or protein is generally needed for the amino acid sequencing due to its comparative low sensitivity. It is often unacceptable, especially for some precious protein or peptide samples.

In order to solve this problem, several other Edman-type reagents have been synthesized, such as UV-based 2-*p*-isothio cyanophenyl-3-phenylindone [6], 4-*N*,*N*-dimethylaminoazobene-4-isothiocyanate [7,8], 4-(*N*-*tert*-butyloxycarbonylaminomethyl) phenylisothiocyanate [9], fluorescence-based fluoresceinisothiocyanate [10], 4-(*N*,*N*-dimethylamino)-1-naphthylisothiocyanate [11], 4-(*N*-1-dimethylamino-napthalene-5-sulfonylamine)phenylisothiocyanate [12], 4-(2-phenanthra-[9,10-*d*] oxazolyl)phenylisothiocyanate [13], 4-(3-isothiocyanatopyrrolidine-1-yl)-7-(*N*,*N*-dimethylamino-sulfonyl)-2,1,3-benzoxadiazole [14], and chemiluminescence (CL)-based 4-isothiocyanatophthalhydrazide [15]. However, still two main drawbacks existed in these methods: the repetitive yield in the Edman procedure is low and the degradation products sometimes interfere with the detection of thiohydantoin-amino acids. Recently, Imai and co-workers developed another two benzofurazan Edman reagents: 7-[(*N*,*N*-dimethylamino)sulfonyl]-2,1,3-benzoxadiazol-4-isothiocyanate [16–18], and 7-methylthio-4-(2,1,3-benzoxadiazolyl)isothiocyanate [19]. Although thiohydantoin-amino acids fluoresced weakly and thiazolinone-amino acids fluoresced strongly, free amino acids were observed due to the decomposition of unstable thiazolinone. Compared with previous methods, however the sensitivity is much higher with a detection limit of 50 fmol–1 pmol.

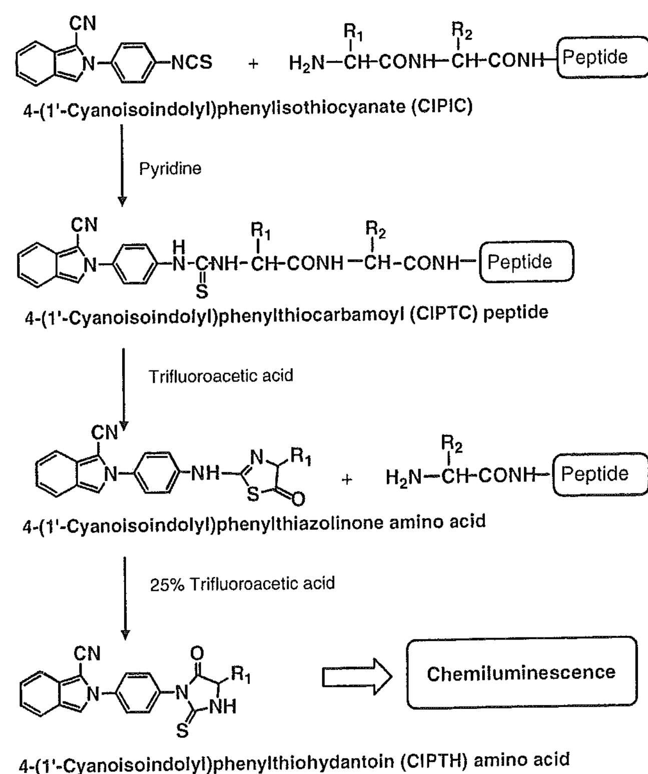

Fig. 1. Edman degradation with CIPIC for peptide sequence.

To overcome the above disadvantages, we reported a novel fluorescent Edman reagent, 4-(1′-cyanoisoindolyl) phenylisothiocyanate (CIPIC) [20]. CIPIC was found to be of high reactivity with amino acid in the Edman procedure. It is well known that CL has been exploited with a wide range of applications in different fields such as biotechnology, pharmacology, molecular biology, and clinical and environmental chemistries. Specifically CL-based analysis offers a simple, low-cost, sensitive means of measuring a variety of compounds. Thus CIPIC was further investigated to see whether it is possible to tune as a sensitive CL-type Edman reagent. Our results show that CIPIC could induce a strong CL after mixing with H_2O_2 in an alkaline solution, and thus can be used for the CL detection of amino acids (Fig. 1).

2. Experimental

2.1. Apparatus

Batch CL measurements were made by using a luminescence reader (BLR-201, Aloka, Japan). Fluorescence and UV measurements were done by employing Hitachi 650-10 and Shimadzu UV-240, respectively. LC system consisted of JASCO PU-980 pump and UV-970 detector. LC–MS measurements were carried out by using M-1000 quadruple mass spectrometer (Hitachi, Japan) with atmospheric chemical ionization at 200 °C, nebulizer temperature at 370 °C, filament current at +8 μA, drift voltage at 110 V and focus voltage at 60 V.

2.2. Materials and chemicals

Buffer solutions for pH 9–13 for CL reaction were prepared by mixing 0.1 M boric acid and 0.1 M sodium borate, 0.1 M boric acid and 0.1 M triethylamine, or 0.1 M $NaHCO_3$ and 0.1 M NaOH. Buffer solution (pH 5.0) for liquid chromatography (LC) was made by mixing 0.1 M acetic acid and 0.1 M triethylamine. CIPIC, CIPIC-thiocarbamoyl-Ala (CIPTC-Ala) and CIPIC-thiohydantoin (CIPTH-Ala) stock solutions, and their working solutions were prepared in acetonitrile. All other reagents and solvents were of analytical or guaranteed reagent grade.

2.3. Synthesis of CIPIC, CIPTC-Ala and CIPTH-Ala

CIPIC was synthesized as reported previously [20]. In order to synthesize CIPTC-Ala, 50 ml of 80 mM L-Ala in acetonitrile and water (4:1, v/v), 10 ml of 10 mM CIPIC in acetonitrile and 50 ml of pyridine and acetonitrile (2:3, v/v) were reacted at 80 °C for 20 min. After cooled to room temperature, 100 ml of 25 mM sodium phosphate buffer (pH 8.5) and 300 ml chloroform were added into the reaction mixture. The aqueous phase was separated from organic phase and kept at 4 °C (solution A). Solution A (80 μl each) was subjected to reversed-phase LC and CIPTC-Ala frac-

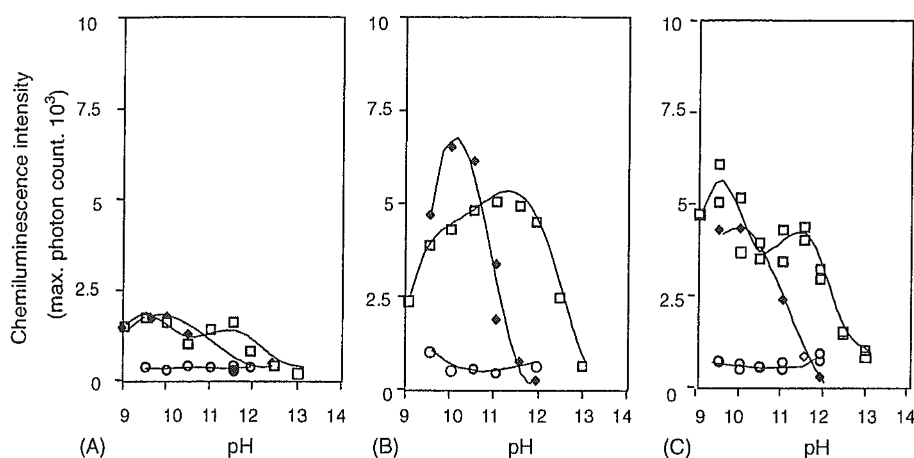

Fig. 2. Effects of pH of several buffers on the chemiluminescence reaction with (A) CIPTH-Ala, (B) CIPTC-Ala, and (C) CIPIC. Buffers: (□) sodium-borate; (◊) triethylamine-borate; (○) $NaHCO_3$-NaOH.

tion (ca. 20 mg) was obtained. To purify CIPTH-Ala, solution A was mixed with 200 ml of 2.5 M HCl, and then heated at 80 °C for 20 min for converting CIPTC-Ala to CIPTH-Ala. The resulting solution was cooled to room temperature and 400 ml chloroform was added. The chloroform phase was concentrated, and the precipitate was recrystallised in acetonitrile and water. CIPTH-Ala (ca. 150 mg) was obtained.

2.4. CL detection procedures

Light producing reactions were carried out in 12 mm × 75 mm disposable culture tubes containing 100 μl of CIPIC, CIPTC-Ala and CIPTH-Ala at different concentrations. Portions of 100 μl of 0.1 M borate buffer (pH 9.6), and 50 μl of acetonitrile were added, and the tubes were placed in the luminescence reader. A 50 μl portion of 1.5 M H_2O_2 was injected and the cover was closed to initiate CL. The signal was displayed and integrated for 30 s. The progress of the CL was monitored on a recorder connected to the luminescence reader.

2.5. Fluorescence and absorbance detections

Fluorescence intensity was detected at Ex = 350 nm and Em = 425 nm for CIPIC in acetonitrile, and at Ex = 350 nm and Em = 410 nm for CIPTH-Ala in acetonitrile. UV absorbance was measured at 275 nm for CIPIC and 255 nm for CIPTH-Ala.

2.6. Synthesis of N-(4-acetylaminophenyl) phthalimide (product B in Fig. 7)

Twelve millilitres of 72 μM phthalic anhydride in acetonitrile and dimethylformamide (2:1, v/v) were mixed with 6 ml of 36 μM p-aminoacetanilide in acetonitrile and dimethylfor-

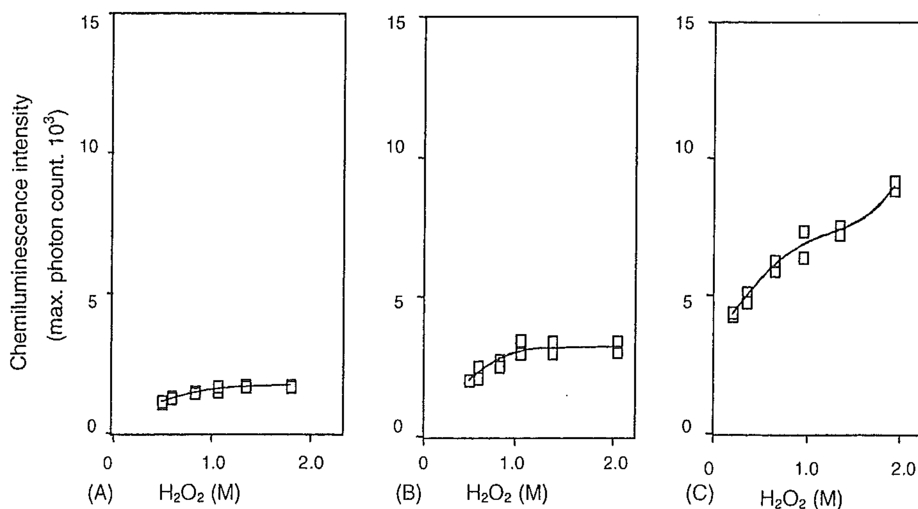

Fig. 3. Effects of concentration of hydrogen peroxide at pH 9.6 and acetonitrile on the chemiluminescence reaction with (A) CIPTH-Ala, (B) CIPTC-Ala, and (C) CIPIC.

mamide (2:1, v/v), and then stirred at room temperature for 30 s. White precipitate (ca. 1.7 g) was formed and filtered. The precipitate (450 mg) was dissolved in 120 ml of tetrahydrofuran and dimethylformamide (1:1, v/v), 6 ml of conc. HCl was added and then the reaction was carried out at 80 °C for 40 min. Water (240 ml) was added into the reaction mixture and then ice-cooled overnight. The product was filtered and recrystalised in tetrahydrofuran (product B left in Fig. 7).

2.7. LC conditions

A reversed-phase column of Cosmosil 3C (100 mm × 4.6 mm i.d., particle size 3 μm) (Nacalai tesque, Japan) was used. 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–0.1 M triethylamine mixture (pH 5.0), and the flow-rate was set at 0.5 ml/min. The column eluate was first monitored by fluorescence and then 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 at each flow-rate of 0.23 ml/min. CL was monitored by intelligent CL detector (JASCO 825-CL, Japan).

3. Results and discussion

3.1. Optimum CL conditions

It is well known that the separation technique plays a key role for the detection of different analytes in complex matrixes. Reversed-phase LC has become dominant as a potential tool for the separation and quantification of amino acids or peptides. Acetonitrile, methanol and tetrahydrofuran (THF) are generally used as organic solvents for elution. Therefore it is very important to see whether these commonly used organic solvents were suitable to be used in the current CL reactions. Surprisingly, we found that these three organic solvents, i.e. acetonitrile, methanol and THF, all enhanced the CL intensity in the current CL reactions. These results indicated that CIPIC-based CL Edman reagent is wholly compatible with currently used reversed-phase LC system. For the detection of CIPIC-conjugated amino acids, H_2O_2 solution was simply mixed with the separated eluates and CL was monitored on a recorder. Acetonitrile was used in the following experiments.

It was found that CL intensity was greatly affected by the reaction pH or buffer type. Indeed CL intensity was much higher in either borate-triethylamine or sodium borate buffer than that in $NaHCO_3$–NaOH buffer (Fig. 2). Thus sodium borate buffer was chosen for CL reactions. As shown in Fig. 3, the CL intensity increased when the concentration of H_2O_2 was increased.

Also as shown in Fig. 4, the shape of the kinetic profile of CL emittance is greatly affected by the reaction pH. At pH < 9.6, the signal is small but the signal decays slowly. At pH > 9.6, the signal is much larger, but it decreases quickly after reaching the maximum. The maximum signal was the highest at pH 9.6 in the borate buffer solutions. Therefore, pH 9.6 of 0.1 M sodium borate buffer and 1.5 M H_2O_2 were selected in the following experiments.

3.2. Comparison of calibration curves and detection limits by UV, FL and CL detections

Since CIPIC and related conjugates all have UV absorbance, fluorescence (FL) and CL emission, thus it is worth to compare with detection installation to provide a best choice. The concentrations of 25–250 nM CIPTH-Ala derivatives were thus exemplified to compare these three detection techniques. As shown in Fig. 5, by using UV detection, a calibration curve is obtained with a poor accuracy due to large error caused by electrical noise. Comparatively, a calibration curve was obtained with a good accuracy by either using FL

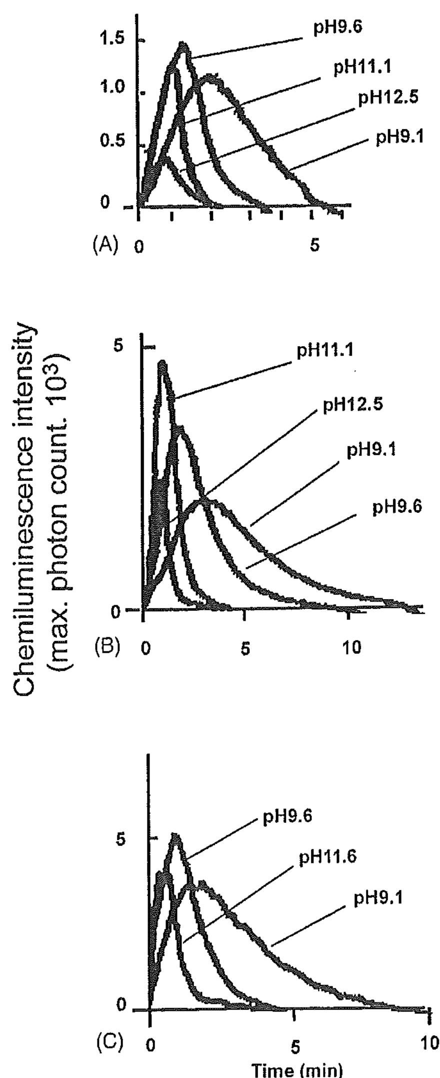

Fig. 4. Time-course of chemiluminescence development from (A) CIPTH-Ala, (B) CIPTC-Ala, and (C) CIPIC depending on pH.

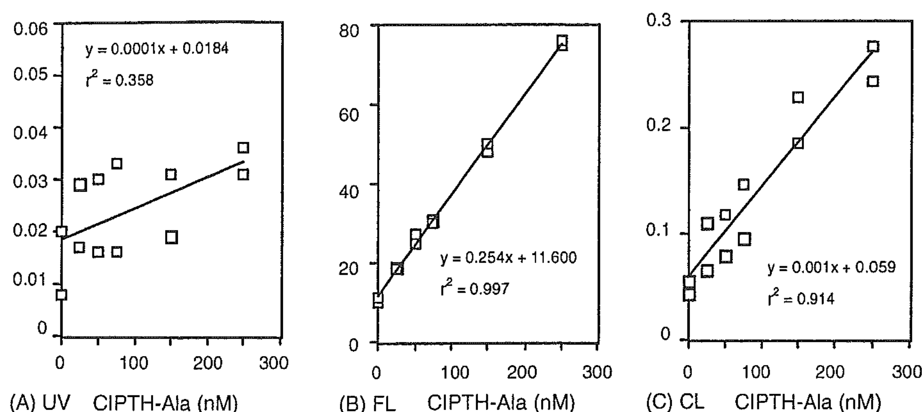

Fig. 5. Calibration graphs of 25–250 nM CIPTH-Ala obtained by (A) UV absorption, (B) fluorometric, and (C) chemiluminometric methods.

or CL detection. These results indicated that CL or FL detection was 20 times more sensitive than UV absorbance.

3.3. Structure elucidation of CL intermediate and emitter

The CIPIC analogues, 4-(1′-cyanoisoindolyl)acetanilide (CIAA) and 4-(1′-cyanoisoindolyl)aniline (CIA) were exemplified to elucidate possible structure of CL intermediates and emitters. CIAA first reacted at room temperature with H_2O_2 in borate buffer (pH 8.0), and then the reaction mixture at a reaction time was separated by reversed-phase LC equipped with UV detector. As shown in Fig. 6, CL reactant and products are eluted within 50 min and well separated from each other. Importantly, peak A was first increased with the reaction time, and then decreased with the increase of peak B, while CIAA and CIA were decreased with the reaction time. The same phenomenon was observed for both CL reactions. These facts indicated that peak A was possible intermediate of this CL reaction and peak B was the CL emitter.

Subsequently, LC–MS was then used to further study the mass spectrometric analysis of these CL intermediates and emitters. For CIAA reaction, both product A (intermediate) and product B (emitter) had a molecular weight of 280. For CIA reaction, both products A and B had a molecular weight of 238. The mass difference between these products obtained by CIAA- and CIA-CL reactions was 42. It should be mentioned that the difference of CIAA and CIA structures is one substituting group with $-NHCOCH_3$ for CIAA and $-NH_2$ for CIA, and its mass difference is exactly 42. This clearly indicates that the CL emission was occurred by the oxidation in the cyanoisoindole part of CIAA and CIA.

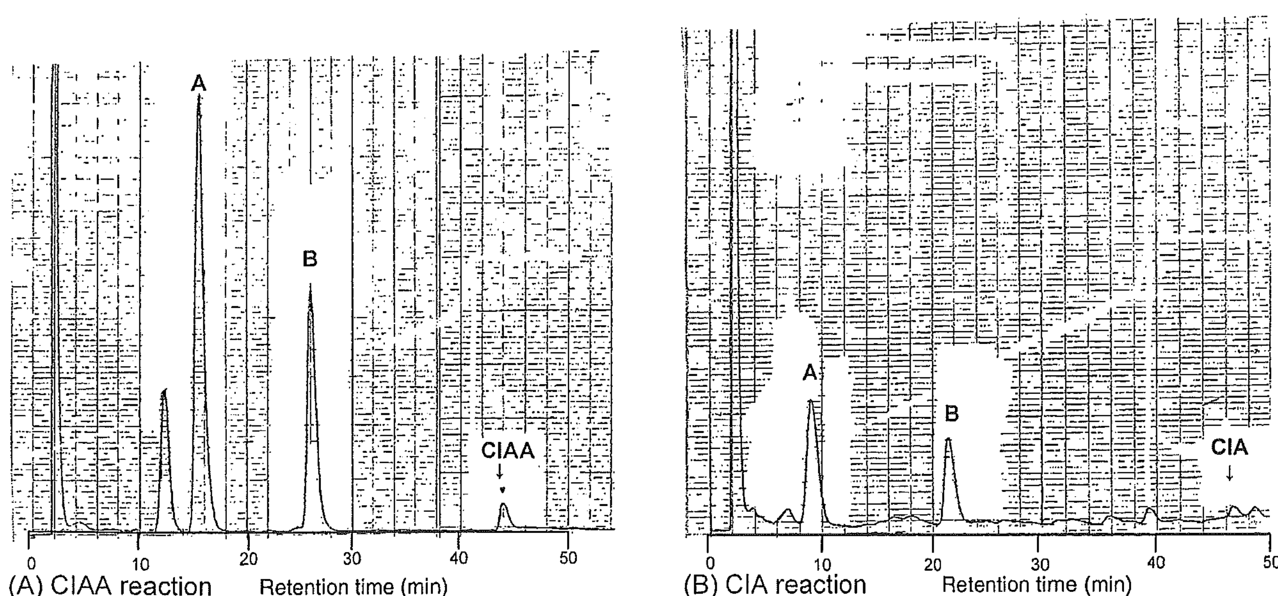

Fig. 6. LC separation and UV detection of chemiluminescent reaction mixtures with (A) CIAA and (B) CIA. LC conditions: column = Cosmosil $3C_{18}$; mobile phase CH_3CN (15–40%) + 0.1 M CH_3COOH–triethylamine, pH 4.9 (10%) + H_2O (75–50%) for 40 min and 50% CH_3CN after 50 min.

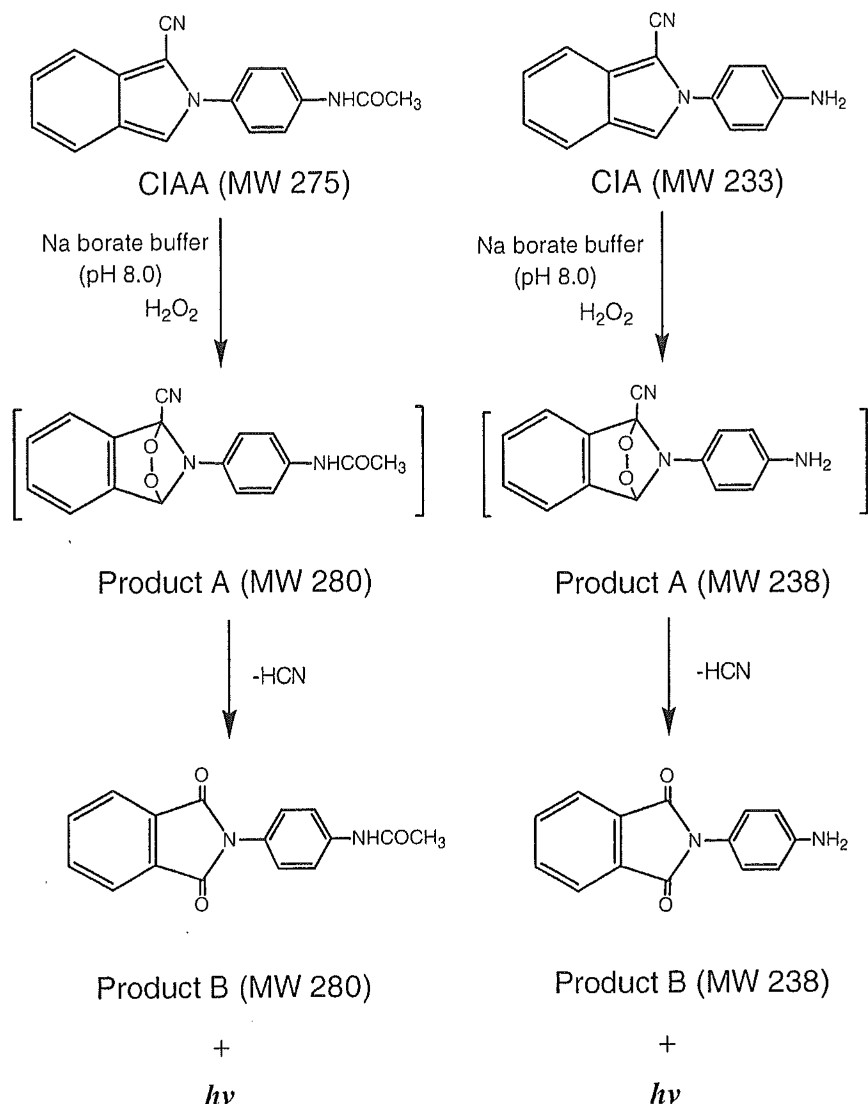

Fig. 7. Products estimated from CIAA and CIA in their mixtures of chemiluminescence reaction.

From the data, possible reaction scheme is shown in Fig. 7. CIAA or CIA first formed a six-membered ring of peroxide (intermediate), and then by the breakage of –O–O– bond, the excited state of phthalimide derivative (emitter) was produced, yielding emission of light.

In order to further confirm that CL emitter was the phthalimide derivative, the product B in Fig. 7 was synthesized from phthalic anhydride and *p*-aminoacetanilide as described in Section 2. The product (melting point, 276 °C) was *N*-(4-acetylaminophenyl)phthalimide, and then its structure was characterized by ^{1}H NMR and ^{13}C NMR. Three or seven peaks from aromatic regions of 7.3–7.9 ppm in the ^{1}H NMR spectrum and 120–140 ppm in the ^{13}C NMR spectrum were observed, respectively. We believed that the data show the symmetric structure of product B. Subsequently CL emitter of CIAA reaction (peak B in Fig. 6) was collected and dried, and then characterized by ^{1}H NMR. It was found that the same ^{1}H NMR spectrum was observed for the peak B as the CL emitter of CIAA reaction. Furthermore, the same retention time was observed for the peak B in Fig. 6 and the synthetic compound (the product B) by the same RP–LC conditions. Overall, it was confirmed that CL emitters of CIAA, CIA and CIPIC are phthalimide derivatives.

3.4. Separation of CIPTH-amino acids

For the detection of CIPTH-amino acids, H_2O_2 solution and borate buffer were simply mixed with the column eluates after separation of 21 amino acid derivatives, and CL was then monitored on a recorder. As shown in Fig. 8, all CIPTH-amino acids were eluted within 30 min. Sixteen CIPTH-amino acids were well separated and were monitored by both FL and CL. Peaks 11, 19, and 20 were also confirmed by directly injecting free CIPIC after derivatization or thiocarbamoyl amino acids into the column. Detection limits of these amino acids at 3 of signal-to-noise ratio were

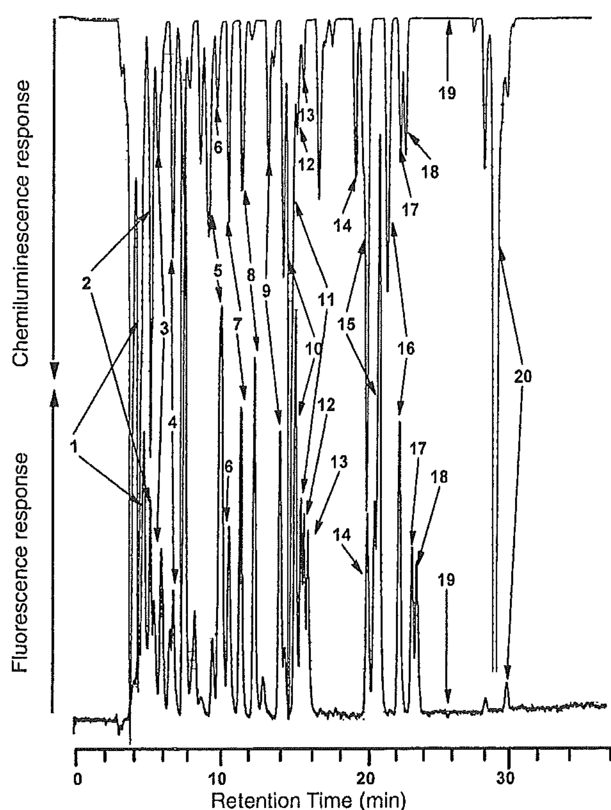

Fig. 8. CL and FL chromatograms of the CIPTH-amino acids. Peaks: (1) Glu and Asp; (2) Arg; (3) Cys; (4) Lys; (5) His and Gln; (6) Asn; (7) Ser; (8) Thr; (9) Gly; (10) Hyp; (11) byproduct from CIPIC; (12) Ala; (13) Tyr; (14) Met; (15) Val, Pro and Trp; (16) Phe; (17) Ile; (18) Leu; (19) CIPIC; (20) CIPIC-byproduct.

0.3–0.8 pmol/injection. The detection limit of CIPTH-amino acids was almost same as those (pico or subpicomole) of hydantoin derivatives formed by other FL reagents [10–14].

4. Conclusion

This work shows that our developed fluorescent Edman-type reagent could be tuned as a sensitive CL Edman reagent. CL reaction conditions were optimized, and the structures of CL intermediate and emitter were elucidated and confirmed by using LC, LC–MS and NMR. Furthermore, this novel CL reaction was wholly compatible with reversed-phase LC for the separation of amino acid conjugates since commonly used organic solvents enhanced CL intensity. However the sensitivity of the present CL detection of CIPTH-amino acids was not much higher than the FL detection. It means that FL detection is simpler and more convenient than this CL detection. Next step, we will synthesize the modified CIPIC reagents as more sensitive CL reagents, and develop the Edman-degradation conditions for the determination of amino acid sequence of protein.

Acknowledgement

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References

[1] P. Edman, Acta Chem. Scand. 4 (1950) 283.
[2] P. Edman, Acta Chem. Scand. 10 (1956) 761.
[3] P. Edman, A.L. Riedesel, G.E. Folk, Nature 177 (1956) 667.
[4] P. Edman, G. Begg, Eur. J. Biochem. 1 (1967) 80.
[5] R. Kobayashi, G.E. Tarr, Tanpakusitu Kakusan Kouso 31 (1986) 991.
[6] C.P. Ivanov, I.N. Mancheva, Anal. Biochem. 53 (1973) 420.
[7] J.Y. Chang, E.H. Creaser, K.W. Bentley, J. Biochem. 153 (1976) 607.
[8] H. Hirano, Tanpakusitu Kakusan Kouso 29 (1984) 374.
[9] Z. Palactz, J. Salnikow, S.-W. Jin, B.W. Liebold, FEBS Lett. 176 (1984) 365.
[10] K. Muramoto, K. Nokihara, A. Ueda, H. Kamiya, Biosci. Biotechnol. Biochem. 58 (1994) 300.
[11] H. Ichikawa, T. Tanimura, T. Nakajima, T. Tamura, Chem. Pharm. Bull. 18 (1970) 1493.
[12] S.-W. Jin, G.-X. Chen, Z. Palacz, B.W. Liebold, FEBS Lett. 198 (1986) 150.
[13] O. Imakyure, M. Kai, Y. Ohkura, Anal. Sci. 9 (1993) 647.
[14] T. Toyooka, T. Suzuki, T. Watanabe, Y.-M. Liu, Anal. Sci. 12 (1996) 779.
[15] S.R. Spurlin, M.M. Cooper, Anal. Lett. 19 (1986) 2277.
[16] H. Matsunaga, T. Santa, K. Hagiwara, H. Homma, K. Imai, Anal. Chem. 67 (1995) 4276.
[17] H. Matsunaga, T. Santa, T. Iida, T. Fukushima, H. Homma, K. Imai, Anal. Chem. 68 (1996) 2850.
[18] Y. Huang, H. Matsunaga, T. Toriba, T. Santa, T. Fukushima, K. Imai, Anal. Biochem. 270 (1999) 257.
[19] A. Toriba, K. Adzuma, T. Santa, K. Imai, Anal. Chem. 72 (2000) 732.
[20] O. Imakyure, M. Kai, Y. Ohkura, Anal. Chim. Acta 291 (1994) 197.

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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-

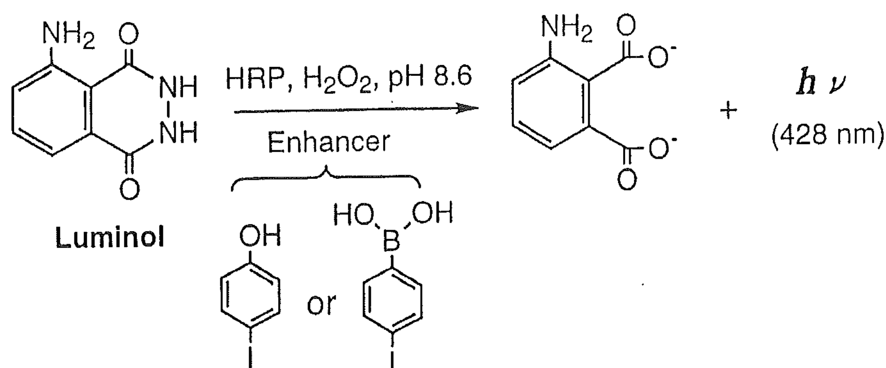


Figure 1 HRP-catalyzed chemiluminescent reaction of luminol.

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

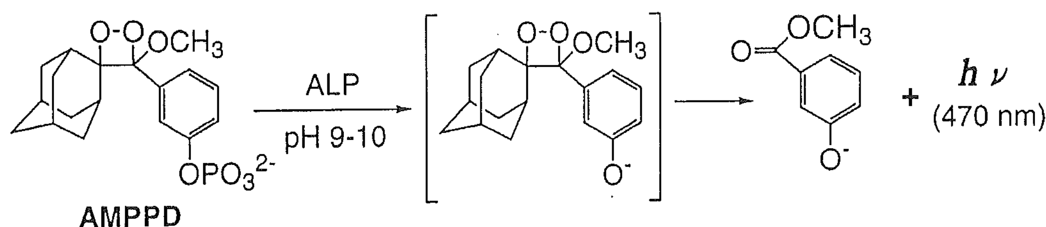


Figure 2 ALP-catalyzed chemiluminescent reaction of AMPPD.

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.

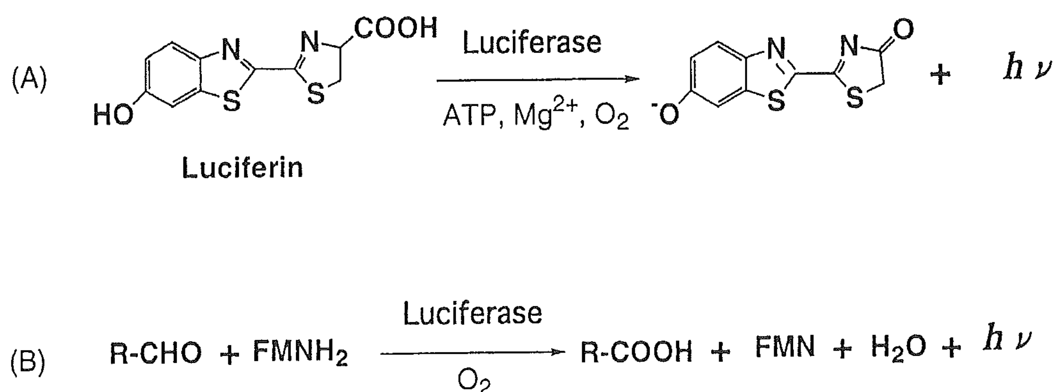
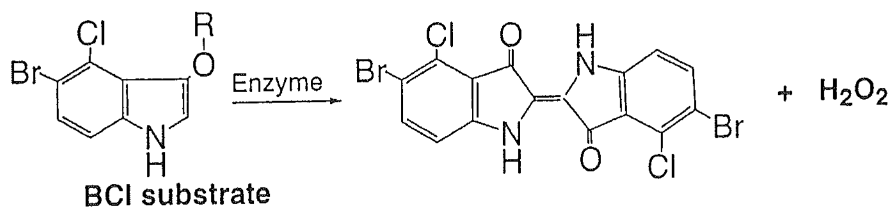


Figure 3 Luciferase-catalyzed bioluminescent reaction with (A) luciferin and (B) an aldehyde compound and $FMNH_2$.



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-

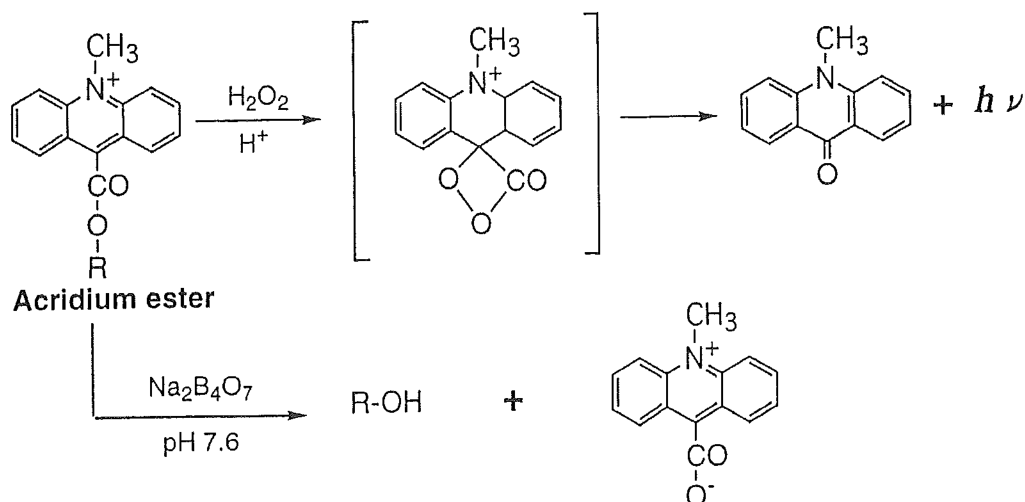


Figure 5 Chemiluminescent reaction of acridinium ester and its hydrolysis.

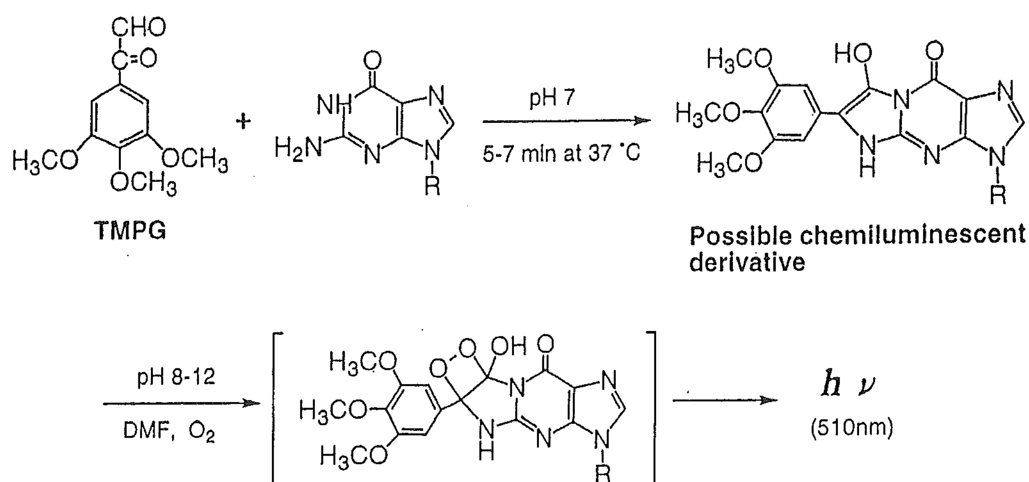


Figure 6 Derivatization and chemiluminescent reactions of guanine-containing compounds with TMPG.

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-

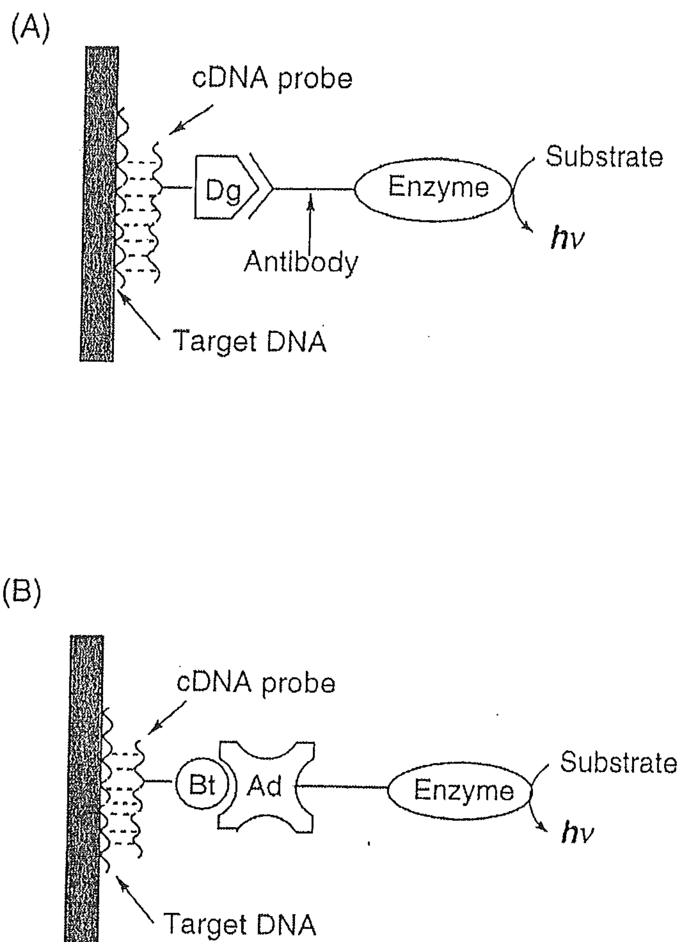


Figure 7 Chemiluminescent detections using (A) digoxigenin- and (B) biotin-binding probes for membrane hybridizations of DNA. Dg, digoxigenin; Bt, biotin; Ad, avidin. Procedures A and B [20]: Membrane hybridization of DNA involves the use of nylon membranes as a solid support onto which various sample 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.

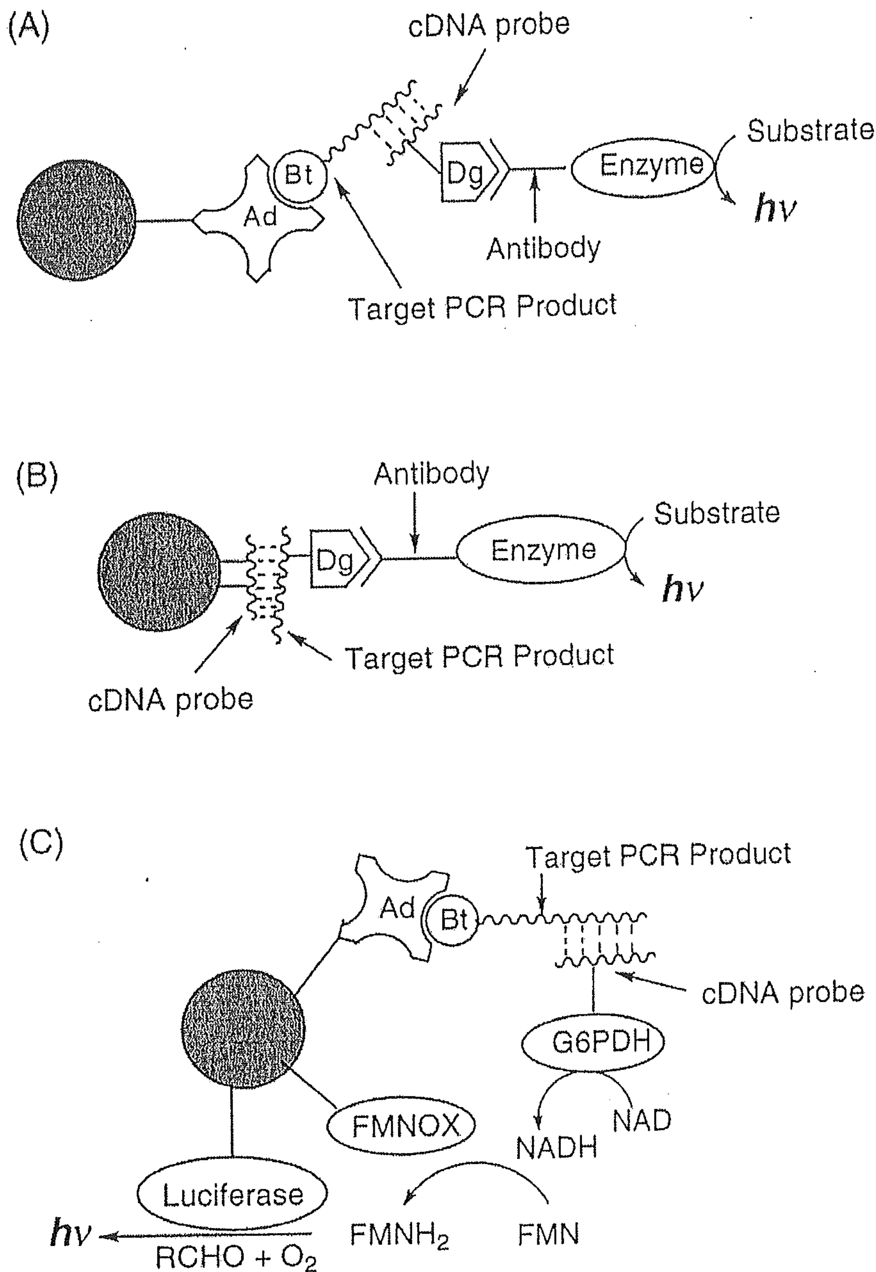


Figure 8 Chemiluminescent (A and B) and bioluminescent (C) detections for immobilized hybridizations of PCR product. Dg, digoxigenin; Bt, biotin; Ad, avidin. Procedure A [30]: Biotin moiety is incorporated into PCR products during the amplification reaction, using one 5′-biotinylated primer. The product 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.

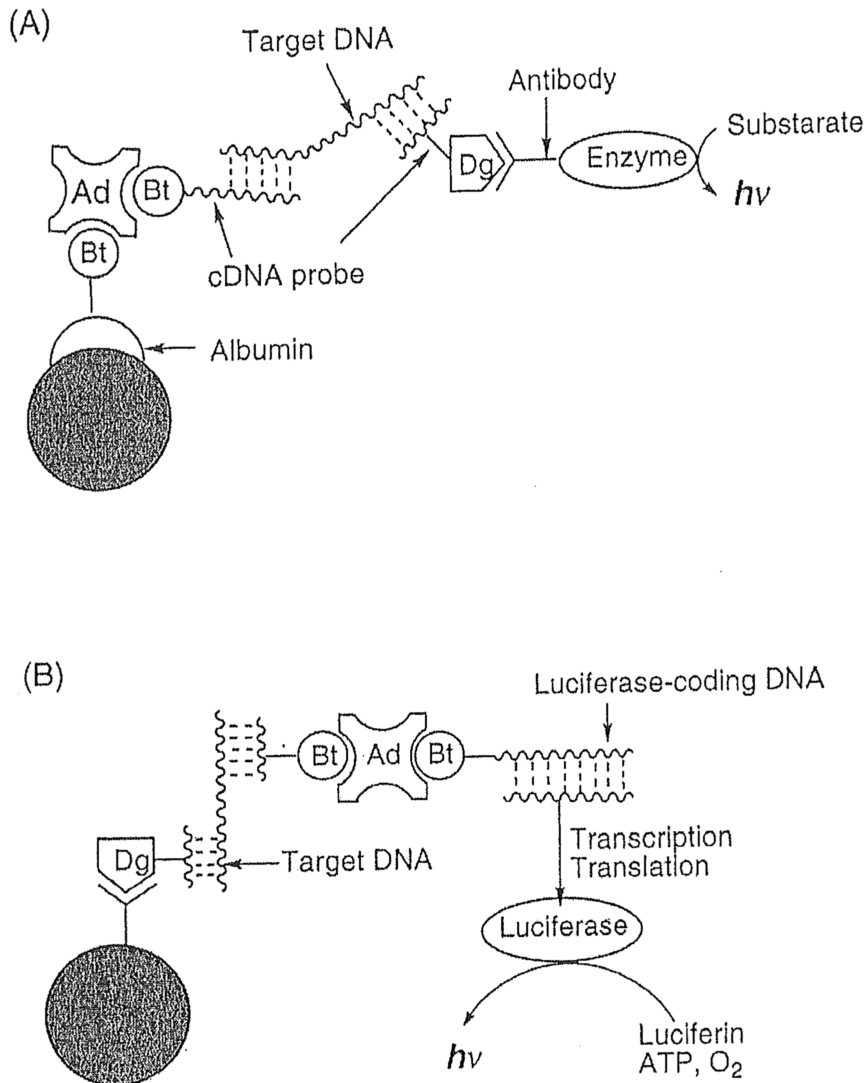

Figure 9 Chemiluminescent (A) and bioluminescent (B) detections for immobilized hybridizations of unmodified DNA target. Dg, digoxigenin; Bt, biotin; Ad, avidin. Procedure A [33]: A Bt-labeled cDNA probe is first bound to a polyvinylchloride microtiter plate well that was previously coated overnight 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.

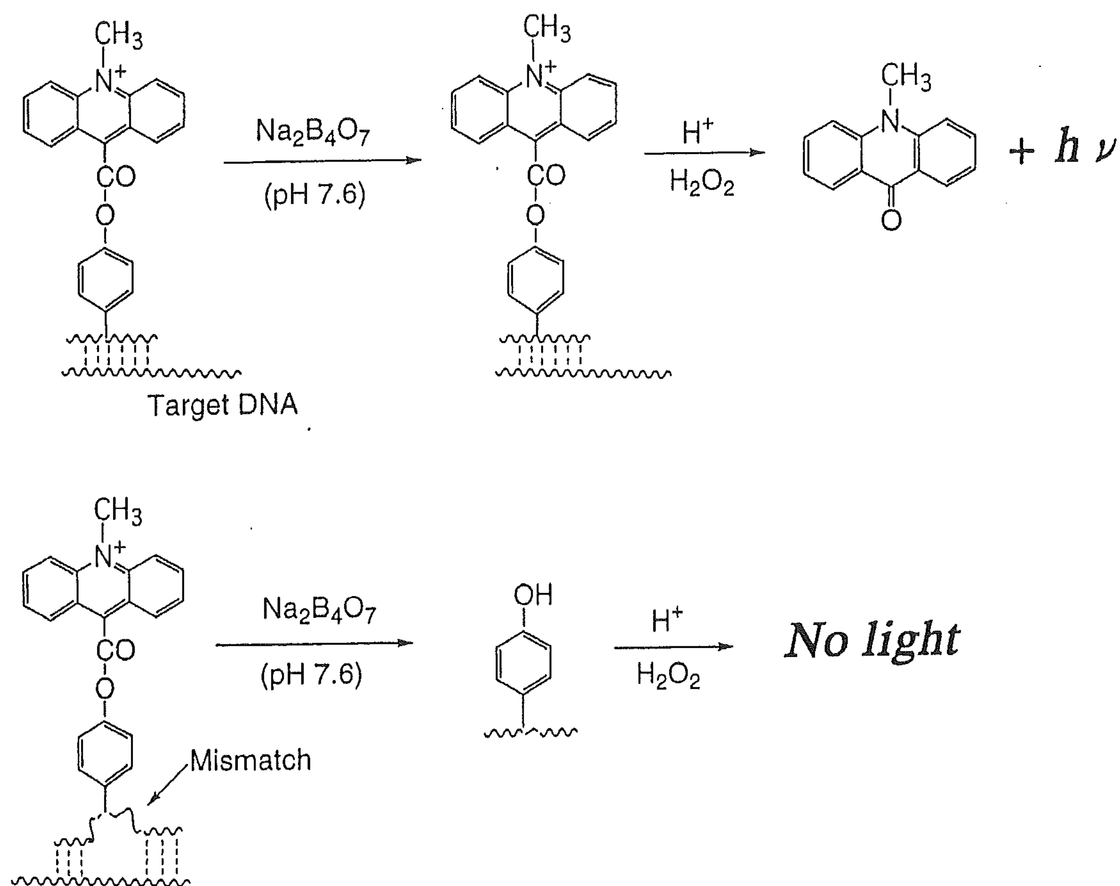

Figure 10 Mismatch detection by using a chemiluminescent AE-labeled cDNA probe. Procedure [9, 11]: Acridinium ester–labeled probes specific for either wild-type or mutant sequence corresponding to a target DNA are hybridized with the sample DNA for 1.0 h at 60°C in a hybridization buffer (pH 5.2). Hybridized 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

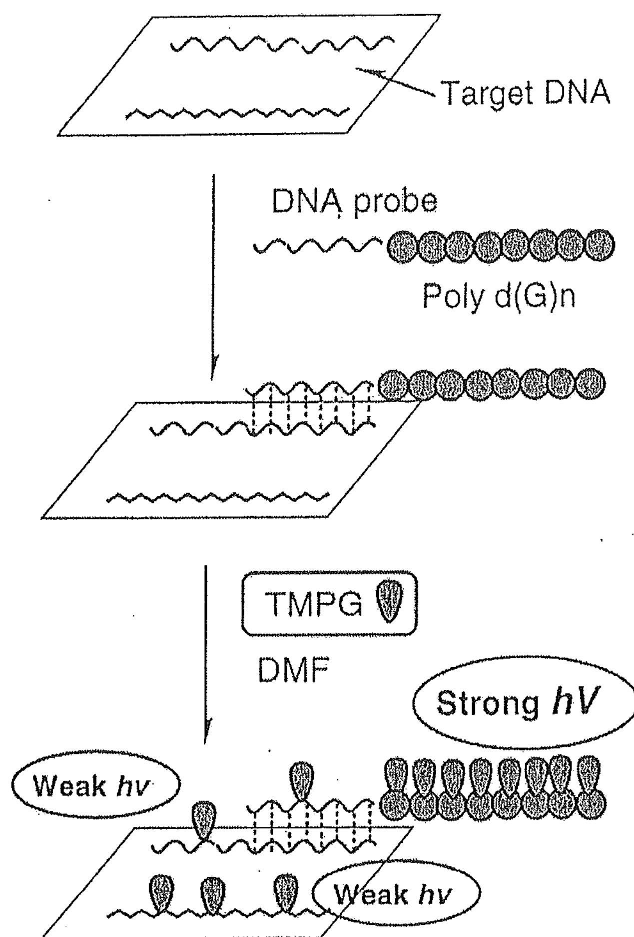

Figure 11 Chemiluminescent detection for membrane hybridization of unmodified DNA target by derivatization reaction with TMPG. Procedure [15]: A portion of the DNA solution is spotted on a nylon membrane. The target DNA is hybridized to its cDNA probe having a -(G)15TT(G)15TT at its 3′ terminus in a 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