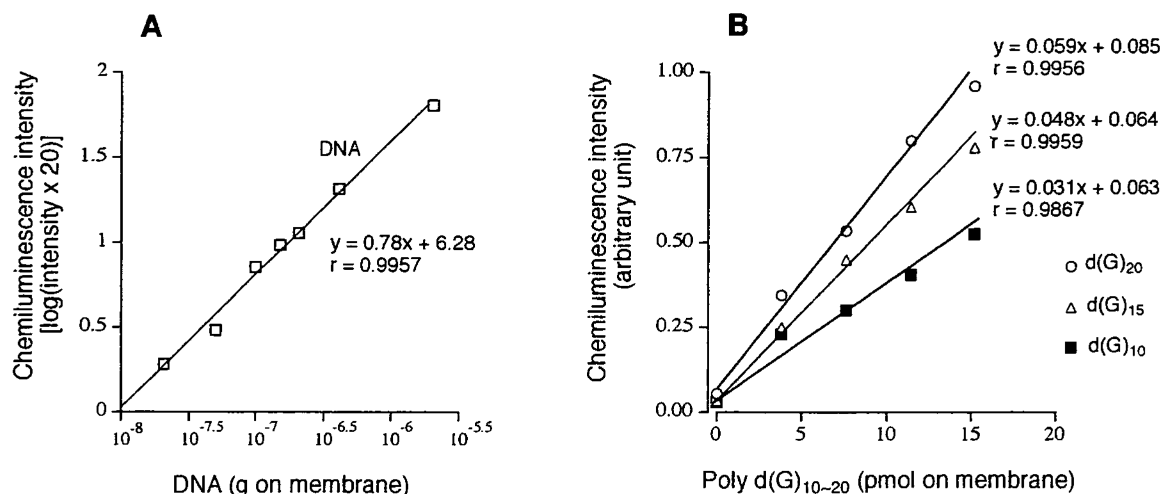

Fig. 6. Calibration graphs for (A) salmon testes DNA and (B) deoxyguanylic acid oligomers $d(G)_{20}$, $d(G)_{15}$ and $d(G)_{10}$ on a nylon membrane. The samples were treated in the same way as described in Fig. 5(B).

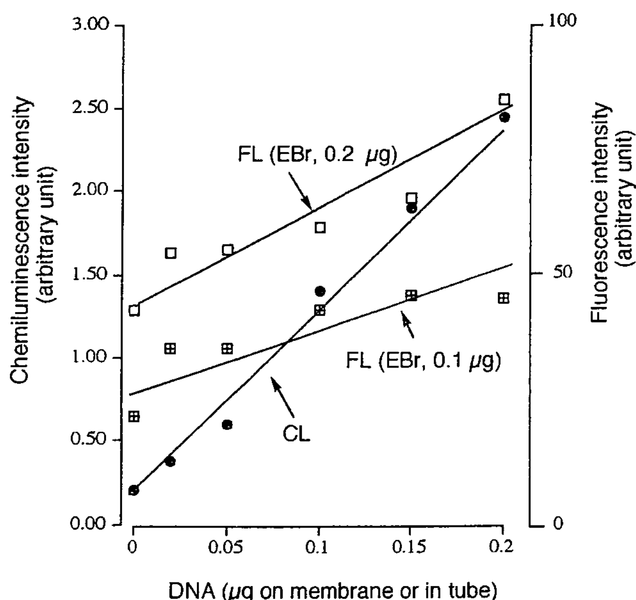

Fig. 7. Calibration graphs of DNA obtained by the present chemiluminescence (CL) method and a fluorescence (FL) method using ethidium bromide (EBr) as an intercalator. For the FL determination, a portion (100 μl) of 0.2–2.0 μg/ml salmon testes DNA was mixed with 100 μl of 1 or 2 μg/ml EBr in 0.2 M NaCl and 0.2 M Tris–HCl buffer (pH 7.5) (1:1, v/v). The fluorescence intensity was measured manually by a Hitachi F-2000 spectrofluorimeter at 480 nm of excitation wavelength and 600 nm of emission wavelength.

cence intensity was relatively high. In comparison, the present method offers more sensitive and reliable signals than the EBr method.

3.3. Detecting the chemiluminescence of hybrid DNA bound to a membrane

The preceding results led to the design of a new hybridization-assay protocol using a cDNA probe with an attached $d(G)_n$ oligomer to increase its chemiluminescence, as illustrated in Fig. 1(B). In this study, a DNA probe with a $d(G)_{30}$ tail covalently attached to its 3′-terminus (5′-CACAATTCCACA-CAAC-$(G)_{15}$TT$(G)_{15}$TT-3′), its complementary DNA target (5′-CTCGTATGTTGTGTGGAATTGTGAGC-GGAT-3′) and a non-target DNA fragment (5′-TCG-TGACTGGGAAAACCCTGGCGTTACCCA-3′) were used to test a fundamental hybridization system.

In the example shown in Fig. 8, the chemiluminescence intensity produced by a hybrid (sample A-2) of the target DNA fragment bound to the $d(G)_{30}$ probe was greater than that of the target DNA alone (sample B-2). This method detected 10 pmol of the target DNA fragement, spotted on a nylon membrane after hybridization with 50 nM of the $d(G)_{30}$ probe, by its intense chemiluminescence. Background chemiluminescence caused by non-specific binding of the probe in the hybridization buffer to the membrane was negligible. In this experiment, the $d(G)_{30}$ probe was also dotted as a reference on the membrane (samples A-1 and B-1) to compare with the non-hybridization image, because the DNA fragments as well as the probe bound to the membrane-bound probe was detected by 0.1 pmol. Therefore, the probe bound to the target DNA frag-

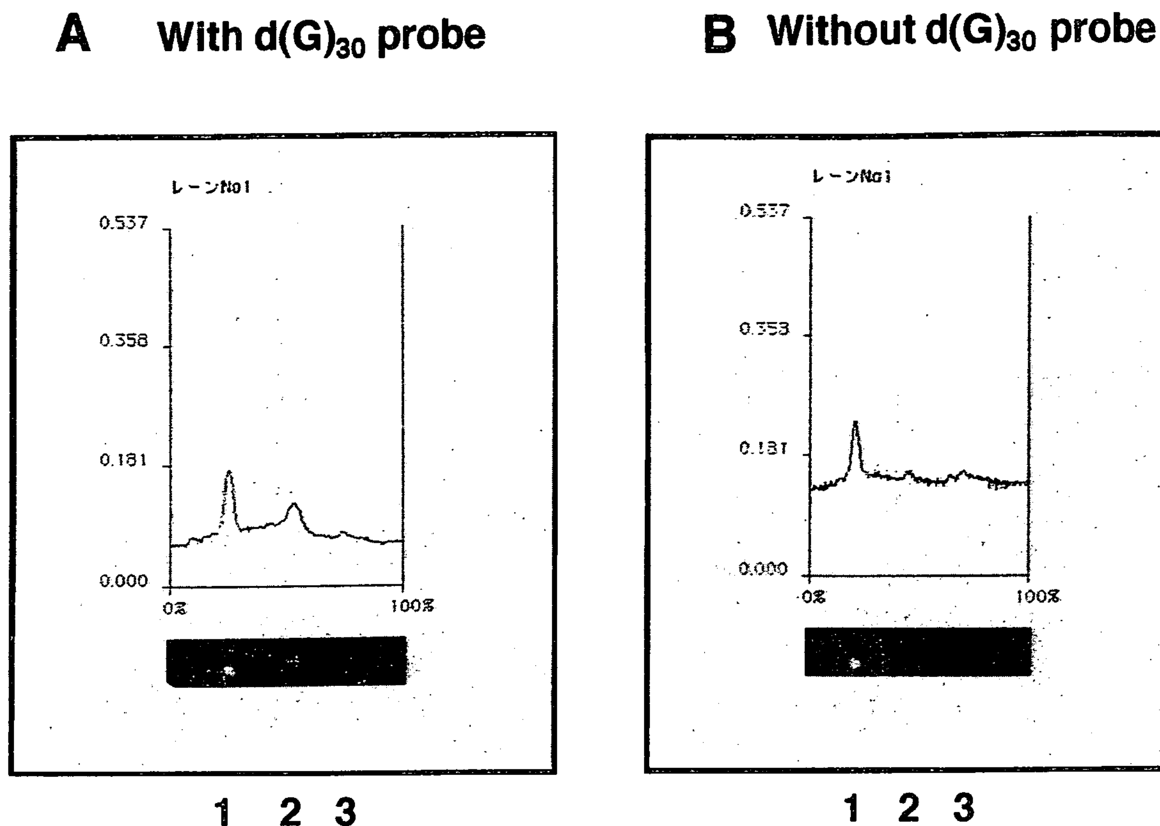

Fig. 8. Hybridization assay of a DNA fragment (30 mer) on a nylon membrane: (A) with a DNA probe with a $(G)_{15}TT(G)_{15}TT$ tail, (B) without the probe. Samples (10 pmol each): (1) probe, (2) target DNA fragment, and (3) non-target DNA fragment. The procedures are described in Section 2.

ment gave a positive and reproducible signal, with a lower detection limit of approximately 1.0 pmol of the target DNA.

In this assay system, a certain amount of the probe hybridized with the target might be removed while washed the membrane after the hybridization reaction, and/or the binding capacity of the probe to the target might be low. With the aim of increasing the detection signal of the hybrid versus its concentration, we will study the effects of much larger amount of $d(G)_n$ in the probe, its binding capacity to the target, and some compounds that enhance chemiluminescence on this assay system.

4. Conclusions

We developed a specific chemiluminescence derivatization (SCD) method for detecting DNA and $d(G)_n$ on a nylon membrane, based on the chemical reaction of guanine moieties of the nucleic acid with TMPG. The SCD method has the advantages of speed and simplicity for the quantitative determination of nucleic acids and DNA probes containing $d(G)_n$. Using the probe with a $d(G)_n$ tail will increase the chemiluminescence in proportion to the amount of guanylic acid in the probe molecule. The SCD technique with TMPG described here may be useful for the in situ detection of guanine-rich regions of the genome. Such regions include telomeres, which consist of hundreds or thousands of tandem repeats of the sequence – TTAGGG – at the end of chromosomes [16].

Acknowledgements

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Chemiluminescence and Bioluminescence in DNA Analysis

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1. INTRODUCTION

Over the last decade, various nonradiochemical methods utilizing chemiluminescent or bioluminescent reactions have been developed to increase the sensitivity and speed of detecting DNA probes or DNA itself. This has permitted the devel-

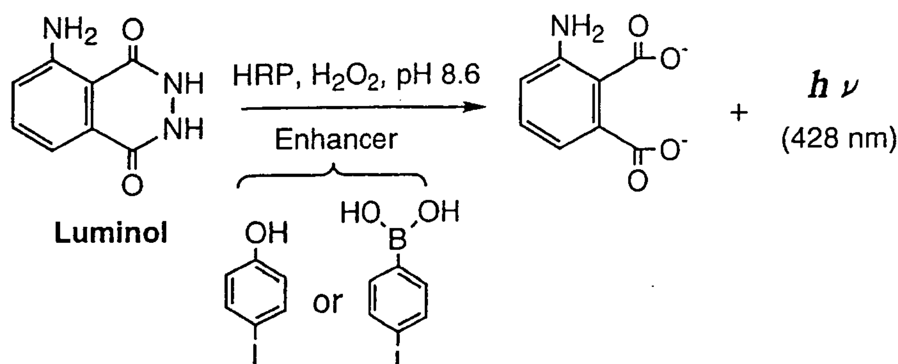


Figure 1 HRP-catalyzed chemiluminescent reaction of luminol.

opment of powerful analytical techniques for obtaining information on gene structure and function.

Recently, two major enzyme-catalyzed chemiluminescent reactions have become popular. These use either luminol as a substrate of peroxidase or 3-(2′-spiroadamantane)-4-methoxy-4-(3″-phosphoryloxy)phenyl-1,2-dioxetane (AMPPD) as a substrate of alkaline phosphatase (ALP).

Luminol is the most popular chemiluminescent compound. Chemiluminescent detection based on horseradish peroxidase (HRP)-catalyzed oxidation of luminol in the presence of hydrogen peroxide requires a halogenated phenol such as 4-iodophenol or 4-iodophenylboronic acid [1] as a potent enhancer (Fig. 1). This method is often called enhanced chemiluminescent detection. In the enhanced reaction, the light emission is increased over 100-fold, permitting detection of HRP at subfemtomole levels. The light has a maximum wavelength of 428 nm and can be captured with high efficiency by blue-light-sensitive X-ray film.

AMPPD is the best chemiluminescent substrate for detecting an ALP-labeled probe [2, 3]. The enhanced sensitivity of the chemiluminescence based on the reaction of AMPPD with ALP depends on the enzymatic reaction time (Fig. 2), because the slow kinetics of the signal decay result in the accumulation

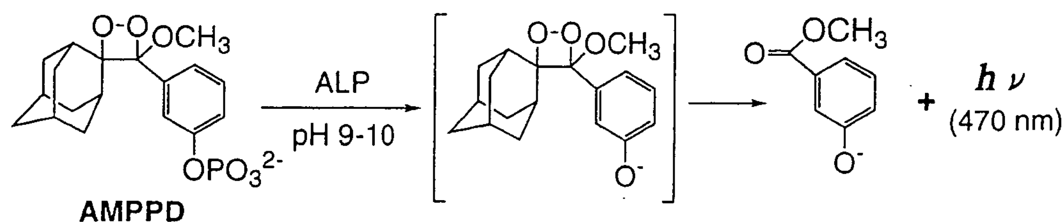


Figure 2 ALP-catalyzed chemiluminescent reaction of AMPPD.

of light (maximum wavelength of 470 nm). This protocol permits highly sensitive detection of enzyme molecules at subattomole levels [2].

Both ALP and HRP are commonly used as signal markers for chemiluminescent detection of complementary deoxyribonucleic acid (cDNA) probes. Recently, marine bacterial luciferase and firely luciferase have been utilized as the marker enzymes of cDNA probes [4, 5]. The light emission by reaction of luciferase with luciferin in the presence of ATP, magnesium(II) ion, and oxygen is called bioluminescence since this reaction is a naturally occurring chemiluminescent reaction (Fig. 3A) [6]. Luciferase also mediates an oxidative reaction of an aldehyde compound in the presence of the reduced type ($FMNH_2$) of flavin mononucleotide (FMN), and then emits a strong and stable light (Fig. 3B) [6, 7]. These enzymatic chemiluminescent methods permit reliable and sensitive nonisotopic detection in immobilized hybridization assays, as well as in common membrane hybridization assays.

5-Bromo-4-chloro-3-indolyl (BCI) substrates can also be used with several commercially available marker enzymes, including ALP, β-D-galactosidase, and β-glucosidase (Fig. 4) [8]. The enzymes hydrolyze the BCI substrate, producing the corresponding chromogenic indigo dye and hydrogen peroxide stoichiometrically in the reaction mixture. Therefore, the enhanced luminol-HRP detection system can detect the enzyme used as a signal marker when high sensitivity is required in hybridization assays.

Acridinium esters have also been utilized for chemiluminescent detection of cDNA probes (Fig. 5) [9–11]. The hydrolysis rate is much faster when the ester is conjugated to single-stranded DNA, rather than to double-stranded DNA. This means that the chemiluminescence from unhybridized acridinium ester–labeled probe is rapidly lost, whereas the chemiluminescence from the hybridized probe is minimally affected. This permits discrimination between hybridized and unhybridized acridinium ester–labeled DNA probes without separation steps.

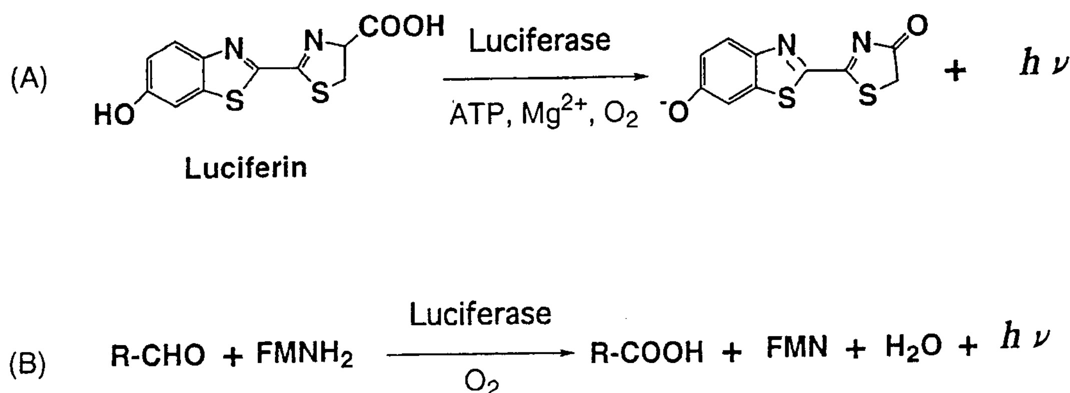
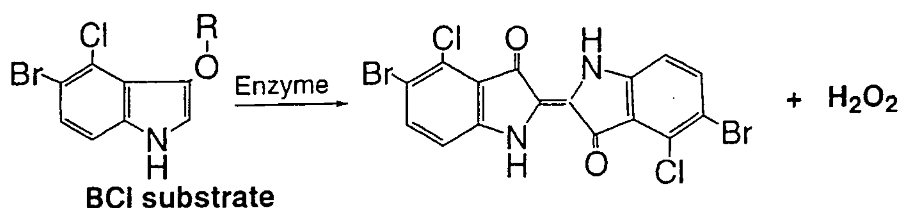


Figure 3 Luciferase-catalyzed bioluminescent reaction with (A) luciferin and (B) an aldehyde compound and $FMNH_2$.



R of substrate	Enzyme
Phosphate	ALP
D-Galactopyranoside	β-Galactosidase
D-Glucoside	β-Glucosidase

Figure 4 Enzymatic reactions of BCI substrates and their chemiluminescent detection by the luminol reaction.

Therefore, chemiluminescent methods using an acridinium ester–labeled cDNA probe allow the discrimination of a mismatched DNA sequence in a homogeneous assay.

On the other hand, few reagents react directly with the target DNA for chemiluminescent detection. Recently, a unique chemical derivatization reagent, 3′,4′,5′-trimethoxyphenylglyoxal (TMPG), has been developed [12–15]. This reagent reacts specifically with guanine bases in nucleic acids to produce a chemiluminescent, fluorescent derivative quickly, under mild reaction conditions (Fig. 6). The derivative emits chemiluminescence (maximum wavelength of 510 nm) un-

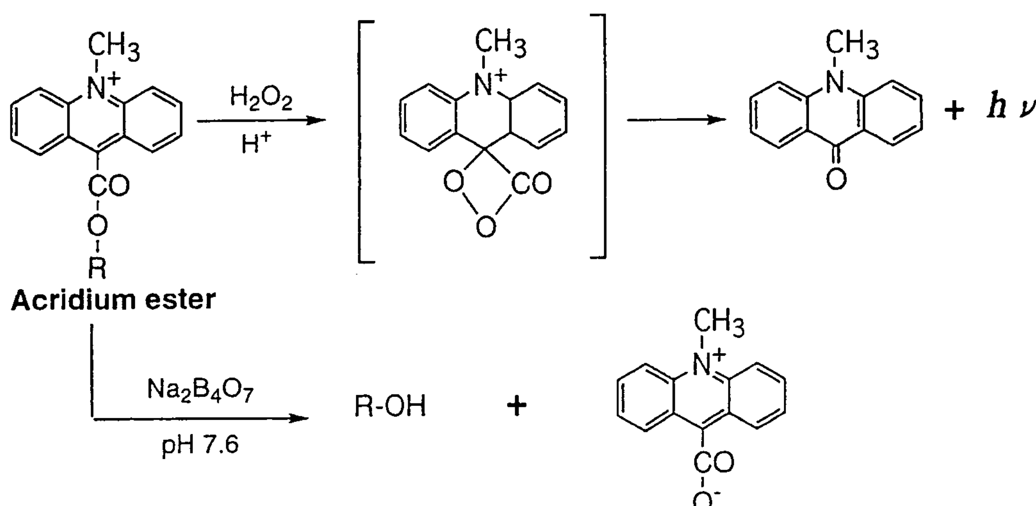


Figure 5 Chemiluminescent reaction of acridinium ester and its hydrolysis.

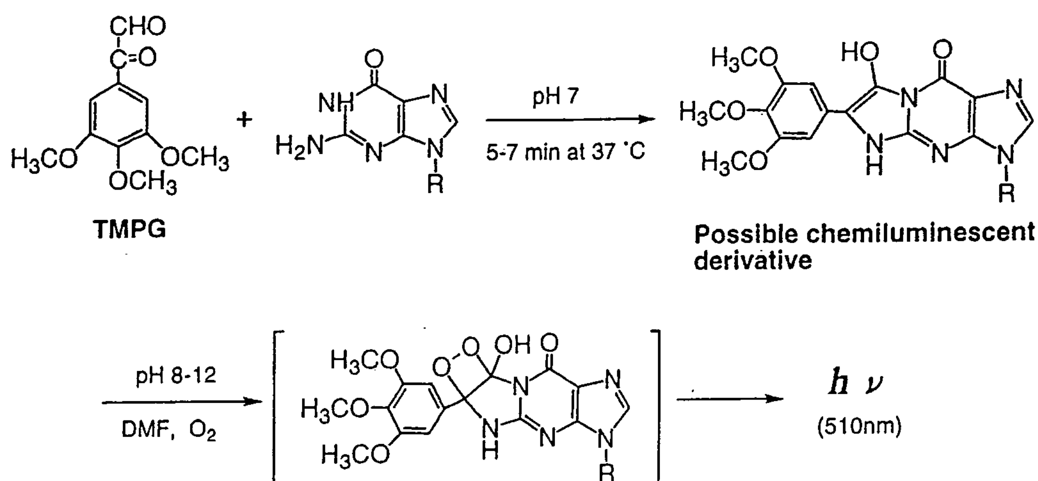


Figure 6 Derivatization and chemiluminescent reactions of guanine-containing compounds with TMPG.

der weakly alkaline conditions in the presence of dimethylformamide (DMF) at room temperature. The TMPG reaction can be used to detect DNA or polydeoxyguanylic acid (d(G)n), both on nylon membranes and in aqueous solution.

This chapter outlines the chemical principles for luminescent detection of target DNA in hybridization and quantitative assays that utilize the above-mentioned chemiluminogenic and bioluminogenic reagents.

2. ENZYMATIC CHEMILUMINESCENT AND BIOLUMINESCENT DETECTIONS

Enzyme-labeled probes have been employed for sensitive chemiluminescent or bioluminescent detection of hybridized target DNA that is bound to a membrane or otherwise immobilized. For these assays, HRP, ALP, and luciferase have been used as the marker enzyme predominantly. These enzymes are far bulkier than isotopic labels, and are also less thermally stable. For labeling, a small molecule such as digoxigenin or biotin is employed to conjugate the enzymes to a DNA hybrid duplex by means of either bioaffinity interaction of avidin or immunochemical interaction of antidigoxigenin antibody [16]. The primary advantage of using biotin or digoxigenin probes is that they are suitable for indirect labeling of many unstable enzymes to cDNA probes.

2.1 Membrane Hybridization

Hybridization assays of membrane-bound DNA are important for characterizing or searching for cloned genes related to a genetic disease, and for identifying

DNA fragments in restriction fragment length polymorphism analyses [16]. These assays usually involve electrophoretic separation of the DNA fragments produced using appropriate restricted enzymes, transferring the resulting band pattern onto a membrane in a process called Southern blotting, and subsequent hybridization with a cDNA probe to detect only the target DNA fragment. In this assay system, a higher detection sensitivity for the hybrid duplex is desirable because less target DNA is necessary for the assay [6, 16].

After hybridization with the target DNA, the enzyme-conjugated complex can be sensitively detected using chemiluminescent reactions with either AMPPD-ALP [17–24] or HRP-enhanced luminol [25, 26] that involves the incorporation of digoxigenin (Fig. 7A) or biotin (Fig. 7B) into cDNA probes. Biotin- or digoxigenin-modified probes can be generated with the polymerase chain reaction (PCR) using digoxigenin- or biotin-labeled nucleotides in the PCR medium. Several modified nucleotide analogs, antidigoxigenin antibody-conjugated enzymes, and biotin-conjugated enzymes are available from commercial suppliers.

These chemiluminescent detection methods have the advantages of high sensitivity and safety. The protocols use a conventional X-ray-film-reading device or an optical instrument such as a cooled charge-coupled device (CCD) camera [27, 28] for acquiring images. If the light emission is increased, the exposure time required for detecting light in methods based on enzymatic amplification can be reduced considerably.

2.2 Immobilized Hybridization

PCR is a technique for in vitro amplification of DNA sequences that involves repeated cycles of denaturation, oligonucleotide annealing, and DNA polymerase extension [29]. The amplified products following PCR cycles contain double-stranded DNA fragments of discrete length. These DNAs are copies of the template DNA that are bounded at the 5′-terminus by the oligonucleotide primer for the sequence extension with a heat-resistant DNA polymerase. In quantitative assays of PCR products, therefore, nonspecific products interfere with the assay.

A target PCR product incorporating biotin or digoxigenin deoxynucleotides can be immobilized to a solid phase such as magnetic beads, Sepharose polymer, or a microtiter well by bioaffinity binding between avidin and biotin or digoxigenin and antidigoxigenin antibody, or by covalently binding a spacer compound to the cDNA probe. The target is hybridized with biotin- or digoxigenin-labeled cDNA probe to capture the marker enzyme that produces the luminescent signal (Fig. 8A and 8B). [30–32]. The sensitivity of immobilization-based hybridization assays of a target PCR product can be increased markedly by utilizing the luminol-HRP or AMPPD-ALP methods.

The protocol shown in Figure 8C is based on a multienzymatic channeling reaction leading to light emission [4]. This method requires three enzymes: glu-

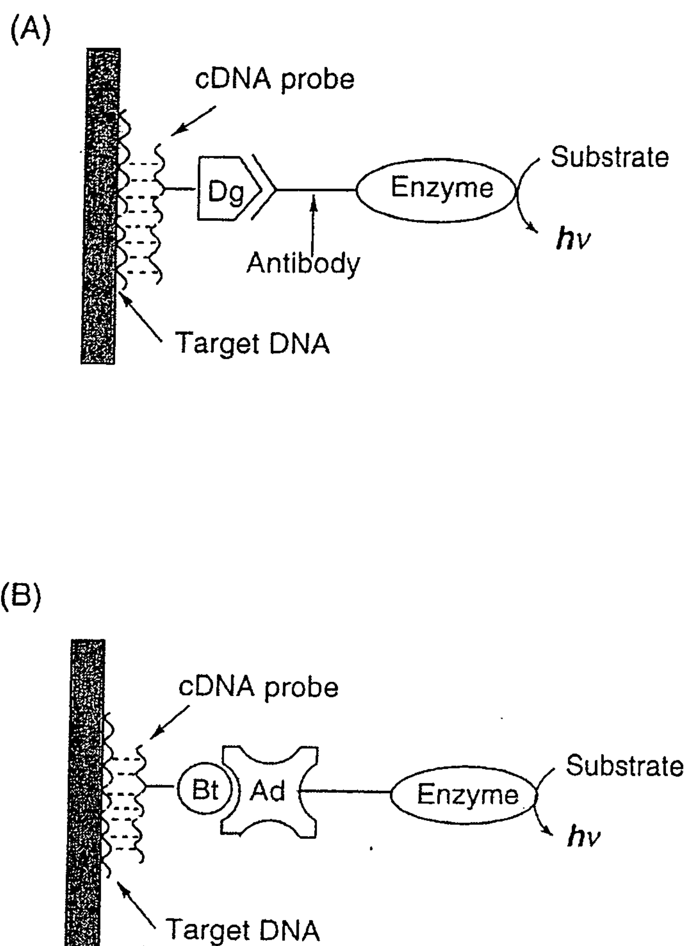


Figure 7 Chemiluminescent detections using (A) digoxigenin- and (B) biotin-binding probes for membrane hybridizations of DNA. Dg, digoxigenin; Bt, biotin; Ad, avidin. Procedures A and B [20]: Membrane hybridization of DNA involves the use of nylon membranes as a solid support onto which various sample DNAs are adsorbed. Of the adsorbed DNAs, the target DNA only can be hybridized to its cDNA probe (12 ng/mL) in which Dg-11-dUTP for protocol A or Bt-11-dUTP for protocol B is incorporated in advance by PCR. After hybridization at 65°C for 16 h, the membrane is washed with an appropriate buffer to remove excess cDNA probe and nonspecifically bound probe, and rinsed with a blocking solution to avoid binding nonspecific antibody or avidin proteins. For membranes using Dg-binding probe, anti-Dg Fab fragment containing ALP is conjugated to the probe for 30 min at 60–70°C. For membranes using Bt-binding probe, a streptavidin-ALP conjugate is used similarly. After conjugate treatment, membranes are rinsed with blocking reagent and washed four times. Each membrane is equilibrated for 5 min in a pH 9.5 substrate buffer, and reacted for 10 min in 0.26 mM AMPPD in fresh substrate buffer. The membrane is then sealed in a polyester/polyethylene bag, and exposed to X-ray film at ambient temperature.

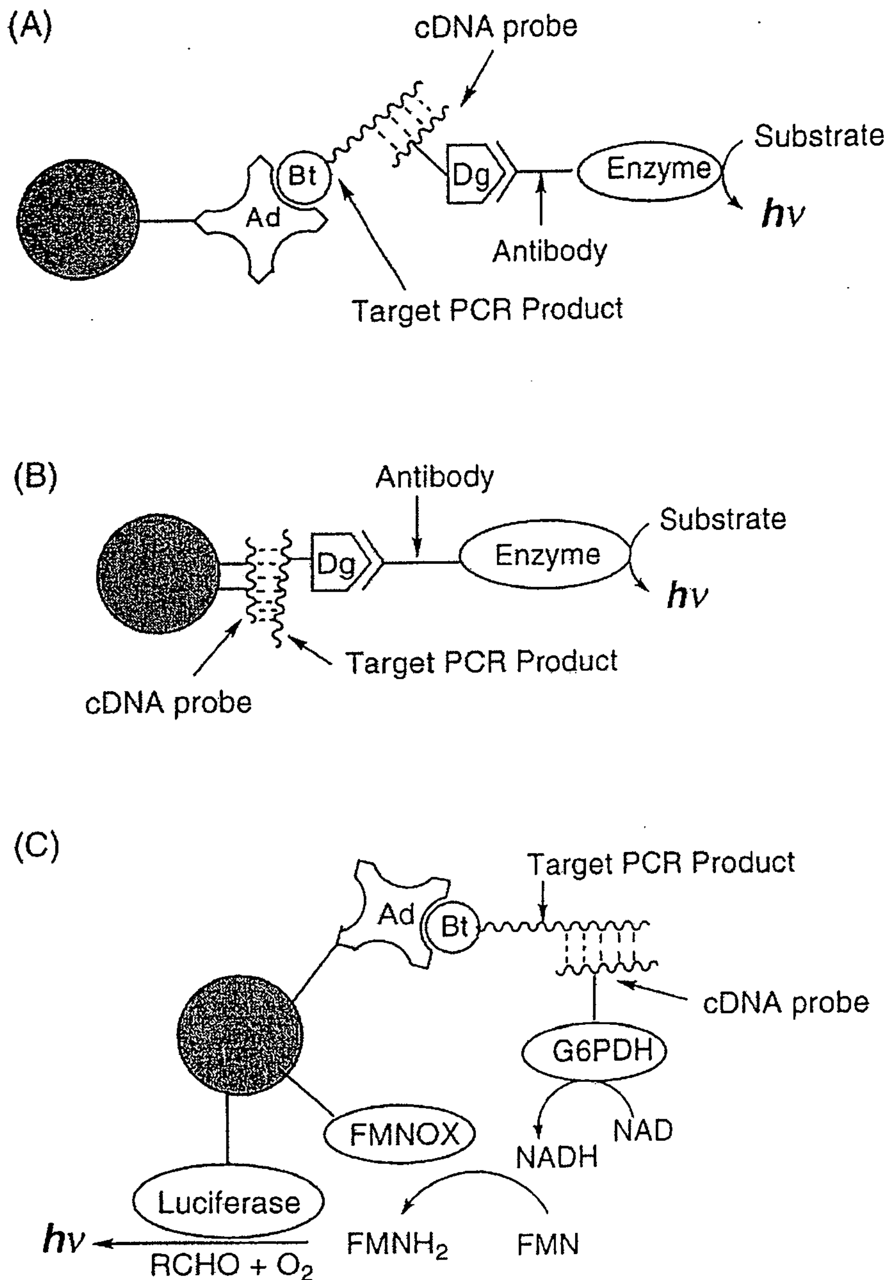


Figure 8 Chemiluminescent (A and B) and bioluminescent (C) detections for immobilized hybridizations of PCR product. Dg, digoxigenin; Bt, biotin; Ad, avidin. Procedure A [30]: Biotin moiety is incorporated into PCR products during the amplification reaction, using one 5′-biotinylated primer. The product is hybridized with a Dg-labeled probe and is immobilized on streptavidin-coated magnetic beads. This capture reaction is carried out for 30 min at 37°C. A permanent magnet is used to sediment the beads during washing to remove unbound DNA. By incubation with the washed beads for 45 min at 37°C, anti-Dg antibody conjugated to HRP enzyme is bound to the Dg-labeled probe, and luminol reaction is performed for CL detection. Procedure B [31]: Wells of a polystyrene microtiter plate are activated with 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide, and then coated with a labeled cDNA probe complementary to an internal region of the target DNA.

cose-6-phosphate dehydrogenase (G6PDH), flavin mononucleotide oxidoreductase (FMNOX), and luciferase. FMNOX and luciferase are immobilized to a suitable solid phase. The hybrid duplex of the target PCR product with a cDNA probe labeled with G6PDH is formed by the interaction of avidin and biotin on a bioluminescent adsorbent. In the assay, NADH produced by G6PDH attached to the cDNA probe bound to the target is used directly by the FMNOX reaction to reduce FMN. The immobilized luciferase produces stable light emission in the presence of $FMNH_2$ and an aldehyde compound. The luminescence signals are proportional to the amount of bound label.

The immobilized-hybridization methods provide simple detection systems that do not require separation of the target PCR product from contaminating DNA. These protocols are also applicable to the detection of an unmodified DNA target [5, 33, 34].

In the method shown in Figure 9A, a biotin-labeled cDNA probe is first immobilized to a polyvinylchloride microtiter plate well that is coated with biotinylated-bovine serum albumin [33]. The target DNA is hybridized in the liquid-phase with a digoxigenin-labeled probe, so that the biotin-labeled probe can capture a marker enzyme. An antibody-conjugated enzyme is then added, followed by a chemiluminescent substrate.

In the method shown in Figure 9B, a firefly luciferase gene is introduced for sensitive bioluminescent detection of target DNA [5]. The luciferase-coding DNA requires no posttranslational modification, and the activity of the luciferase produced can be readily measured in the transcription/translation mixture without prior purification. In this assay system, the digoxigenin-labeled probe is first immobilized to polystyrene wells coated with antidigoxigenin antibody. The target

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Dg-labeled PCR products are added to the wells, and hybridized for 4 h at 65°C. The hybridized wells are washed and rinsed with a blocking agent. For chemiluminescent detection, an anti-Dg antibody conjugated to ALP and AMPPD are added to the wells at ambient temperature. Procedure C [4]: PCR product contains biotin moiety by the PCR reaction in the presence of Bt-dUTP. The target PCR product and its cDNA probe, which is labeled covalently with G6PDH by using 4-(*N*-maleimidomethyl)cyclohexane-1-carbonate and dithiobis(propionate *N*-hydrosuccinimide ester), are hybridized for 2 h at 37°C. In the solid phase, the enzyme produces NADH from NAD. The produced NADH is directly used by the FMN oxidoreductase to reduce FMN. The immobilized luciferase uses the $FMNH_2$ plus an aldehyde to produce a stable light emission. In the supernatant, the unbound cDNA probe produces also NADH, which is oxidized by lactate dehydrogenase plus pyruvate that are previously added to the reaction mixture. This process does not require any separation step since the unbound enzyme does not lead to light emission. Luminescence values are related to the amount of the bound cDNA probe.

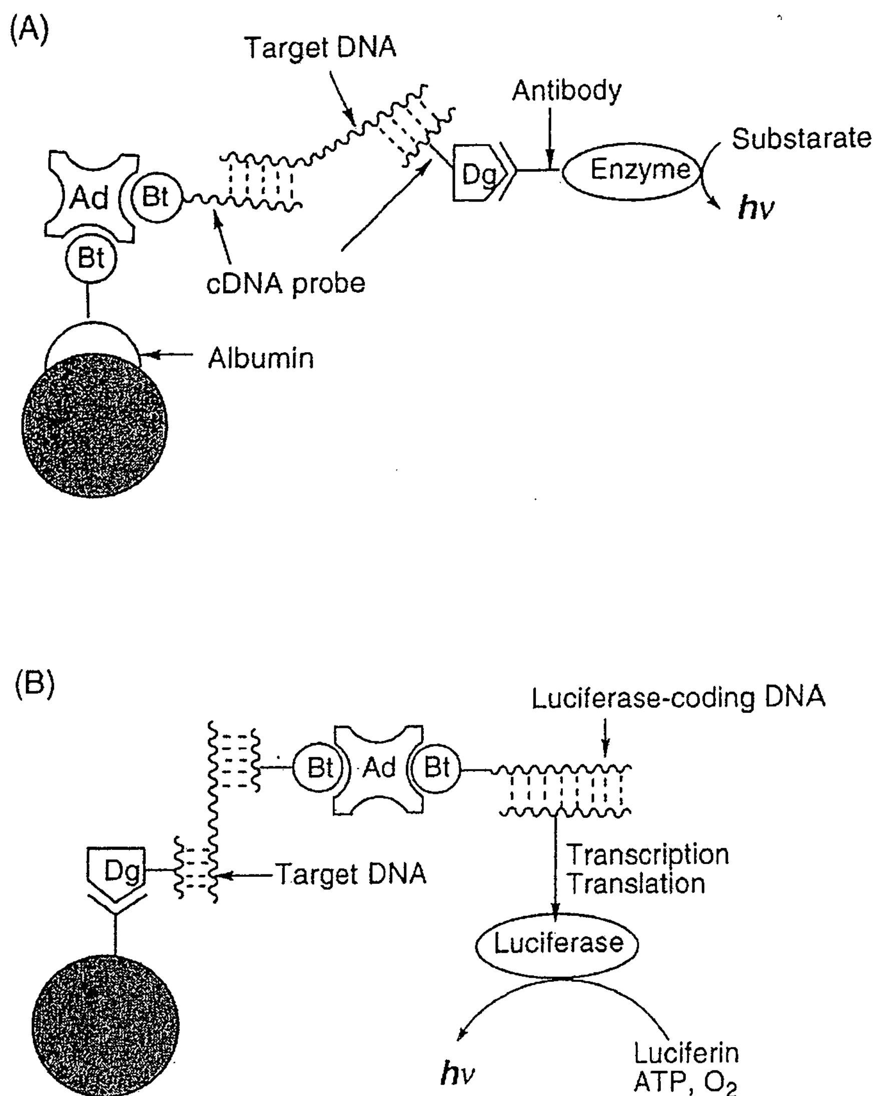

Figure 9 Chemiluminescent (A) and bioluminescent (B) detections for immobilized hybridizations of unmodified DNA target. Dg, digoxigenin; Bt, biotin; Ad, avidin. Procedure A [33]: A Bt-labeled cDNA probe is first bound to a polyvinylchloride microtiter plate well that was previously coated overnight at 4°C with a biotinylated-bovine serum albumin, and saturated with streptavidin for 1 h at 37°C. The test sample containing an unmodified target DNA is hybridized in liquid-phase at 55°C for 1 h with the Dg-labeled cDNA or cRNA probe, and then the mixture is added to the well to be captured at 55°C for 1 h by the Bt-labeled probe in the well. Anti-Dg Fab conjugated with alkaline phosphatase is then reacted at 37°C for 1 h. The well is washed sufficiently, and followed by AMPPD reaction for chemiluminescent detection. Procedure B [5]: An unmodified target DNA is hybridized with its two different sequencing cDNA probes for 1 h at 42°C. One of the probes is immobilized in a microtiter well, via Dg and anti-Dg antibody interaction that is physically adsorbed to the polystyrene well, and the other probe is biotinylated. After completion of the hybridization, the hybrids are reacted with streptavidin and a biotinylated luciferase-coding DNA (2.1 k base pair) for 20 min. After washing of the well, the solid-phase bound luciferase-coding DNA is expressed by adding the transcription-translation solution for 90 min at 30°C. The synthesized luciferase in the well is transferred to a tube, and mixed with the luciferin substrate to detect the luminescence.

DNA is then hybridized with the probe, and also another biotin-labeled cDNA probe is hybridized to capture the luciferase gene. The complex is prepared by mixing biotinylated luciferase-coding DNA with a large excess of avidin. The assay is completed by expressing the solid-phase bound DNA label and measuring the activity of the luciferase synthesized. Expressed luciferase, which mediates the luminescence reaction of luciferin as the substrate, has been exploited to detect target DNA in the hybridization assay. The chemiluminescent signal obtained depends on a linear relationship between the target DNA (5–5000 amol) and the activity of the luciferase produced.

3. CHEMILUMINESCENT DETECTION OF ACRIDINIUM ESTER–LABELED PROBE

The acridinium ester (AE) in an AE-labeled cDNA probe hybridized to target DNA is less likely to be hydrolyzed than in the unhybridized conformation (Fig. 10) [9–11]. Single-base mismatches in the duplex adjacent to the site of AE attachment disrupt this protection, resulting in rapid AE hydrolysis [11]. Hydrolysis by a weak base renders AE permanently nonchemiluminescent. After hydrolysis, it is possible to use the remaining chemiluminescence as a direct measure of the amount of hybrid present. This selective degradation process is a highly specific chemical hydrolysis reaction, which is sensitive to the local environment of the acridinium ester. The matched duplex can be detected and quantified readily, whereas the mismatched duplex produces a minimal signal.

Therefore, a homogeneous hybridization assay for detecting a DNA target not mismatched with its AE-labeled cDNA probe sequences can be performed. The AE-containing probe is synthesized using protected alkylamine linker arms at any location within the synthetic DNA probe. The method is very simple and sensitive, and is completed in 30–60 min. In this method, the background produced by unhybridized probe is sufficiently low. Therefore, this assay is useful for searching for genetic disorders [10, 11].

4. DERIVATIZATION-BASED CHEMILUMINESCENT DETECTION

Methods currently available for chemiluminescent detection of nucleic acids are not based on derivatization techniques that directly recognize one of the nucleic acid bases or nucleotides. For chemical derivatization-based chemiluminescent detection, the specific reactivity of alkyl glyoxals and arylglyoxals with adenine or guanine nucleotides has been investigated.

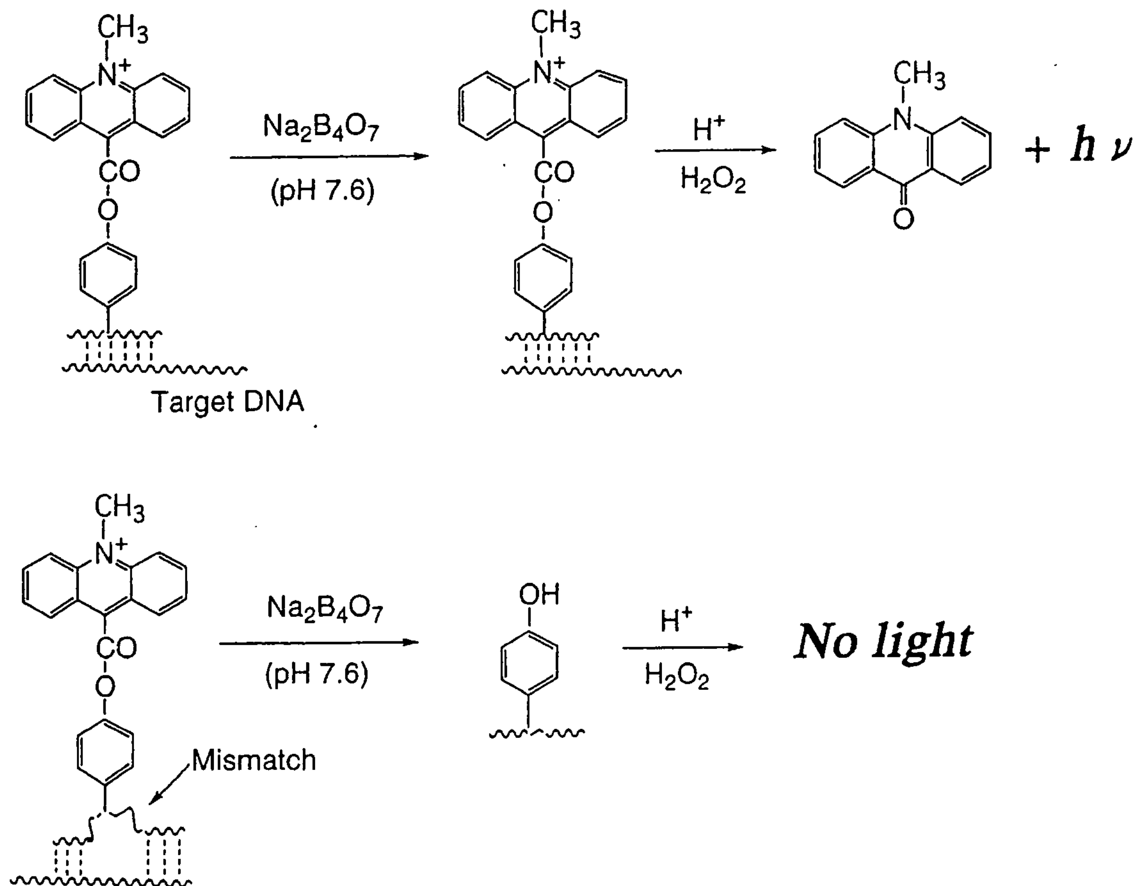

Figure 10 Mismatch detection by using a chemiluminescent AE-labeled cDNA probe. Procedure [9, 11]: Acridinium ester–labeled probes specific for either wild-type or mutant sequence corresponding to a target DNA are hybridized with the sample DNA for 1.0 h at 60°C in a hybridization buffer (pH 5.2). Hybridized and nonhybridized probes are discriminated by the hydrolysis reaction for 12 min at 62.5°C in the presence of $Na_2B_4O_7$ (pH 8.5) and Triton X-100. The chemiluminescence of each sample is then measured in a luminometer.

4.1 DNA Quantification

Phenylglyoxal and alkoxyphenylglyoxals react selectively with the guanine moiety of nucleosides and nucleotides in phosphate buffer (pH 7.0) at 37°C for 5–7 min to give the corresponding fluorescent derivatives [12–15], as shown in Figure 6. Other nucleic acid bases and nucleotides (e.g., adenine, cytosine, uracil, thymine, AMP, CMP) do not produce derivatives under such mild reaction conditions. The fluorescent derivative emits chemiluminescence on oxidation with dimethylformamide (DMF) and H_2O_2 at pH 8.0–12 [14, 15].

Of the alkoxyphenylglyoxals, 3′,4′,5′-trimethoxyphenylglyoxal (TMPG) produces the most intense chemiluminescence for both DNA and guanine nucleotides in aqueous solution [15]. TMPG also reacts readily with DNA absorbed to a nylon membrane at pH 9–10 for 30–60 s at room temperature, and the derivatives produced on the membrane emit chemiluminescence in the presence of DMF [15]. The sensitivity for detecting DNA samples bound to a nylon membrane is approximately 4 ng when reacted with TMPG and detected with a CCD camera cooled to −25°C. This is equivalent to a genome size of about 3×10^9 base pair or a molecular weight of about 2×10^{12}, and corresponds to zmol (10^{-21} mol) levels. This method is highly sensitive for nucleic acids containing a large amount of guanine.

On the other hand, glyoxals and phenylglyoxal also react with adenine and DNA under strongly acidic conditions when heated at 100°C for approximately 1 h [35–37]. The products then emit chemiluminescence under strong alkaline conditions. With the method using methylglyoxal, the detection limit for adenine-containing DNA is 5 ng. Under these drastic reaction conditions, however, guanine and guanine nucleotides do not produce any chemiluminescence.

4.2 Detection of Polydeoxyguanilic Acid (d(G)n)–Labeled Probe

The TMPG reagent can be used for chemiluminescent detection of d(G)n on a blotting membrane [15]. The chemiluminescence can be detected with a densitometric luminometer equipped with a cooled CCD camera. This method is used to detect a hybrid with a DNA probe containing a d(G)n oligomer to increase its chemiluminescence (Fig. 11). In Figure 11, the chemiluminescence produced by the hybrid of target DNA bound to the $d(G)_{30}$ probe is greater than that of the target DNA alone.

This protocol permits detection of 10 pmol of target DNA dotted on a nylon membrane after hybridization with the $d(G)_{30}$ probe [15]. The background chemiluminescence caused by nonspecific binding of the probe in the hybridization buffer to the membrane is negligible in this assay system. However, both the target DNA and the cDNA probe bound to the membrane are detected solely

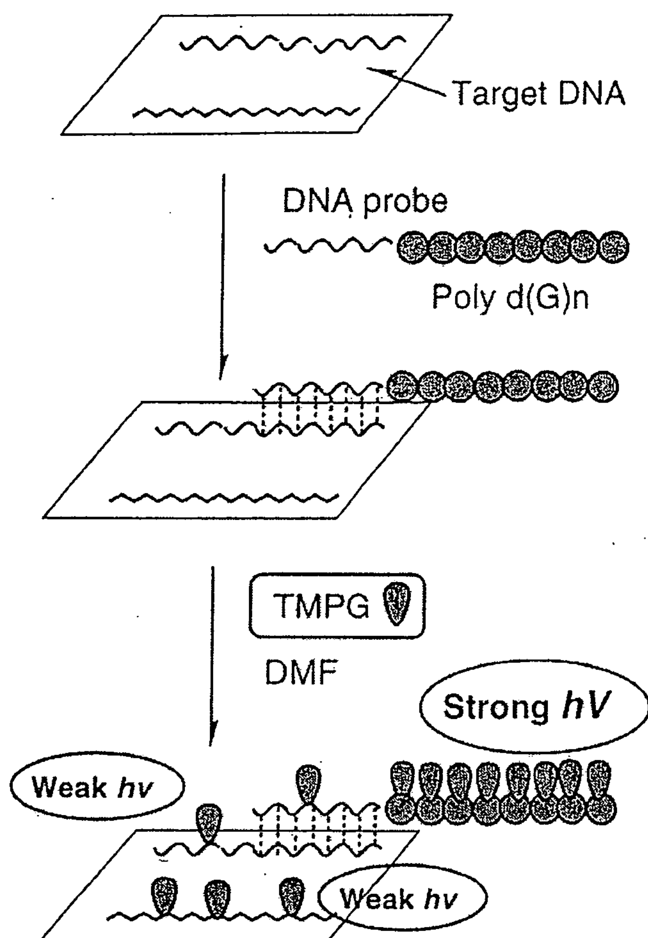

Figure 11 Chemiluminescent detection for membrane hybridization of unmodified DNA target by derivatization reaction with TMPG. Procedure [15]: A portion of the DNA solution is spotted on a nylon membrane. The target DNA is hybridized to its cDNA probe having a -(G)15TT(G)15TT at its 3′ terminus in a hybridization buffer (pH 7.0) at 42°C for 2 h. After washing, the membrane is moistened with sodium phosphate solution (pH 10) for a few seconds, and then immersed in 0.2 M TMPG dissolved with dimethyl sulfoxide for 0.5 min at ambient temperature. The moist membrane is then dipped in dimethylformamide for a few seconds, and the luminescence is detected for 0.5 min.

by the guanine they contain. This hybridization gives a positive, reproducible signal, with a lower detection limit of approximately 1.0 pmol of target DNA.

5. CONCLUSIONS

The advantages of detecting cDNA probes by chemiluminescence and bioluminescence include high sensitivity and simple protocols, using either manual film

reading or sophisticated instrumentation such as CCD and photon-counting cameras for processing image data. In addition, the luminescent reagents are considerably more stable than radioisotopes, and adaptable for detecting hybridization on membranes or with immobilized DNA.

The chemiluminescent derivatizing method for detecting DNA and d(G)n on a nylon membrane, based on the chemical reaction with TMPG, has the advantages of speed and simplicity for the quantitative determination of nucleic acids and DNA probes containing d(G)n. Using a d(G)n probe enhances the chemiluminescence in proportion to the amount of guanylic acid in the probe molecule. The TMPG reaction is useful not only for in situ detection of guanine-rich regions of the genome, but also for immobilized hybridization assays.

Current methods for DNA detection usually require enzymatic amplification of the target DNA sequence prior to analysis. For example, the PCR technique selectively increases the concentration of the target sequence relative to unrelated sequences. PCR methods, however, introduce ambiguities resulting from contamination by different DNA sequence. Therefore, a definitive method is required for the analysis of a single, original DNA sequence. To achieve this objective, the sensitivity and speed of the chemiluminescent enhancement techniques described in this chapter must be improved.

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A Chemiluminescence Reaction between Hydrogen Peroxide and Acetonitrile and Its Applications

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Here we report a new chemiluminescence reaction between basic aqueous H_2O_2 and acetonitrile. Its ultraweak chemiluminescence could be greatly enhanced by luminol, isoluminol-labeled streptavidin, and an Edman-type fluorescent reagent. Light emission was intense and long-lived, and this facilitated the initiation of the reaction and the measurement of the light emission. The present results permit us to propose a series of convenient, highly sensitive, and enzyme-free techniques for the detection and quantification of luminol, related conjugates, acetonitrile, and amino acids. Overall, this new chemiluminescence reaction will be quite promising for numerous applications in immunoassay, DNA hybridization, environmental monitoring, and postcolumn chromatographic detection.

Chemiluminescence (CL) has been exploited with a wide range of applications in different fields such as biotechnology, pharmacology, molecular biology, and clinical and environmental chemistries.[1–9] The most frequently used CL reactions are luminol–peroxidase and 3-(2′-spiroadmantane)-4-methoxy-4-(3″-phosphoryloxy)phenyl-1,2-dioxetane (AMPPD)–alkaline phosphatase systems. These CL reactions offer the possibility of a high sensitivity and a lasting light signal, making more accurate measurements possible and simplifying the measurement procedure. However, the use of enzyme can be problematic, as enzymes may be unstable or cause background interference especially in serum samples as a result of hemoglobin release during red blood cell lysis. Thus, it is a great challenge for the chemists to develop new CL reactions with wide applications, though it would also be important to extend the applications of classical CL reactions.

Here we report such a new CL reaction between basic aqueous hydrogen peroxide and acetonitrile. Although this reaction itself only emitted an ultraweak CL, the light could be greatly enhanced by luminol, related conjugates, and our recently developed Edman-type fluorescent reagent, 4-(2′-cyanoisoindonyl)phenylisothiocyanate (CIPIC).[10] In addition to obtaining a high sensitivity, a longer light signal was observed for routine applications especially with a simple setup. Moreover, the mixing of CL reagents could be generally performed outside the measuring device, making convenient and reproducible CL measurements possible. Based on this fact, the potential use of this reaction was demonstrated in the fields of labeled protein quantification, organic solvent monitoring, and postcolumn amino acid detection.

EXPERIMENTAL SECTION

Materials. Luminol and isoluminol-labeled streptavidin were obtained from Sigma. Acetonitrile, propanenitrile, benzonitrile, and other organic compounds were purchased from Wako. CIPIC was synthesized as described.[10]

Batch CL Measurements. Light-producing reactions were carried out in 12 × 75 mm disposable culture tubes containing 10 μL of different concentrations of luminol or isoluminol-labeled streptavidin or CIPIC. A total of 100 μL of 0.1 M Na_2CO_3 and 20 μL of 0.1 M H_2O_2 were added. Then 20 or 50 μL of distilled water was added to adjust the total volume, and the tubes were placed in the luminescence reader (BLR-201, Aloka). The 50 or 20 μL of acetonitrile was injected, and the cover was closed to initiate CL. The signal was displayed and integrated for a 10-s interval, in arbitrary units. Kinetics of the CL was monitored on a recorder connected to the luminescence reader.

HPLC Conditions. The HPLC system consisted of a Tosoh (Tokyo, Japan) HLC-803D high-performance liquid chromatograph, with a Rheodyne model 7125 syringe-loading sample injector (100-μL loop). A reversed-phase column of TSKgel ODS-80T$_M$ (150 × 4.6 mm i.d., particle size 5 μm) (Tosoh) was used. The column temperature was ambient (25 ± 4 °C). The separation of the CIPIC-conjugated amino acids was carried out with a mobile phase of acetonitrile (45–90%) and 0.1 M acetic acid–triethylamine (pH 5.0), and the flow rate was set at 0.5 mL/min. The column eluate was introduced into the postcolumn reactor. In the system, 1.0 M H_2O_2 and 0.1 M sodium borate buffer (pH 11) were added to the eluate stream with two reagent-delivery pumps each at a flow rate of 0.23 mL/min. CL was monitored by intelligent CL detector (Jasco 825-CL).

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