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Quantitative Western blot analysis and spot immunodetection using time-resolved fluorometry

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We describe a new method of staining and quantification of proteins blotted or spotted on nitrocellulose. Blotted or spotted proteins are first reacted with specific antibodies followed by reaction with biotinylated secondary antibodies. The immunocomplex is then reacted with a streptavidin-based macromolecular complex labeled with the fluorescent europium chelate of 4,7-bis(chlorosulphophenyl) 1,10-phenanthroline-2,9-dicarboxylic acid (BCPDA). The fluorescent spots or bands can then be assessed by visual inspection under UV illumination, by instant photography or quantified by scanning with a time-resolved fluorometer. The method does not involve enzyme detection, is simple, sensitive and gives sharp bands which remain fluorescent for long periods of time (months to years).

Key words: Quantitative Western blot analysis; Time-resolved fluorometry; Europium chelate; Non-isotopic detection

Introduction

Western blot analysis is a widely used technique for the characterization of proteins. It involves protein separation by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and subsequent transfer of the separated proteins to a solid support like nitrocellulose or nylon by electroelution (Towbin et al., 1979; Towbin and Gordon, 1984).

The transferred proteins are then detected on the support by using specific antibodies and a suitable detection system. Some detection systems include radionuclides like ¹²⁵I, enzymes, fluorescent, chemiluminescent or colorimetric probes (Burnett, 1981; Blake et al., 1984; Hsu, 1984; Diamandis, 1990; Fernandez and Kopchick, 1990; Gillespie and Hudspeth, 1991).

Time-resolved fluorometry, with europium chelates as labels, is an established immunoassay technique (Diamandis, 1988; Diamandis and Christopoulos, 1990). The Delfia time-resolved fluorescence immunoassay method uses Eu³⁺ as the immunological label. Although this system has been applied in some non-immunological applications, i.e., dot-blot nucleic acid hybridization (Syvanen et al., 1986; Dahlen, 1987; Sund et al., 1988) it is not suitable for either Southern, Northern or Western blot analysis because the final measurement is carried out in solution after

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Abbreviations: SBMC, streptavidin-based macromolecular complex; BCPDA, 4,7-bis(chlorosulphophenyl) 1,10-phenanthroline-2,9-dicarboxylic acid; BSA, bovine serum albumin; GAMIg, goat anti-mouse immunoglobulin; RAGIg, rabbit anti-goat immunoglobulin; AFP, α -fetoprotein; CEA, carcinoembryonic antigen; HSA, human serum albumin.

Eu³⁺ extraction. We have recently described a streptavidin-based macromolecular complex (SBMC) multiply labelled with the europium chelate of 4,7-bis(chlorosulphophenyl) 1,10-phenanthroline-2,9-dicarboxylic acid (BCPDA) (Morton and Diamandis, 1990). This reagent was used successfully for highly sensitive immunological assays (Christopoulos et al., 1990) and for nucleic acid hybridization assays (Christopoulos et al., 1991). The advantage of the above reagent, which is used in combination with biotinylated antibodies or nucleotides is that the final fluorescent complex is measured on the solid phase thus being suitable for either spot immunodetection or Western blot analysis. In this paper we describe the first application of the SBMC for Western blot analysis and dot immunodetection. The final fluorescent complex can be seen under UV illumination, photographed with an instant camera or scanned on a time-resolved fluorometric scanner for quantitation. This new method is sensitive, non-isotopic and quantitative and may find many applications in biotechnology.

Materials

Instrumentation

For protein electrophoresis and electroelution, we have used the Mini-Protean II electrophoresis cell and Mini Trans-Blot electrophoretic transfer cell from Bio-Rad Laboratories, Richmond, CA 94804. The system was used according to the manufacturer's instructions. For nitrocellulose fluorometric scanning, we have used the CyberFluor 615 Immunoanalyzer, a time-resolved fluorometer. This instrument was modified so that it can be used as a high-resolution scanner as described elsewhere (Christopoulos et al., 1991).

Reagents and solutions

The streptavidin-based macromolecular complex (SBMC) stock solution (15 µg/ml in streptavidin) and its diluent were obtained from CyberFluor, Toronto, Canada. The SBMC is formed by incubating at 55°C, a precise amount of Eu³⁺ with (a) the conjugate of streptavidin with BCPDA-labeled thyroglobulin and (b) with free BCPDA-labeled thyroglobulin, as described else-

where (Morton and Diamandis, 1990). The SBMC is composed of one molecule of streptavidin, about three molecules of thyroglobulin and about 480 molecules of the chelate of Eu³⁺ with BCPDA. Its approximate molecular weight is 3×10^6 . The working SBMC solution was prepared just before use by diluting the stock solution 50-fold in its diluent. For membranes $\sim 6.5 \times 10$ cm, we use ~ 50 ml of working staining solution. The wash solution concentrate was prepared by dissolving 900 g of NaCl, 60 g of Tris base and 50 ml of Tween 20 in 4 liters of water and adjusting the pH to 7.80 with concentrated HCl. The working wash solution was prepared by diluting the concentrate five-fold in water. A general blocking/diluent solution is a 6% (w/v) bovine serum albumin (BSA) solution in a 50 mM Tris buffer of pH 7.80.

The antigens α -fetoprotein (AFP), carcinoembryonic antigen and ferritin were from Scripps Laboratories, San Diego, CA 92131. All other antigens were from Sigma Chemical Co., St. Louis, MO. All antibodies used are available from CyberFluor. Affinity purified goat anti-mouse and rabbit anti-goat antibodies were from Jackson Immunoresearch, West Grove, PA 19390. Sulfosuccinimidyl 6-(biotinamido) hexanoate (NHS-LC-biotin) purchased from Pierce Chemical Co., Rockford, IL was used for all biotinylations as described elsewhere (Diamandis et al., 1989). The monoclonal anti-digoxigenin antibody (code 45-20) is described elsewhere (Mudgett-Hunter et al., 1982, 1985) and obtained from Dr. M. Mudgett-Hunter. The digoxigenin labelling reagent, digoxigenin-3-*o*-methylcarbonyl- ϵ -aminocaproic acid-*N*-hydroxysuccinimide was from Boehringer-Mannheim Canada, Montreal, PQ. Biotinylated protein mol. weight markers were obtained from Pierce and Sigma. Acrylamide, bisacrylamide, *N,N,N',N'*-tetramethylethylenediamine (TEMED) and ammonium persulfate were from Bio-Rad. All other chemicals were from Sigma.

Membranes

We routinely used supported nitrocellulose, Hybond-C-Extra, from Amersham International, Arlington Heights, IL 60005. Supported and native nitrocellulose membranes supplied by other

companies gave comparable results. Nylon membranes also work but they exhibit about 3–5 times higher background signals. Cationized nylon membranes are unsuitable because they exhibit very high background signals.

Methods

Protein electrophoresis and electroelution

We have prepared 10% polyacrylamide minigels and run them under reducing conditions (mercaptoethanol) at 200 V for approximately 45 min. Electroelution was performed at 100 V for 90 min.

Spot immunodetection of biotinylated antibody

Affinity purified goat anti-mouse immunoglobulin (GAMiG) was biotinylated with NHS-LC-biotin as previously described (Diamandis et al., 1989). The biotinylated GAMiG was then diluted in a 50 mM Tris buffer, pH 7.40, containing 10 μ g/ml of bovine serum albumin (BSA) as carrier protein. All pipet tips and glass tubes were blocked with 1% BSA solution and washed with H₂O, to avoid any loss of protein. Nitrocellulose membranes were wetted in the above Tris buffer not containing BSA just before use. Membrane strips, approximately 9 \times 0.8 cm were cut and spotted with 1 μ l of protein solution. About 6–8 different protein concentrations could be spotted per strip. After spotting, the strips were dried at 45°C under vacuum for 1 h. The strips were then blocked by incubation for 1 h to overnight, at room temperature, with continuous rotary mixing, in tubes containing 6% (w/v) BSA.

Detection of the spotted biotinylated GAMiG was accomplished by incubating the strips in the working SBMC solution for 3–4 h with continuous rotary mixing, at room temperature. The strips were then washed \times 3 with the wash solution. The strips were further soaked for 1 h in the above solution at RT (optional) with continuous rotary mixing. The strips were then dried with a stream of hot air (hair dryer). The fluorescence on the strips was detected as described later under ‘membrane visualisation and scanning’.

Spot immunodetection of mouse IgG

A stock mouse IgG solution (1 mg/ml) was diluted, spotted and the membrane processed as described above up to the point of blocking. After blocking, the strips were immersed into 15 ml tubes filled with biotinylated GAMiG (stock biotinylated GAMiG was 1 mg/ml; it was diluted 2000-fold in a 6% BSA solution) and incubated for 1–2 h with continuous rotary mixing. After washing \times 3 with the wash solution, the dots were detected with the SBMC and further processed exactly as described under the previous heading.

Spot immunodetection of α -fetoprotein, ferritin and carcinoembryonic antigen

Stock solutions of human α -fetoprotein (AFP), ferritin and carcinoembryonic antigen (CEA) were diluted, spotted and the membrane strips processed as described under “spot immunodetection of biotinylated antibody”. After blocking, the strips were immersed into 15 ml tubes filled with detection antibodies as follows: For AFP, we used either a monoclonal mouse anti-AFP antibody (code 5108) or a goat affinity purified polyclonal anti-AFP antibody. For ferritin, we used a mouse monoclonal anti-ferritin antibody (code A027) and for CEA a monoclonal mouse anti-CEA antibody (code 5914). All antibodies are available from CyberFluor and they were used at a working concentration of 500 ng/ml in 6% BSA diluent. Incubation was for 1–2 h at RT with continuous rotary mixing. After washing \times 3 with the wash solution, the strips were incubated for 1–2 h in 15 ml tubes filled with biotinylated GAMiG (500 ng/ml in 6% BSA) or with biotinylated rabbit anti-goat immunoglobulin (RAGiG) (500 ng/ml in 6% BSA) for the polyclonal anti-AFP antibody. After washing \times 3 as above, the dots were detected with the SBMC and further processed exactly as described under “spot immunodetection of biotinylated antibody”.

Western blot analysis of digoxigenin-labeled proteins

Bovine IgG, ovalbumin, bovine serum albumin and lysozyme were labeled with digoxigenin as follows. We prepared a 1 mg/ml solution of the proteins in 0.1 M carbonate buffer, pH 9.1 and added per mg of protein, \sim 0.2 mg of the *N*-hy-

droxysuccinimide ester of digoxigenin dissolved in 20 μ l of dimethylsulfoxide. After incubation for 2 h at room temperature, the labeled proteins were electrophoresed on SDS-PAGE under reduced conditions and transferred to nitrocellulose as described above. Detection on nitrocellulose was accomplished by reaction with the biotinylated monoclonal anti-digoxigenin antibody followed by staining with the SBMC, under the principles described above. The biotinylated monoclonal anti-digoxigenin antibody stock solution (1 mg/ml) was diluted 5000-fold in a 6% BSA diluent and incubated for 1 h at room temperature with continuous shaking.

Western blot analysis of proteins

Mouse IgG, human serum albumin (HSA), somatotropin and α -fetoprotein were loaded on minigels and separated by electrophoresis under reduced conditions as described above. These proteins were then transferred to nitrocellulose by electroelution. The proteins were visualized on the nitrocellulose membranes by reaction with primary antibody, i.e., biotinylated polyclonal goat anti-mouse IgG, non-biotinylated monoclonal anti-HSA, non-biotinylated monoclonal anti-somatotropin or non-biotinylated goat polyclonal anti-AFP, respectively. After washing, a biotinylated GAMIg was added (for HSA and somatotropin) or biotinylated RAGIg (for AFP). All proteins were then visualized with the SBMC as described above. All detection antibodies were dissolved in the 6% BSA diluent.

Membrane visualization and scanning

The finished nitrocellulose membranes can be evaluated in three different ways: (a) by visual observation under UV illumination using a conventional transilluminator (in this case and in case (b) below, the side of the membrane containing the immunospots is put facing down); (b) the membrane can be photographed with a conventional Polaroid instant camera in a manner identical to that of photographing ethidium bromide stained gels. Filters used are the same (a cutoff filter for wavelengths shorter than 350 nm and a bandpass filter absorbing between 380 nm and 580 nm) but exposure time must be optimized; we found that best results could be obtained with

10–13 s exposures; (c) the membrane can be scanned on the modified Cyberfluor 615 Immunoanalyzer to obtain a quantitative measure of the fluorescence intensity of the bands or spots. In this case the side of the membrane containing the immunospots is facing up.

Results and discussion

The streptavidin-based macromolecular complex, multiply labelled with the europium chelate of BCPDA is a sensitive, stable and easy to use general reagent for diverse applications where biotinylated complementary reagents are used. Up to now, the SBMC has primarily been used for immunological assays and nucleic acid hybridization assays. Here, we apply the same reagent for Western blot analysis. To achieve quantification, we have previously modified the CyberFluor 615 Immunoanalyzer so that it can scan nitrocellulose membranes and quantify the fluorescent spots and bands (Christopoulos et al., 1991).

In Fig. 1 we detect biotinylated goat anti-mouse IgG immobilized on supported nitrocellulose. The instrument can detect down to approx. 25 pg of biotinylated antibody. Precision studies revealed that CVs of 10–15% can be achieved with this method, in the range of 400–10,000 pg/ 1μ l spot of biotinylated antibody. In Fig. 2 we present similar data for the spot immunodetection of unlabeled mouse IgG which is first reacted with biotinylated GAMIg and then visualized with the SBMC. In this case, the sensitivity of detection is ~ 10 pg of mouse IgG. In both Fig. 1 and 2, there is a near-linear relationship between the fluorescence measured by the instrument and the amount of spotted protein. Other similar experiments for the spot immunodetection of α -fetoprotein, carcinoembryonic antigen and ferritin have also been performed. About 5–10 pg or more of these proteins can be detected. Data for alpha-fetoprotein are shown in Fig. 3.

We have further analyzed, by Western blot, proteins covalently linked to digoxigenin. The detection was accomplished with a biotinylated anti-digoxigenin antibody followed by the SBMC. Partial data are shown in Fig. 4. Although there

is significant smearing in these blots, the major bands can be seen, visualized close to the position predicted by their mol. weights. The digoxigenin-labeled proteins tested are bovine IgG, ovalbumin (shown in Fig. 4), bovine serum albumin and lysozyme (data not shown).

Some other unlabeled proteins (mouse IgG, human serum albumin, α -fetoprotein and somatotropin) could also be visualized on Western blots by use of monoclonal or polyclonal primary antibodies and biotinylated polyclonal detection

antibodies. Partial data for mouse IgG are shown in Fig. 5. Single, sharp bands are obtained with the proposed method. In one case, involving Western blot analysis of α -fetoprotein, multiple bands were visualized, presumably due to the presence of more than one immunoreactive protein form.

Comparative Western blot analysis was carried out using similar methods but instead of using the SBMC for visualization, we used a streptavidin alkaline phosphatase conjugate. Visualization of

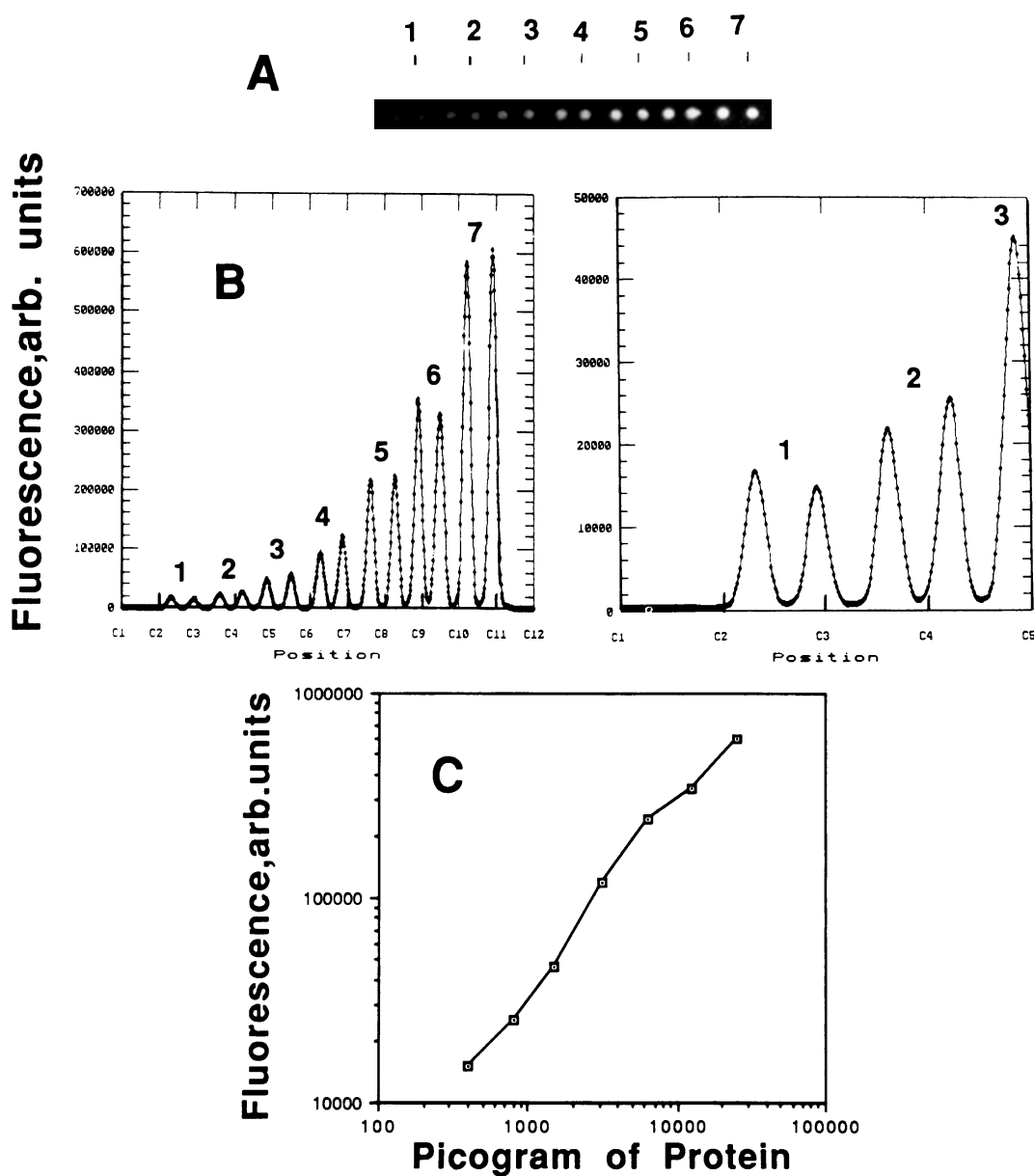


Fig. 1. Detection of biotinylated goat anti-mouse immunoglobulin (GAMiG) spotted to nitrocellulose ($1 \mu\text{l}/\text{spot}$, in duplicate) with the streptavidin-based macromolecular complex (SBMC) labeled with the europium chelate of BCPDA. The amount of spotted protein (pg) was 400 (1); 800 (2); 1500 (3); 3100 (4); 6250 (5); 12,500 (6) and 25,000 (7). *A*: instant photograph of the membrane with a Polaroid camera under UV illumination. *B*: time-resolved fluorometric scan of the whole strip (left) or of the area up to 1500 pg (right). *C*: a plot of fluorescence vs. amount of protein spotted, revealing a near linear relationship. The detection limit of biotinylated GAMiG is around 25 pg, when the instrument is used for detection. Background signal is less than 1000 arbitrary units.

bands was then accomplished with the chromogenic BCIP-NBT reagent. The comparative data suggested that our new method has similar sensitivity on Western blots with the BCIP-NBT method (color development time $\sim 1-2$ h). We also confirmed that in the case of digoxigenin-labeled proteins, the smearing effect was also present to the same extent when staining was done with the BCIP-NBT method.

One of the limitations of the Western blot technique is that the proteins on nitrocellulose are denatured and it may be difficult for monoclonal and to a lesser degree polyclonal antibod-

ies to recognize them. This was evident in the analysis of human serum albumin where the signal obtained was weaker in comparison to the other proteins detected.

In recent years, a number of non-isotopic methodologies suitable for Western blot analysis have been reported. Some of these methods are based on either chemiluminescence or enhanced luminescence and are more sensitive than the method described here. However, the proposed method is simpler, does not involve exposure on X ray films and the fluorescence emitted can be quantified with a commercially available instru-

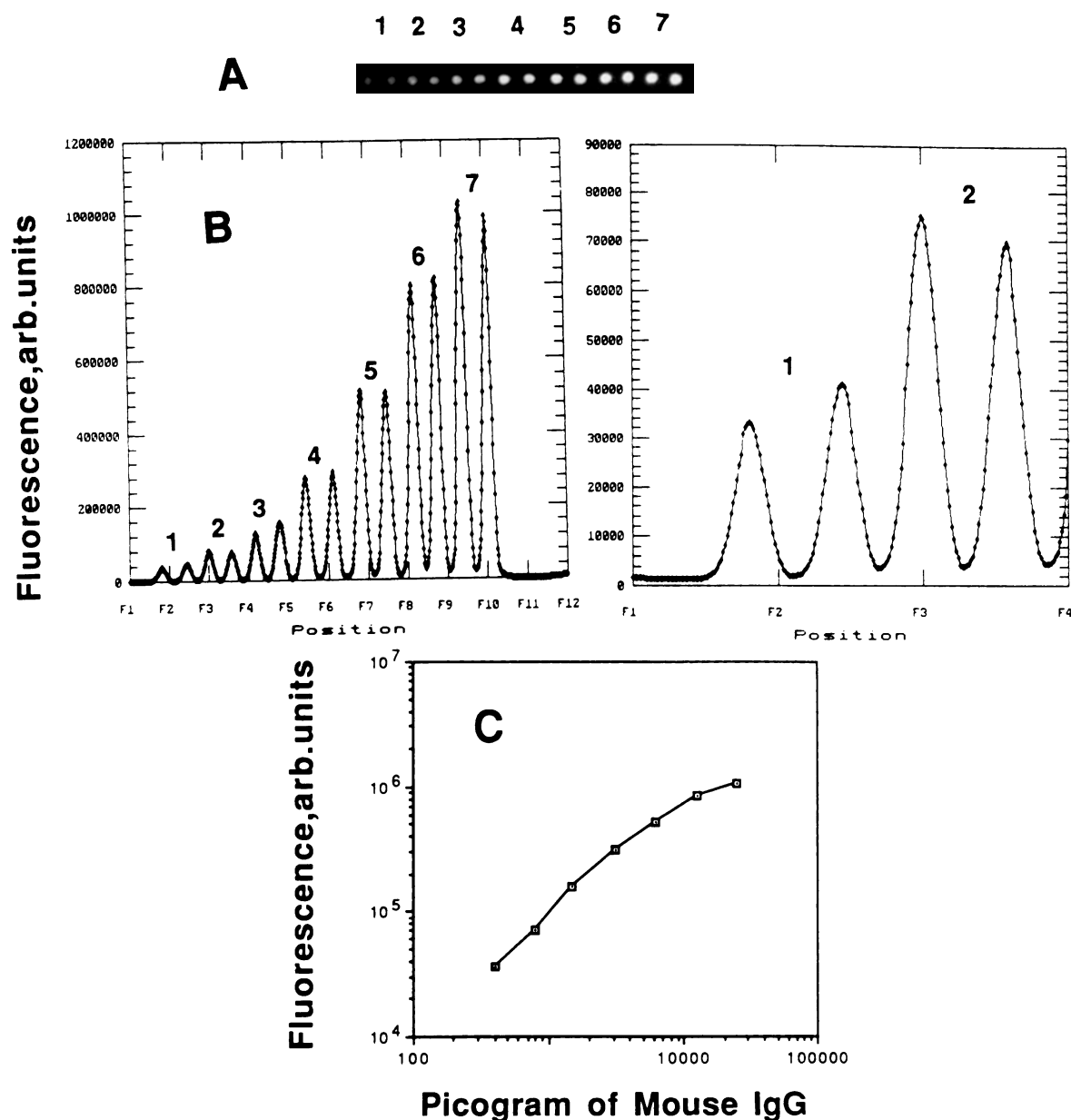


Fig. 2. Immunodetection of mouse IgG spotted on nitrocellulose ($1 \mu\text{l}/\text{spot}$, in duplicate). Immobilized mouse IgG was detected by reaction with biotinylated GAMiG and then visualized with the SBMC. For more details see methods section. The amount of spotted protein (pg) was 400 (1); 800 (2); 1,500 (3); 3100 (4); 6250 (5); 12,500 (6) and 25,000 (7). See legend of Fig. 1 and text for other details.

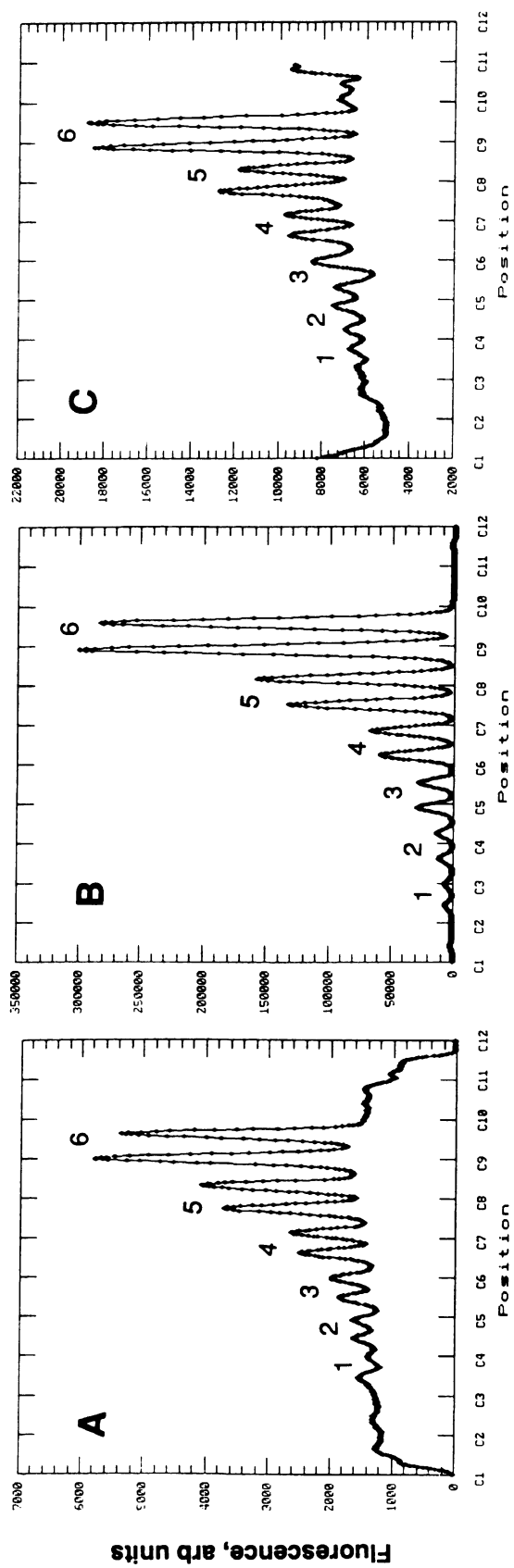


Fig. 3. Time-resolved fluorometric scan of nitrocellulose strips spotted ($1 \mu\text{l}$) with α -fetoprotein (AFP) and then reacted with a mouse monoclonal (A, B) or goat polyclonal (C) anti-AFP antibody, followed by biotinylated goat anti-mouse immunoglobulin (A, B) or biotinylated rabbit anti-goat immunoglobulin (C). The spots were then visualized with the SBMC as described under "spot immunodetection of α -fetoprotein". The amount of AFP spotted (pg) was 5 (1); 10 (2); 20 (3); 40 (4); 80 (5); 160 (6) in cases A and C and 312 (1); 625 (2); 1250 (3); 2500 (4); 5000 (5); 10,000 (6); in case B.

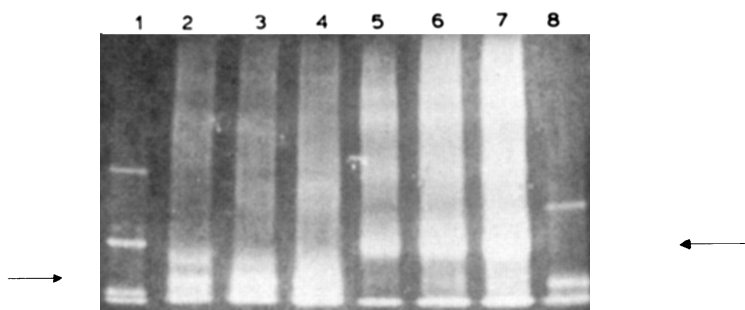


Fig. 4. Western blot analysis of digoxigenin-labeled proteins. Lane 1: biotinylated mol. weight markers (Sigma) with M.W. 97,400 (phosphorylase *b*) 58,100 (catalase), 39,800 (alcohol dehydrogenase) and 29,000 (carbonic anhydrase). Lane 8: biotinylated mol. weight markers (Pierce) with M.W. of 81,000 (transketolase), 40,500 (creatine phosphokinase) and 29,000 (phosphoglycerate mutase). Lanes 2, 3, 4: digoxigenin-labeled ovalbumin (M.W. ~ 45,000) with loadings per lane of 60, 250 and 1000 ng, respectively. The position of the major band is indicated by an arrow on the left side. Lanes 5, 6, 7: digoxigenin-labeled bovine IgG (MW ~ 160,000) with loadings per lane of 60, 250 and 1000 ng, respectively. The position of the major band (monomeric heavy chain) is indicated by an arrow on the right side. All samples were run under reduced conditions. Detection was with biotinylated anti-digoxigenin antibody followed by the streptavidin-based macromolecular complex. Markers were 1 μ g total protein per lane.

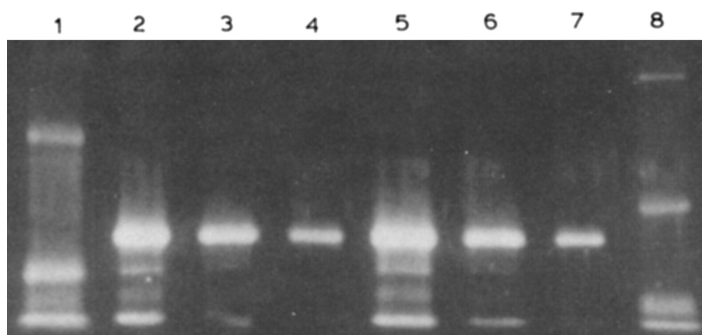


Fig. 5. Western blot analysis of mouse IgG (M.W. ~ 160,000). Lanes 1 and 8 contain 1 μ g of biotinylated M.W. markers from Pierce and Sigma, respectively (see also Fig. 4). Lanes 2, 3, and 4 contain 1000, 250 and 60 ng of mouse IgG, respectively. Lanes 5, 6 and 7 are duplicates of lanes 2, 3 and 4, respectively. For more details on detection see text.

ment. Additionally, the method gives sharp bands and does not involve use of enzyme substrates. We expect that this method, which is the first that utilize fluorescent europium chelates as labels for Western blot analysis will find applications in the field of protein characterization and serve as a prototype for the development of even more sensitive fluorescence detection techniques.

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