A Simple, Two-Color Fluorescence Detection Method for Membrane Blotting Analysis Using Alkaline Phosphatase and Horseradish Peroxidase

Yasumitsu KONDOH,1,3 Satoshi FUJITA,1,4 Naoto KAGIYAMA,1 and Michihiro C. YOSHIDA2,3,*

AISIN COSMOS R&D CO., LTD., Kariya, Aichi 448-8650, Japan,1 Chromosome Research Unit, Faculty of Science, Hokkaido University, Nishi-8, Kita-10, Kita-ku, Sapporo, Hokkaido 060-0810, Japan,2 Laboratory of Cytogenetics, Division of Bioscience, Graduate School of Environmental Earth Science, Hokkaido University, Sapporo, Hokkaido 060-0810, Japan,3 and Department of Applied Chemistry, Nagoya Institute of Technology, Nagoya 466-8555, Japan4

(Received 13 April 1998; revised 14 May 1998)

Abstract

We have developed a one-step, two-color fluorescence detection method using simultaneously two fluorogenic substrates for both Southern and Western blots on nylon membranes. For this enzyme-mediated reporter system, a mixture of (i) 3-hydroxy-Ar-2'-biphenyl-2-naphthalenecarboxamide phosphate ester (HNPP), a substrate for alkaline phosphatase and (ii) Ar-(4-amino-5-methoxy-2-methylphenyl)benzamide (AMMB), a fluorogenic substrate for horseradish peroxidase was used. The reaction with these substrates produces blue (HNPP) and yellow (AMMB) fluorescent signals under ultraviolet light (302 nm). Therefore, this simple method allows the simultaneous visualization of two different targets on a single nylon membrane, e.g. nucleic acids or proteins.

Key words: alkaline phosphatase; horseradish peroxidase; Southern blotting; Western blotting; substrate

Non-radioisotopic (non-RI) detection methods have recently been developed to analyze nucleic acids or proteins by Southern or Western blotting. Signals of the hybridization site depend on the signal-generating system, using either of the two main types: fluorochromes and enzymes. Fluorochromes are mostly used for in situ hybridization and the site of hybridization can be visualized directly by its fluorescence.1 However, this system is not applicable to membrane blotting analysis such as Southern or Western analysis due to its low sensitivity. Enzyme-mediated reporter systems work by catalyzing precipitation of a visible product at the hybridization site on a nylon membrane. The enzymes horseradish peroxidase (PO) and alkaline phosphatase (APase) are generally used as reporters.2–5

Recently, several chemifluorescent substrates have been developed for the APase reaction, resulting in fluorescence products on nylon membranes.6–8 Fujita et al.9 recently developed a fluorescent substrate, 5-(4-biphenylcarboxamido)-3'-O-(1-naphthyl)methylfluorescein phosphate ester (BNFP). In combination with another substrate like HNPP,7 BNFP enables one to distinguish two different contrast fluorescent colors, namely green by BNFP and blue by HNPP, on a single nylon membrane. In this method, HNPP was reacted first with APase-conjugated anti-digoxigenin antibody coupled to a digoxigenin-labeled probe, followed by inactivation of APase. Subsequently, BNFP was reacted with APase-conjugated anti-biotin antibody coupled to a biotin-labeled probe. Since this method involves stepwise incubation of HNPP and BNFP with two kinds of APase-antibody conjugates, the method is quite labor intensive. A one-step procedure using two different kinds of enzymes, like APase and PO, is undoubtedly more convenient than this two-step methodology. However, conventional fluorogenic substrates for PO, such as 3-(4-hydroxyphenyl)propionic acid (HPPA) and 2',7'-dichlorofluorescin diacetate,10,11 are water-soluble and can not be used to detect a hybridization site on a membrane due to diffusion without deposition of their products on the membrane.

Therefore, as an alternative approach, we have developed a simple, two-color fluorescence detection method on nylon membranes using a newly found fluorogenic substrate for the reaction with PO. Among the nineteen aromatic amine derivatives screened, only the N-(4-amino-5-methoxy-2-methylphenyl)benzamide (AMMB) substrate produced a discrete yellow fluorescent product on a ny-
Table 1. Components in the detection and reaction systems for Southern blotting.

| Probe                        | Conjugated antibody            | Substrate   |
|------------------------------|-------------------------------|-------------|
| DIG labeled λDNA/ Eco RI probe | Horseradish peroxidase-conjugated anti-DIG antibody | AMMB        |
| Fluorescein labeled Col E1 and pBR322 anti-fluorescein antibody | Alkaline phosphatase-conjugated | HNPP        |

Table 2. Components in the detection and reaction systems for Western blotting.

| Target         | Primary antibody               | Secondary antibody                                             | Substrate |
|----------------|--------------------------------|---------------------------------------------------------------|-----------|
| Human albumin | Mouse anti-human albumin       | Goat anti-mouse lgG (H+L) antibody-horseradish peroxidase conjugate | AMMB      |
| Human lgG     | Rabbit anti-human lgG (whole) antibody | Goat anti-rabbit lgG antibody-alkaline phosphatase conjugate | HNPP      |

1. Screening of Aromatic Amine Derivatives

We reacted aromatic amine derivatives with PO bound to both nitrocellulose and nylon membranes in order to examine its reactivity with PO and substantivity (affinity to membranes). A series of different amounts of PO were spotted on each nitrocellulose and nylon membrane, and incubated with aromatic amine derivatives in the presence of H₂O₂. As shown in Fig. 1, 19 of the screened aromatic amine derivatives are classified into three types: (i) aniline derivatives (Type 1), (ii) 4-aminoazobenzene derivatives (Type 2), (iii) N-(4-aminophenyl)benzamide derivatives (Type 3). After incubation with PO, each spot of Type 1 derivatives and Type 2 derivatives was stained in brown or reddish purple, but non-fluorescent. Only Type 3 derivatives fluoresced by PO oxidation. One of Type 3 derivatives, N-(4-amino-5-methoxy-2-methylphenyl)benzamide (AMMB) was found to give high fluorescent intensity and substantivity, resulting in the highest sensitivity among all tested compounds.

The detection limit of the method using the substrate AMMB was compared with that of a conventional method using the colorimetric substrate 3,3′-diaminobenzidine (DAB) on Southern blotting. The amounts of HindIII-digested λDNA applied on lanes were 200, 40 and 8 pg, respectively. DIG-labeled λDNA/HindIII was hybridized to membrane-transferred λDNA and bound with PO-conjugated anti-DIG antibody. AMMB was reacted with probe-bound PO, and 0.7 pg of DNA was detected as yellow fluorescence on Southern blotting. The detection method using AMMB is about ten times more sensitive than the method using DAB (7.7 pg). After the enzymatic reaction with PO, the substrate AMMB produced a yellow fluorescent signal (λ_ex = 298 and 418 nm, λ_em = 530 nm) on a nylon membrane.

2. Two-Color Fluorescence Southern Blotting

For Southern hybridization, we studied the simultaneous detection of two different DNA sequences using AMMB for PO and HNPP for APase. As shown in Table 1, PO was bound to the digoxigenin-labeled λDNA/EcoRI probes by the anti-digoxigenin antibody,
while the anti-fluorescein antibody APase conjugate was bound to the fluorescein-labeled ColEI/EcoRI and pBR322/EcoRI probes. Figure 2 (A) shows that the ADNA/EcoRI appeared as five yellow bands of 21, 7.4, 5.8/5.6, 4.9 and 3.5 kb, respectively, due to the enzymatic reaction between PO and AMMB, where the bands of 5.8 and 5.6 kb appeared as one unseparated band. And the mixture of the ColEI/EcoRI and pBR322/EcoRI appeared as two blue bands (6.6 and 4.4 kb), which were produced by the APase and HNPP enzymatic reaction. It is thus possible to simultaneously detect two different target DNAs as yellow and blue fluorescence by irradiation with ultraviolet light (UV, 302 nm).

3. Two-Color Fluorescence Western Blotting

For Western blotting, we examined the simultaneous detection of two different proteins using AMMB and HNPP on a nylon membrane. Both human albumin and immunoglobulin G (IgG) in human serum were targets of the two-color fluorescence detection method in Western blotting. The electrophoresed human serum on the

Figure 2. (A) Simultaneous detection of two different DNAs on a single membrane by Southern blotting. The ADNA/EcoRI appeared as five yellow bands and a mixture of ColEI/EcoRI and pBR322/EcoRI as two blue bands. The targets studied were ADNA/EcoRI consisting of six fragments (21 kb, 7.4 kb, 5.8 kb/5.6 kb, 4.9 kb, 3.5 kb: where kb stands for 10^3 base pairs) and a mixture of plasmids, ColEI and pBR322 (6.6 kb, 4.7 kb). Both plasmids were linearized by digestion with EcoRI. The ADNA/EcoRI was labeled with DIG-11-dUTP using a random primed labeling kit (Boehringer Mannheim, Mannheim, Germany), while the mixture of EcoRI-digested plasmids was labeled with fluorescein-11-dUTP. A mixture of 500 pg of ADNA/EcoRI and 100 pg each of linearized plasmids was electrophoresed, depurinated by 0.25 N HCl and denatured by 0.5 N NaOH, and subsequently transferred onto a nylon membrane (Biodyne® PLUS, 0.45 μm, Pall, Port Washington, NY, USA) using a conventional method. After fixation of DNA, pre-hybridization was carried out using DIG Easy Hyb (Boehringer Mannheim) for 30 min at 42 °C. Hybridization was then performed for 16 hr at 42 °C with a hybridization mixture of DIG-labeled DNA and fluorescein-labeled pBR322 and ColEI (50 ng/ml in DIG Easy Hyb). After post-hybridization washing and blocking, each membrane was immersed in a solution containing PO-conjugated anti-DIG antibody (Boehringer Mannheim) and APase-conjugated anti-fluorescein antibody (Vector Laboratories, Burlingame, CA, USA) for coupling for 30 min with hybrid DNAs at room temperature. Then, the membrane was treated with a substrate solution, consisting of 0.24 mM HNPP (Aisin Cosmos R&D, Kariya, Aichi, Japan), 1 mM AMMB, 100 mM Tris-HCl (pH 8.0), 100 mM NaCl, 10 mM MgCl2, 0.01% H2O2. After incubation for 2 hr at room temperature, the membrane was rinsed with distilled water and dried in the air. Yellow and blue signals appeared under ultraviolet light (302 nm) and recorded on 35 mm color film (Kodak) using a cut filter (400 nm). (B) Simultaneous detection of two different proteins on a single Western blot. Human albumin (65 kDa) appeared as yellow band, and the heavy chain of human IgG (50 kDa) appeared as blue band. As target proteins we used whole human serum (Organon Teknika-Cappel, Durham, NC, USA) containing human albumin and human immunoglobulin G (IgG). Primary antibodies to human albumin and human IgG were respectively mouse anti-human albumin monoclonal antibody (Cedarlane Laboratories, Hornby, ON, Canada) and rabbit anti-human IgG (whole) antibody (Organon Teknika-Cappel). The secondary antibody to the mouse monoclonal antibody and the rabbit polyclonal antibody were respectively goat anti-mouse IgG (H+L) antibody-PO conjugate (Bio-Rad, Hercules, CA, USA) and goat anti-rabbit IgG antibody-APase conjugate (Bio-Rad). The substrates for APase and PO were HNPP and AMMB, respectively. Whole human serum containing 125 ng of total protein was diluted in a solution of 50 mM Tris-HCl (pH 6.8), 2% SDS, 6% β-mercaptoethanol, 10% glycerol (1× sample buffer) and electrophoresed on a 10% SDS-polyacrylamide gel with 25 mM Tris-HCl, 192 mM glycine, 0.1% SDS. After electrophoresis, proteins were electro-blotted onto a nylon membrane (Biodyne® PLUS, 0.45 μm, Pall) at 60 V for 3 hr using an electrophoresis solution (25 mM Tris-HCl, 192 mM glycine and 20% methanol). The membrane was then washed two times for 5 min each with phosphate-buffered saline (PBS), followed by blocking with 1% Blocking reagent (Boehringer Mannheim), 100 mM maleic acid (pH 7.5), and 150 mM NaCl. Subsequently, the membrane was immersed in PBS containing mouse anti-human albumin monoclonal antibody (1:5000) and rabbit anti-human IgG (whole) antibody (1:5000) for 1 hr at room temperature, followed by rinsing with PBS. The membrane was then immersed in PBS containing PO-conjugated goat anti-mouse IgG (H+L) antibody (1:5000) and APase-conjugated goat anti-rabbit IgG antibody (1:5000) for 1 hr at room temperature, followed by rinsing with PBS. Finally, the membrane was incubated with the substrate solution containing two fluorogenic substrates (HNPP and AMMB) for 2 hr at room temperature. After the enzymatic reaction, the membrane was washed with distilled water for 10 min and dried at 50 °C. Fluorometric detection was carried out under UV light (302 nm), and the result was recorded as described previously.
SDS-polyacrylamide gel was electro-transferred onto a nylon membrane. Human albumin and IgG were detected following the scheme in Table 2. Figure 2 (B) shows a yellow human albumin band (65 kDa) produced by the PO and AMMB reaction, and a blue human IgG band (50 kDa) produced by the APase and HNPP reaction. The band of human IgG was a heavy chain fragment produced by the action of a reducing agent in the loading buffer. Yellow and blue bands are clearly distinguishable, and thus two different proteins can be simultaneously detected on a single blotting membrane.

Interestingly, a mixture of PO-conjugated antibodies catalyzed with AMMB (yellow) and APase-conjugated antibodies catalyzed with HNPP (blue) was visualized as a mixture of both fluorochromes resulting in green fluorescence (data not shown). Therefore, three-color Southern or Western blotting might also be available using this method.

Although PVDF membranes are often used in Western blotting, signals produced by AMMB did not fluoresce on a PVDF membrane, which might be due to concentration quenching. However, such quenching did not occur using a nylon membrane by the PO and AMMB enzymatic reaction. Moreover, after the nylon membrane was dried, further intense AMMB fluorescent signals were obtained.

We further noticed that bromophenol blue (BPB) should not be used as marker in electrophoresis, due to its red fluorescence emittance under UV light resulting in background noise on the membrane.

The present novel AMMB substrate can be used in a two-color fluorescence detection method for Southern and Western blottings on a single blot membrane with high efficiency. This method requires only one-step incubation of mixed solution of HNPP and AMMB with APase and PO, demonstrating the simplicity and rapidity of the two-color hybridization method on nylon membranes.

In addition, the present two-color method may also be applicable for in situ hybridization histochemistry and cytochemistry. Recently, simultaneous detection of mRNA and proteins in early embryogenesis of Drosophila was demonstrated using fluorescein isothiocyanate and HNPP.12 Although the present AMMB/HNPP two-color method has not yet been investigated for in situ hybridization, the usefulness of the method described in this paper for the visualization of in situ hybridization is currently under study.

References

1. Pinkel, D., Straume, T., and Gray, J. W. 1986, Cyto- genetic analysis using quantitative, high-sensitivity, fluorescence hybridization, Proc. Natl. Acad. Sci. USA, 83, 2934–2938.
2. Bronstein, L., Edwards, B., and Voyta, J. C. 1989, 1,2- Dioxetanes: novel chemiluminescent enzyme substrates. Applications to immunoassays, J. Biolumin. Chemilum., 4, 99–111.
3. Domingo, A. and Marco, R. 1989, Visualization under ultraviolet light enhances 100-fold the sensitivity of peroxidase-stained blots, Anal. Biochem., 182, 176–181.
4. Hsu, U. M. and Soban, E. 1982, Color modification of diaminobenzidine (DAB) precipitation by metallic ions and its application for double immunohistochemistry, J. Histochem. Cytochem., 30, 1079–1082.
5. Leary, J. J., Brigati, D. J., and Ward, D. C. 1983, Rapid and sensitive colorimetric method for visualizing biotin-labeled DNA probes hybridized to DNA or RNA immobilized on nitrocellulose: bio-blots, Proc. Natl. Acad. Sci. USA, 80, 4045–4049.
6. Cano, R. J., Torres, M. J., Klein, R. E., and Palomes, J. C. 1992, DNA hybridization assay using ATTOPHOS™, a fluorescent substrate for alkaline phosphatase, BioTechniques, 12, 264–269.
7. Kagiyama, N., Fujita, S., Momiyama, M., Saito, H., Shirahama, H., and Hori, S. H. 1992, A fluorescent detection method for DNA hybridization using 2-hydroxy-3-naphthoic acid-2'-phenylanilide phosphate as a substrate for alkaline phosphatase, Acta Histochem. Cytochem., 25, 467–471.
8. Larison, K. D., BreMiller, R., Wells, K. S., Clements, I., and Haagland, R. P. 1995, Use of a new fluorogenic phosphatase substrate in immunohistochemical applications, J. Histochem. Cytochem., 43, 77–83.
9. Fujita, S., Toru, T., Kondoh, Y., Momiyama, M., Kagiyama, N., and Hori, S. H. 1997, A novel fluorogenic substrate for the use of nucleic acid hybridization, Acta Histochem. Cytochem., 30, 165–172.
10. Ferrer, A. S., Santema, J. S., Hilhorst, R., and Visser, A. J. W. G. 1990, Fluorescence detection of enzymatically formed hydrogen peroxide in aqueous solution and in reversed micelles, Anal. Biochem., 187, 129–132.
11. Zaitsev, K. and Ohkura, Y. 1980, New fluorogenic substrates for horseradish peroxidase: rapid and sensitive assays for hydrogen peroxide and the peroxidase, Anal. Biochem., 109, 109–113.
12. Goto, S. and Hayashi, S. 1997, Cell migration within the embryonic limb primordium of Drosophila as revealed by a novel fluorescence method to visualize mRNA and protein, Dev. Genes Evol., 207, 194–198.