-coupled device (CCD) coupled to a lens system, a controller, and a digital image processor. Since most of these camera systems are capable of imaging in two dimensions, micro- and macroscopic luminescent specimens can be analyzed spatially and temporally. The Argus-100/CL (Hamamatsu Corporation, Photonic Microscopy, Inc., Oak Brook, IL) is a photon-counting imaging device that has been used in the detection of blotted proteins (Hauber *et al.*, 1988). The Star I CCD cooled camera system (Photometrics Ltd., Tucson, AZ) exhibits very low dark current background and a wide dynamic range and has been used successfully to detect protein and nucleic acid analytes in solution and on membranes (Martin and Bronstein, 1993, 1994.).

IV. CHEMILUMINESCENCE ASSAYS FOR VIRUS DETECTION

The combination of DNA hybridization assays with chemiluminescence detection methods has enabled the development of rapid, sensitive, quantitative, nonradioactive assays that are amenable to automation. DNA hybridization technology is becoming accepted as a reliable clinical laboratory technique for the identification of infectious organisms and has fueled the need for more rapid, sensitive, and automated assay formats. Culture assay methods are laborious, time-consuming, and costly, and sometimes impossi-

ble to use. Antigen-based detection assays including fluorescent antibody and immunoassay techniques, although faster and automatable, are often less sensitive than culture techniques. With the advent of technologies such as PCR, DNA probe methods offer rapid, easy, and highly sensitive assay formats. DNA hybridization assays using radioactive labels are sensitive and are easily quantified, but health, environmental, disposal, and cost concerns render these systems less than ideal as widely used clinical assays.

Chemiluminescence methods for the detection of viral agents as well as other microorganisms have become widely used (Table 1), and continued development will certainly expand their applications in research and clinical diagnostic tests. More traditional immunoassays have also been developed and used with chemiluminescence for the detection of various viral antigens and the assessment of immune status with respect to viruses (selected references in Table 1). A survey of commercially available products that incorporate chemiluminescence or bioluminescence techniques and reagents for specific assays and nonspecific detection systems is available (Stanley, 1993a,b).

A. DNA Hybridization Assay Formats

Several DNA hybridization assay formats including membrane-based, solution, and *in situ* hybridization have been coupled with chemiluminescence for the detection of viruses and other infectious agents. Membrane-based chemiluminescent hybridization assays have employed either 1,2-dioxetane substrates for alkaline phosphatase or the enhanced chemiluminescence reaction of luminol and HRP, and are imaged on X-ray or photographic films or imaged directly and quantified using a CCD camera system. Solution hybridization assays are performed with 1,2-dioxetanes, luminol, and AE labels, and the emitted light signal is measured in a luminometer. Electrochemiluminescent labels are also used for solution hybridization assays and are detected with an instrument combining an electrochemical flow cell, a potentiostat, and a photomultiplier tube. *In situ* hybridization has been performed using both 1,2-dioxetanes and enhanced luminol with either photographic film detection or a CCD camera system.

B. Chemiluminescence Detection Systems

1. Dioxetanes

Alkaline phosphatase–dioxetane chemiluminescence systems have been used in a wide variety of DNA hybridization assays for detection of infectious

TABLE 1
Selected Studies That Have Used Chemiluminescence to Detect Viruses, *Chlamydia trachomatis*, and Other Microorganisms

Agent	Assay ^a	Reference
Barley yellow dwarf virus	MH	Fouly <i>et al.</i> (1992) (DX)
Bluetongue virus	PCR/H	Akita <i>et al.</i> (1993)
Bovine enteric coronavirus	MH	Collomb <i>et al.</i> (1992) (LU)
Bovine immunodeficiency-like virus	IA	Jacobs <i>et al.</i> (1992)
Bovine leukosis virus	IA	Miliukiene <i>et al.</i> (1991)
Bursal disease virus	H	Akin <i>et al.</i> (1993) (AP)
Chicken anemia virus	PCR/MH	Tham and Stanislawek (1992a,b) (DX)
Cytomegalovirus	MH	Musiani <i>et al.</i> (1991a,1992) (DX); Yang <i>et al.</i> (1991) (DX)
Dengue virus	PCR/H	Henchal <i>et al.</i> (1991) (DX)
Enterovirus (poliovirus)	MH	Fuchs <i>et al.</i> (1993) (DX)
Epstein-Barr virus	MH	Yang <i>et al.</i> (1991) (DX)
	PCR/MH	Vlieger <i>et al.</i> (1992) (LU)
Feline infectious peritonitis virus	MH	Martinez and Weiss (1993) (AP)
Grapevine closterovirus	IA	Pollini <i>et al.</i> (1993) (LU)
Hepatitis B virus	MH	Bronstein <i>et al.</i> (1989c) (DX); Farmar and Castaneda (1991) (DX); Yang <i>et al.</i> (1991) (DX)
	PCR/MH	Escarceller <i>et al.</i> (1992) (DX)
	SH	Urdea <i>et al.</i> (1987,1990) (LU; LU, DX)
	IA	Khalil <i>et al.</i> (1991a,b) (AE); Bouveresse and Bourgeois (1992) (AP); Boxall (1992) (LU); McCartney <i>et al.</i> (1993) (LU)
Hepatitis C virus	IM	Ireland and Samuel (1989) (LU); Robertson <i>et al.</i> (1991) (AE); Weare <i>et al.</i> (1991) (AE)
	PCR/H	Geiger and Caselmann (1992)
	IA	Khalil <i>et al.</i> (1991b) (AE)
Herpes simplex virus	MH	Bronstein and Voyta (1989) (DX)
	ISH	Bronstein and Voyta (1989) (DX)
	PCR/H	Puchhammerstoekl <i>et al.</i> (1993) (AP)
	IA	Pronovost <i>et al.</i> (1981) (ILU); Dalessio and Ashley (1992) (LU)
Human immunodeficiency virus	PCR/MH	Conway <i>et al.</i> (1990) (AP); Zachar <i>et al.</i> (1991) (DX)
	PCR/SH	Ou <i>et al.</i> (1990) (AE); Blackburn <i>et al.</i> (1991) (EL); Schmidt (1991) (AE); Schmidt and Gschnait (1991); Gudihande <i>et al.</i> (1992) (EL); Kenten <i>et al.</i> (1992) (EL); Suzuki <i>et al.</i> (1992) (DX); Rapiet <i>et al.</i> (1993) (AE); Wages <i>et al.</i> (1993) (EL)
	PCR	Bettens <i>et al.</i> (1991) (DX)
	BH	Ishii and Ghosh (1993) (AP)

	ISH	Bronstein <i>et al.</i> (1989b) (DX)
	RT	Suzuki <i>et al.</i> (1993) (DX)
	IA	Khalil <i>et al.</i> (1991b) (AE); Jacobs <i>et al.</i> (1992)
Human papilloma virus	PCR/SH	Balaguer <i>et al.</i> (1991b) (BL); Kenten <i>et al.</i> (1991) (EL)
	MH	Sarkar <i>et al.</i> (1993)
	ISH	Hawkins and Cumming (1990) (LU)
Human T-cell leukemia virus	IA	Khalil <i>et al.</i> (1991b) (AE)
	IM	Kuroda <i>et al.</i> (1992) (LU)
Human T-cell lymphotropic virus	IA	Papsidero <i>et al.</i> (1992) (LU)
Influenza virus	MH	McKimm-Breschkin (1992) (DX)
	IM	Arenkov <i>et al.</i> (1991)
Lentivirus	RT	Cook <i>et al.</i> (1992) (DX)
Parvovirus	MH	Musiani <i>et al.</i> (1991a,b) (DX)
	IA	O'Neill and Coyle (1992) (LU)
Potato virus Y, potato spindle tuber viroid	MH	Welnicki and Hiruki (1993) (AP)
Potato, pome fruit viroid	MH	Podleckis <i>et al.</i> (1993) (AP)
Respiratory syncytial virus, rotavirus	IM	Hornsleth <i>et al.</i> (1988) (LU)
Rubella virus	IM	Chanteloup <i>et al.</i> (1992) (AP)
Varicella zoster virus	PCR/H	Eis-Hubinger <i>et al.</i> (1992) (AP)
Bacteria	MH	Gustafarro and Persing (1992) (LU)
	?	Daly <i>et al.</i> (1991)
	SH	Clyne <i>et al.</i> (1989) (DX); Urdea <i>et al.</i> (1989) (DX); Gratton <i>et al.</i> (1990) (AE); Mercer <i>et al.</i> (1990) (AE); Iwen <i>et al.</i> (1991) (AE)
<i>Chlamydia trachomatis</i>	IA	Neman-Simha <i>et al.</i> (1991); Dumornay <i>et al.</i> (1992) (AE); Jang <i>et al.</i> (1992) (AE); Scieux <i>et al.</i> (1992a,b) (AE)
Mycobacteria	SH	Bull and Shanson (1992) (AE)
	SD/SH	Donahue <i>et al.</i> (1993) (DX)
	PCR/MH	Sriharan and Barker (1991) (DX)
<i>Neisseria gonorrhoeae</i>	SH	Urdea <i>et al.</i> (1989) (DX); Vlaspolder <i>et al.</i> (1993) (AE)
<i>Plasmodium falciparum</i>	PCR/MH	Barker <i>et al.</i> (1992) (DX)
<i>Toxoplasma gondii</i>	PCR/MH	Stauber <i>et al.</i> (1991) (LU)

^a Assay formats include: BH, bead-based hybridization; H, hybridization; IA, immunoassay; IM, immunoassay; ISH, *in situ* hybridization; MH, membrane-based hybridization; PCR, polymerase chain reaction; RT, reverse transcriptase; SD, strand displacement; SH, solution hybridization.

^b Chemiluminescent (CL) methods employed (if known): AE, acridinium ester; AP, alkaline phosphatase (most likely with dioxetane substrate); BL, bioluminescence; DX, dioxetane; EL, electrochemiluminescence; ILU, isoluminol; LU, enhanced luminol.

agents. Membrane-based hybridization assays have been used for the detection of HBV (Bronstein *et al.*, 1989c; Yang *et al.*, 1991; Escarceller *et al.*, 1992), herpes simplex virus (HSV-1) (Bronstein and Voyta, 1989), CMV (Musiani *et al.*, 1991a,1992; Yang *et al.*, 1991), HIV-1 (Zachar *et al.*, 1991), and other viral agents (Fouly *et al.*, 1992; Tham and Stanislawek, 1992a,b; Fuchs *et al.*, 1993). Solution hybridization assays include those for HBV (Urdea *et al.*, 1990), HIV-1 (Suzuki *et al.*, 1992), and *Chlamydia* (Clyne *et al.*, 1989; Urdea *et al.*, 1989). *In situ* hybridization assays have been performed with both HSV-1 infected cells (Bronstein and Voyta, 1989) and HIV-infected cells (Bronstein *et al.*, 1989b). Finally, assays for retroviruses based on the detection of reverse transcriptase activity can be coupled with chemiluminescence detection by measuring the enzymatic incorporation of digoxigenin-labeled nucleotides with anti-digoxigenin alkaline phosphatase and a dioxetane substrate (Suzuki *et al.*, 1993).

Commercially available detection systems incorporating dioxetanes include the AmpliProbe® system (ImClone Systems) for membrane-based hybridization assays for HBV, CMV, and EBV (Yang *et al.*, 1991), the Hybrid Capture™ System HBV DNA Assay (Murex Diagnostics Ltd., Kent, UK), and solution hybridization assay systems for *Chlamydia trachomatis* and HBV detection (Chiron Corporation; Clyne *et al.*, 1989; Urdea *et al.*, 1989,1990).

2. Luminol

DNA hybridization assays using the ECL system with direct HRP-labeled probes include detection of bovine enteric coronavirus in a slot blot hybridization assay (Collomb *et al.*, 1992) and a solution-phase hybridization assay for HBV DNA (Urdea *et al.*, 1987,1990). *In situ* hybridization for detection of human papillomavirus (HPV) type 16 has been performed with an indirect labeled probe (Hawkins and Cumming, 1990). ECL systems have also been used for the immunoassay detection of several viruses, including grapevine closterovirus (Pollini *et al.*, 1993) and parvovirus B 19 (O'Neill and Coyle, 1992).

3. Acridinium Esters

DNA hybridization assays incorporating AE-labeled probes have been developed for detection of several infectious agents from clinical samples, including *C. trachomatis*, *Neisseria gonorrhoeae*, fungal pathogens, mycobacteria, and several common bacterial pathogens (Nelson and Kacian, 1990). These assay systems, called PACE 2™ and ACCUPROBE™, are

available commercially through Gen-Probe, Inc. (San Diego, CA). The Gen-Probe system for screening for *Chlamydia* has been compared with both culture and nonculture antigen detection methods including enzyme immunoassays and immunofluorescent antibody tests (Gratton *et al.*, 1990; Mercer *et al.*, 1990; Iwen *et al.*, 1991). The PACE 2™ system can provide a rapid, reliable alternative to culture and immunoassay methods for the detection of *Chlamydia* from cervical samples (Iwen *et al.*, 1991). Solution hybridization (hybridization protection) assays with AE-labeled probes have been used for the detection of PCR-amplified HIV-1 DNA (Ou *et al.*, 1990; Schmidt, 1991; Rapiet *et al.*, 1993).

In addition, AEs have also been used to label antibodies that have been incorporated into automated immunoassay formats for the detection of infectious agents and antibody screening from clinical samples (Khalil *et al.*, 1991a,b).

4. Electrochemiluminescence

Electrochemiluminescence detection has been used in both manual (Blackburn *et al.*, 1991; Gudibande *et al.*, 1992; Kenten *et al.*, 1992) and automated (QPCR System 5000; Wages *et al.*, 1993) post-PCR amplification DNA hybridization assays for the detection of HIV-1 and HPV (Kenten *et al.*, 1991).

5. Bioluminescence

Detection of asymmetric amplified papillomavirus sequences using solution-phase hybridization with a G6PDH-labeled oligonucleotide and solid-phase capture has been performed using a bioluminescence assay (Balaguer *et al.*, 1991b).

V. CHEMILUMINESCENCE DETECTION PROTOCOLS

A. Hepatitis B Virus

Hepatitis B “core antigen” DNA, immobilized on nylon membrane, is hybridized with an alkaline phosphatase-labeled oligonucleotide probe. Hybridized probe is then detected with the 1,2-dioxetane substrate AMPPD (Bronstein *et al.*, 1989c).

1. Materials

Hepatitis B core antigen plasmid DNA and alkaline phosphatase-labeled probe, included in a SNAP® Hybridization System, and GeneScreen Plus™ nylon membrane were obtained from NEN/DuPont (Boston, MA). AMPPD and CSPD are from Tropix.

2. Target DNA Preparation and Probe Hybridization

HBV “core antigen” (HBVc) plasmid DNA (100 ng; 1.2×10^{10} copies) was dissolved in 25 μ l sterile deionized H₂O and serially diluted with 0.3 M NaOH to produce target DNA samples ranging in concentration from 4.88×10^3 to 0.98×10^8 copies/ μ l. Blots were prepared as described here:

1. Incubate diluted DNA samples at room temperature for 15 min to denature, and spot 1 μ l of each dilution onto dry membrane strips (1 \times 8 cm).
2. Rinse blots with 2 M NH₄OAc and then with 0.6 M NaCl, 0.08 M sodium citrate, pH 7.0.
3. Prehybridize with 3 ml hybridization buffer [0.75 M NaCl, 0.075 M sodium citrate (5X SSC), 0.5% bovine serum, 0.5% polyvinylpyrrolidone, 1% sodium dodecyl sulfate (SDS), pH 7.0] for 15 min at 55°C.
4. Hybridize with hybridization buffer containing 1.0 nM alkaline phosphatase-labeled oligonucleotide probe for 30 min at 55°C.
5. Wash sequentially for 5 min each in:

1X SSC, pH 7.0, 1% SDS at room temperature

1X SSC, pH 7.0, 1% Triton X-100 at 55°C

1X SSC, pH 7.0, at room temperature

3. Chemiluminescence Detection

1. Wash hybridized blots with 0.1% bovine serum albumin (BSA), 0.05 M sodium carbonate, pH 9.5.
2. Saturate blot with 100 μ l 1.6 mM AMPPD in 0.1% BSA, 0.05 M sodium carbonate, 1.0 mM MgCl₂, pH 9.5.

NOTE: *Alternatively, an improved buffer (0.1 M diethanolamine, 1.0 mM MgCl₂, pH 10.0) can be substituted for this wash, using 0.25 mM AMPPD or CSPD in this buffer for substrate incubation.*

3. Place blots in a plastic pouch and image light emission in a camera

luminometer with Polaroid Instant Black and White Type 612 (ASA 20,000) photographic film.

NOTE: *Alternatively, blots can be imaged on standard X-ray film.*

- Digitize photographic film image using a black and white RBP Reflectance Densitometer (Tobias Associates, Inc., Ivyland, PA).

4. Results

Figure 2 shows a time course of the chemiluminescent DNA hybridization assay for HBVc antigen DNA. Serial dilutions of plasmid DNA were hybridized with alkaline phosphatase-labeled oligonucleotide probe, incubated with chemiluminescent substrate, and imaged on photographic film. Each photograph corresponds to a 30-min exposure. With this chemiluminescence assay, 1.18×10^6 copies of HBVc DNA can be detected within 30 min of substrate incubation. After a 2-hr incubation, 4.39×10^4 copies can be detected. In contrast, with the colorimetric bromochloroindolyl phosphate/nitro blue tetrazolium (BCIP/NBT) substrate system, 9.8×10^7 and 1.07×10^7 copies can be detected after 30 min or 2 hr of substrate incubation,

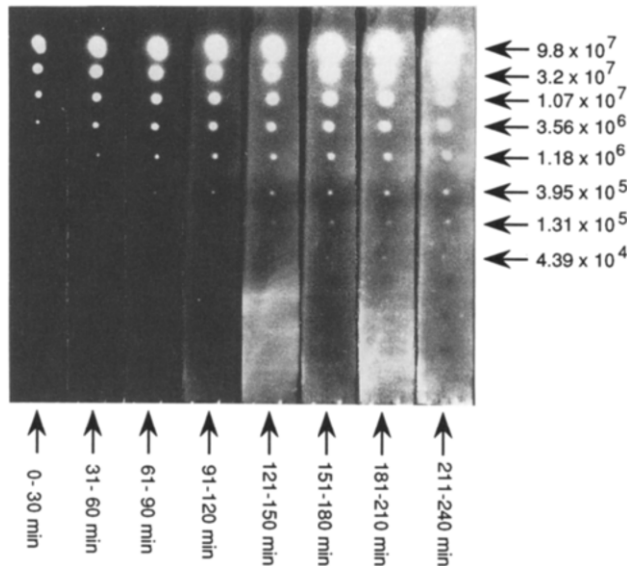


Figure 2 Chemiluminescent detection of hepatitis B "core antigen" plasmid DNA with AMPPD substrate in alkaline phosphatase-based DNA hybridization assay. Reprinted with permission from Bronstein *et al.* (1989c).

respectively (results not shown). Quantitative results were obtained by measuring reflection densities from the imaged photographic film using a black and white reflection densitometer (Fig. 3). These values could be used to establish a dose–response curve for the reflection densities as a function of HBVc plasmid concentration, from which HBVc DNA levels in clinical specimens could be determined. Use of the improved chemiluminescence detection protocol, incorporating the diethanolamine substrate buffer and CSPD chemiluminescent substrate, results in even greater sensitivity for DNA hybridization assays and would increase the sensitivity of this HBV DNA assay. Imaging and quantification of this membrane-based assay with rapidly evolving CCD camera systems will likely provide even greater sensitivity and a greater linear dynamic range than that achieved with densitometry.

5. Summary

Chemiluminescent detection of HBV DNA has also been performed with the AmpliProbe® system (ImClone Systems). This signal amplification probe system incorporates multiple target-specific primary and multiple secondary probes, alkaline phosphatase-labeled oligonucleotides that hybridize to the primary probes, in a two-step hybridization system (Yang *et al.*, 1991). Chemiluminescence detection is performed with a dioxetane substrate. With

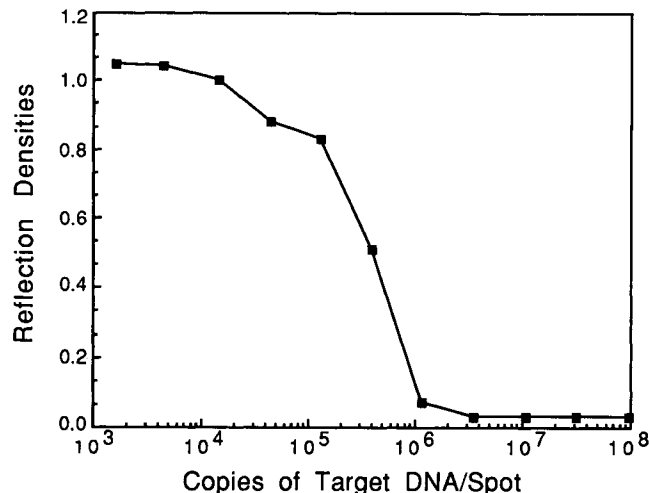


Figure 3 Hepatitis B virus “core antigen” plasmid DNA hybridization assay. Reflection density vs. number of copies of target DNA. Densitometric analysis of the Polaroid instant black and white photographic film image [0.00 (white)–2.00 (black)]. Reprinted with permission from Bronstein *et al.* (1989c).

this system, 0.4 pg (1×10^5 copies) purified target HBV genomic DNA can be detected in a chemiluminescent slot blot assay (Farmer and Castaneda, 1991; Yang *et al.*, 1991). Identical assays performed with serum samples (25 μ l) demonstrated that this chemiluminescent DNA hybridization system has the same specificity and sensitivity as immunoassays and is more sensitive than a ^{32}P -labeled nick-translated probe (Yang *et al.*, 1991).

Escarceller *et al.* (1992) report the use of digoxigenin-labeled probes, anti-digoxigenin alkaline phosphatase, and AMPPD for the direct detection of HBV sequences in human serum samples. These investigators achieved a limit of sensitivity of 2–5 pg, which was equivalent to that obtained with both colorimetric detection and a ^{32}P -labeled probe. These researchers also used digoxigenin-labeled oligonucleotide primers for PCR amplification of HBV DNA purified from human serum, followed by immunological detection of the digoxigenin label (as described), a method that can be used in conjunction with alternatively labeled primers for multiple amplifications.

A chemiluminescent assay incorporating a solution-phase hybridization of synthetic oligonucleotides to target DNA, followed by solid-phase capture, labeling, and detection with either HRP or alkaline phosphatase-labeled oligonucleotides and chemiluminescent substrates has been used to achieve the detection of 0.2 pg (6×10^4 copies) HBV DNA in human serum samples in 4 hr. This solution DNA hybridization method includes novel labeling and amplification schemes and has been performed with both polystyrene bead and microtiter well capture systems (Urdea *et al.*, 1987,1990).

Chemiluminescence techniques have also been used in the development of automated enzyme immunoassay systems for the detection of HBV in human sera (Khalil *et al.*, 1991a,b; Bouveresse and Bourgeois, 1992).

B. Herpes Simplex Virus

Two chemiluminescent DNA hybridization assays for HSV, dot blot hybridization and *in situ* hybridization, are described here as originally reported by Bronstein and Voyta (1989). In these assays, HSV-1 plasmid DNA, immobilized on nylon membrane, or HSV-1-infected Vero cells, fixed and mounted on microscope slides, were hybridized with an alkaline phosphatase-labeled HSV-1 oligonucleotide probe and detected with AMPPD.

1. Materials

HSV-1 plasmid DNA and alkaline phosphatase-labeled oligonucleotide probe, included in a SNAP® Hybridization System, and GeneScreen Plus nylon membrane were obtained from NEN/DuPont. HSV-1-infected Vero

cells were provided by Drs. J. Kershner and E. Jablonski (Molecular Biosystems, San Diego, CA). AMPPD and Emerald™ luminescence-amplifying material are from Tropix.

2. Dot Blot Hybridization and Chemiluminescence Detection

This membrane hybridization protocol is similar to that described for HBV detection.

1. Serially dilute HSV-1 plasmid DNA in 0.3 M NaOH, denature, and spot 1- μ l aliquots onto dry membrane strips.
2. Prehybridize blots with hybridization buffer (0.5% BSA, 0.5% polyvinylpyrrolidone, 1% SDS) for 15 min at 55°C.
3. Hybridize with hybridization solution (containing alkaline phosphatase-labeled HSV-1 oligonucleotide probe) for 30 min at 55°C.
4. Wash sequentially for 5 min each in:

2X SSC, 1% SDS at room temperature

1X SSC, 1% Triton X-100 at 55°C

1X SSC, 1% Triton X-100 at room temperature

1X SSC at room temperature

5. Wash hybridized blots with 0.05 M sodium carbonate/bicarbonate, 1 mM MgCl₂, pH 9.5 (substrate buffer).
6. Saturate blot with 1.6 mM AMPPD (in substrate buffer) for 5 min.

NOTE: As described for HBV detection, the diethanolamine buffer and 0.25 mM AMPPD or CSPD can be substituted in Steps 5 and 6 for increased sensitivity.

7. Image blots with Polaroid Type 612 Instant Black and White film.

NOTE: Alternatively, blots can be imaged on X-ray film.

3. In Situ Hybridization and Chemiluminescence Detection

1. Infect Vero cells with HSV-1 (MacIntyre strain) for 1 hr at room temperature.
2. Harvest cells with trypsin/versene after the addition of 2% fetal calf serum at 0, 2, 4, 6, 8, 10, 12, 24, and 48 hr.
3. Pellet cells, fix in 95% ethanol, and mount on glass microscope slides.

4. Treat mounted slides with 0.2 M HCl for 2 min, rinse with deionized water, and immerse in 70% ethanol. Prior to hybridization, remove slides from ethanol and dry.
5. Immerse slides in 0.1% BSA, 5X SSC for 15 min at 70°C. Treat with 0.3 M NaOH for 1 min at room temperature. Rinse with phosphate-buffered saline (PBS).
6. Hybridize cells with the alkaline phosphatase-labeled HSV-1 oligonucleotide probe at a concentration of 5 nM in 0.1% BSA, 5X SSC for 20 min at 60°C.
7. Wash slides briefly in hybridization buffer at 60°C, and then extensively with 1X SSC at 50°C.
8. Wash with 0.05 M sodium carbonate/bicarbonate, 1 mM MgCl₂, pH 9.5 (substrate buffer).
9. Incubate with 0.8 mM AMPPD, 10% Emerald in substrate buffer for 5 min.
10. Place slides in a camera luminometer and expose to Polaroid Type 612 Instant Black and White film.

4. Results

With the dot blot hybridization assay for HSV-1 plasmid DNA, detection limits achieved with the chemiluminescent substrate AMPPD are 1.3×10^5 and 1.4×10^4 copies of target HSV-1 DNA, with a 30-min exposure performed 1 hr after substrate addition and a 45-min exposure performed 4 hr after substrate addition, respectively (results not shown). The sensitivity achieved with AMPPD is 25- to 100-fold higher than that obtained with the colorimetric BCIP/NBT substrate system (results not shown). Fig. 4 shows the time course of viral infection assayed by *in situ* DNA hybridization with chemiluminescence detection. Use of the AMPPD chemiluminescent substrate enables the detection of HSV-1-infected cells within 6 hr postinfection. Again, with this assay format, CCD detection and imaging may provide even greater sensitivity than that achieved with photographic film.

5. Summary

In situ hybridization with chemiluminescence detection has also been used to detect HIV-infected cells (Bronstein *et al.*, 1989b) and HPV type 16 in a cervical carcinoma cell line (Hawkins and Cumming, 1990). The latter protocol involved the use of biotinylated HPV 16 DNA probes (Enzo Diagnostics, New York), a streptavidin–HRP conjugate, and ECL detection re-

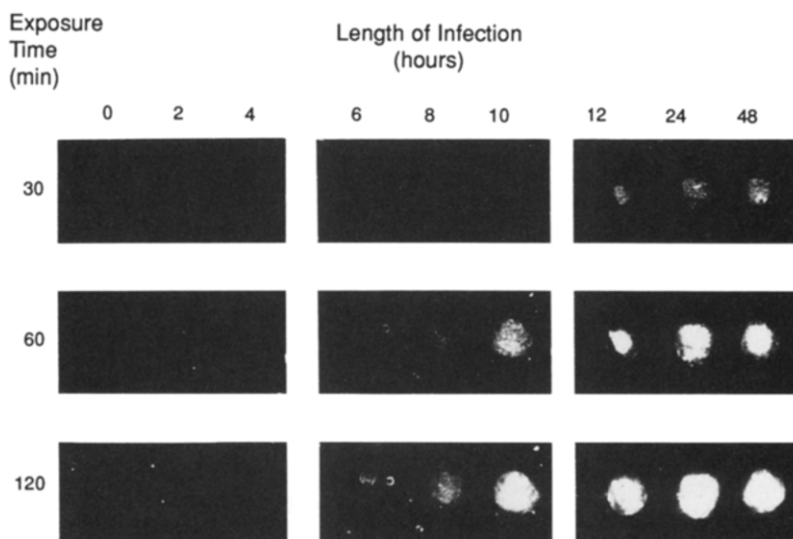


Figure 4 Chemiluminescent detection of *in situ* DNA hybridization of herpes simplex virus I-infected Vero cells: time course of infection. Reprinted from Bronstein and Voyta, *Clinical Chemistry* (1989), **35**, 1856–1857, Courtesy of the American Association for Clinical Chemistry, Inc.

agents coupled with a CCD imaging system. Detection of fewer than 10 HPV-positive cells (containing 600 copies of HPV 16 DNA per cell) among 10,000 HPV-negative cells on a single slide was achieved. However, this detection level is not necessarily the limit of sensitivity; with improved optical instrumentation, *in situ* hybridization coupled with CCD detection may provide a valuable diagnostic tool for the rapid and automated identification of viral sequences within cells.

VI. CONCLUSION

Chemiluminescence detection technologies combined with DNA hybridization methods provide rapid, sensitive, nonradioactive, automatable assay formats for the clinical diagnosis of infectious agents, as well as for research use. Rapidly evolving chemiluminescent enzyme substrates and labels, techniques, and assay and detection instrumentation, coupled with continued advances in DNA hybridization technologies, will further refine and improve the specificity and sensitivity of chemiluminescent DNA detection methods, bringing them into more widespread use.

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