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Simultaneous immunoblotting analysis with activity gel electrophoresis in a single polyacrylamide gel

We describe here that a simple diffusion blotting method can couple immunoblotting analysis with another biochemical technique in a single polyacrylamide gel. The efficiency of protein transfer was evaluated by serial dilutions of nephrosin, a metalloproteinase of the astacin family, and by immunodetection. It is estimated that diffusion blotting produces 25–50% of the signal intensity compared to the classical electrophoretic transfer method. However, with diffusion blotting it is possible to generate several replicas from a single gel. In addition, a protein blot can be obtained from a sodium dodecyl sulfate (SDS)-polyacrylamide gel for zymography assay or from a native polyacrylamide gel for electrophoretic mobility shift assay (EMSA). In this regard, a particular signal in zymography or EMSA can be confirmed by simultaneous immunoblotting analysis with a corresponding antiserum. Therefore, diffusion blotting allows a direct comparison of signals between gels and replicas in zymography assay and EMSA. These advantages make diffusion blotting desirable when partial loss of transfer efficiency can be tolerated or be compensated by a more sensitive immunodetection reaction using enhanced chemiluminescence substrates.

Keywords: Western blotting / Zymography / Multiple blotting / Proteinase / Electrophoretic mobility shift assay

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1 Introduction

Electrophoresis in polyacrylamide gels is a widely used technique for protein analysis due to the high resolving power and the large variety of modifications suitable for different purposes. After electrophoretic separation, proteins can be transferred onto a solid matrix and detected by a specific antiserum, a procedure named immunoblotting [1, 2]. Several techniques have been developed for the transfer of proteins (blotting), *e. g.,* diffusion blotting, capillary blotting and electrophoretic blotting [3]. Diffusion blotting is originally developed for isoelectric focusing gels or ultrathin gels [4–8], which requires laying a blotting membrane on the gel surface and a stack of dry filter paper on top of the blotting membrane. Usually a glass plate and an object carrying certain weight are further stacked on the filter to facilitate the diffusion process. Capillary blotting is very similar to diffusion blotting [9]. However, the diffusion process in capillary blotting is driven by unidirectional solvent movement. Electrophoretic blotting, either by tank buffer transfer or by semidry transfer, is most widely used in immunoblotting analysis, particularly after SDS-PAGE. The main advantage of electrophoretic transfer is the high transfer efficiency. However, for certain applications such as electrophoresis on ultrathin gels or gels bound on plastic sheets [5–7], and multiple blotting from a single gel [4, 8, 10], diffusion blotting and capillary blotting are more suitable.

In activity gel electrophoresis, visualization of protein bands is assisted by the biochemical activity associated with the proteins [11]. For example, hydrolytic enzymes such as proteases and nucleases can be renatured *in situ* after SDS-PAGE in substrate-containing gels. The hydrolytic enzymes are then revealed in the gel by the disappearance of substrate in discrete areas within the gels after substrate staining. This technique is also called zymography assay. Another important example of activity gels is gel retardation assay or electrophoretic mobility shift assay (EMSA) designed to study the interaction between DNA and protein [12, 13]. In the activity gel electrophoresis, several positive signals are frequently observed, which requires an additional immunoblotting analysis in another gel to examine a particular protein involved. In this manuscript, we demonstrate that a protein blot can be obtained from the activity gel by diffusion blotting onto a PVDF membrane and be processed for immunoblotting analysis. The remaining gel after blotting can then be used for the routine "activity staining".

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Abbreviations: AP, alkaline phosphatase; **EMSA**, electrophoretic mobility shift assay; **HRP**, horseradish peroxidase

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Because the blot and the activity staining are derived from the same gel, the localization of signals in the gel and the replica can be easily aligned for comparison.

2 Materials and methods

2.1 Gel electrophoresis

SDS-PAGE was performed using a Tris-Tricine system as described previously [14]. The gel concentration was 7.5%, and the bisacrylamide to acrylamide ratio was 6. For proteolytic zymography assay, Tricine SDS-polyacrylamide gel was prepared with 0.2% gelatin in the separating gel. After diffusion blotting, the gel was incubated at 28°C with 2% Triton X-100 in 20 mM Tris-HCl, pH 8.0, Tris-HCl buffer, and Tris-HCl buffer containing 0.1 mM $ZnCl_2$, each for 90 min. The gel was then stained with 0.2% Coomassie Brilliant Blue R-250 in 40% methanol, 7% acetic acid and destained with 30% methanol/7% acetic acid. For silver stain, the simplified Merril's procedures [15] were adapted in this study.

2.2 Diffusion blotting

PVDF membrane was soaked in methanol for 10 min, rinsed with deionized water for several times and kept in deionized water until use. Immediately after electrophoresis, excess cathode buffer was removed, the top glass plate was lifted and the stacking gel was removed by a razor blade. The separating gel was left on the bottom plate. A piece of PVDF membrane was pressed between two sheets of paper tower with investigator's hands to absorb excess water and the membrane was then placed carefully onto the separating gel to avoid air bubbles. The PVDF membrane was left on the gel for 1 h at room temperature to allow diffusion to occur. If a second blot was necessary, the separating gel was blotted with a second piece of PVDF membrane for another hour. After diffusion blotting, the remaining gel was subjected to silver staining, proteolytic zymography assay, or ther applications. For comparison, tank electrophoretic transfer was conducted at 50 V for 4 h using the transfer buffer containing 50 mM Tris, 380 mM glycine, 0.1% SDS and 20% methanol.

2.3 Immunodetection

Immunoblotting analysis of nephrosin was carried out using guinea pig anti-nephrosin antiserum (1:2000) and horseradish peroxidase (HRP)-conjugated second antibody (1:1000) after transfer to PVDF membrane [16]. Immunoreactive bands were detected by the $NiCl_2$ enhancement method [17].

2.4 EMSA

To prepare the NF*K*B subunit (p50)-enriched nuclear extracts, the human 293 cells were transfected with the p50 expression plasmid, pRcCMVp50, by calcium phosphate coprecipitation [18]. Nuclear extracts were prepared as described by Dignam *et al*. [19]. For EMSA, end-labeled oligonucleotide FPIII probe (0.5 ng; (0.5–1) $\times$ 10^5 cpm) were incubated in the binding reaction buffer (10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 0.05% NP-40, 0.5 mM DTT, and 10% glycerol) containing 1 μg of poly(dI-dC) and 12 μg of p50-enriched nuclear extracts. After incubation at room temperature for 20 min, the reaction mixtures were analyzed by electrophoresis on 5% nondenaturing polyacrylamide gels. For competition, unlabeled fragments were used at an 100-fold molar excess and incubated with nuclear extracts for 20 min on ice before adding the radiolabeled probe. For the supershift assay, an antibody against p50 was preincubated with nuclear extracts for 20 min on ice before the addition of the radiolabeled probe. The same binding conditions described above were also used for EMSA with the unlabeled FPIII fragment. After electrophoresis, the gel was diffusion-blotted onto a PVDF membrane. The gel was then dried and analyzed by autoradiography. On the other hand, the membrane was incubated with a rabbit polyclonal p50-specific antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and subsequently an alkaline phosphatase (AP)-conjugated goat anti-rabbit IgG secondary antibody. The detection of immunocomplex was visualized using CDP-Star as a chemiluminescent substrate (Tropix, Foster City, CA). Oligonucleotides used in EMSA were as follows, FPIII: 5'-GCCTGGGAAAAAACTC; m8: 5'-GGGCACTGTGGGAACGGAAA; NFκB: 5'-CAGAGG GGACTTTCCGAGAG.

3 Results

We have used nephrosin, a 23-kDa metalloproteinase purified from carp head kidney [16], to determine the immunodetection sensitivity of protein blots derived from both the classical electrophoretic transfer and the simple diffusion blotting. Ten ng of carp nephrosin and its 2 × serial diluents were applied to SDS-PAGE and transferred onto PVDF membranes. For diffusion blotting, two consecutive replicas were made to evaluate the transfer efficiency. Immunoblotting analysis with the DAB/$NiCl_2$/H_2O_2 color reaction can detect the presence of nephrosin down to 0.156 ng (lane 7 in Fig. 1A) using the electrophoretic transfer. By diffusion blotting, down to 0.156 ng (lane 7 in Fig. 1B) and 0.625 ng (lane 5 in Fig. 1C) of nephrosin were detected on the first and second replicas, respectively. Therefore, the first diffusion blot exhibited

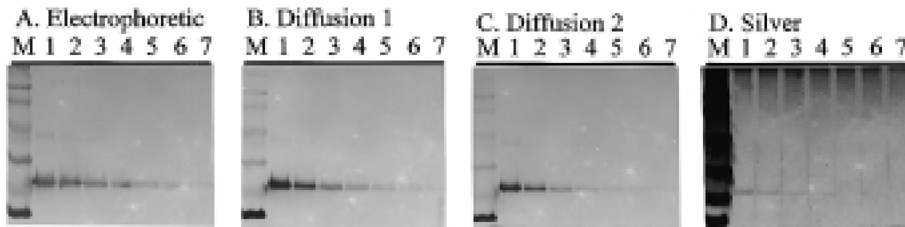

Figure 1. Immunoblotting analysis and silver staining of purified carp nephrosin. Ten ng of carp nephrosin (lane 1) and its 2 × serial diluents (lanes 2–7) were applied to SDS-PAGE. After electrophoresis, the proteins were blotted by (A) electrophoretic tank transfer, (B, D) first diffusion transfer, or (C) second diffusion transfer after the first diffusion transfer. Protein blots from (A, B, C) were subjected to immunoblotting analysis using an anti-nephrosin antiserum and HRP-conjugated second antibodies. (D) Remaining gel after diffusion blotting was stained by Merril's silver staining procedure. M, prestained markers of 200, 97, 68, 43, 30, 20, 14 kDa; lane 1, 10 ng; 2, 5 ng; 3, 2.5 ng; 4, 1.25 ng; 5, 0.625 ng; 6, 0.312 ng; 7, 0.156 ng.

100% of detection sensitivity as compared with that generated by the electrophoretic transfer, but only about 25% of detection sensitivity was achieved by the second blot. The experiments were repeated three times, two of our first blots exhibited 50% assay sensitivity (data not shown) and one blot exhibited 100% sensitivity (Fig. 1B). However, all three second blots exhibited 25% assay sensitivity (Fig. 1C). After diffusion blotting, the remaining gel can also be visualized by protein staining procedures. With 10 ng and less protein used in our experiments, we treated the gel with Merril's simplified silver stain procedure [15]. The silver stain detected nephrosin down to 2.5 ng (lane 3 in Fig. 1D) even though the gel had been blotted once with the PVDF membrane.

We then evaluated whether diffusion blotting can allow direct comparison of signals produced by proteolytic zymography and immunoblotting analysis. Proteolytic activity of nephrosin can be demonstrated in a gel containing 0.2% gelatin given appropriate renaturation and incubation processes [16]. After gel electrophoresis, but before renaturation, a protein blot was made from the gelatin-containing gel by diffusion blotting for 1 h and then processed for immunoblotting analysis. The remaining gelatin-containing gel was subjected to renaturation and incubation as in a typical gelatinolytic zymography assay. The resulting immunoblotting analysis detected nephrosin down to 0.312 ng (lane 6 in Fig. 2A), which is around 50% of the detection sensitivity generated by the electrophoretic transfer from a normal gel (Fig. 1A). The gelatinolytic assay detected nephrosin down to 0.625 ng in zymography gel with (lane 5 in Fig. 2B) or without (lane 5 in Fig. 2C) a diffusion blot taken from the gel. This suggests that diffusion blotting does not significantly reduce the sensitivity of zymography assay. Because the immunoblot in Fig. 2A and zymogram in Fig. 2B were derived from the same gel, the signals for nephrosin in the gel and in the replica can be easily aligned from their positions relative to those of the prestained markers.

Typically the proteolytic zymography assay is carried out after SDS-PAGE and molecular weight of a protease can be estimated from the relative electrophoretic mobility. However, most biological samples display more than one proteolytic zone in the proteolytic zymography assay. It is difficult to ascertain the identity of a protease simply from the estimated molecular weight in such cases. Therefore, a separate immunoblotting blotting analysis is needed to identify the presence of a particular protease. To give an example, we performed the proteolytic zymography assay using crude tissue extracts of carp brain, gill, head kidney, intestine, kidney, liver, spleen and testis. Most proteolytic zones were in the range of 20–30 kDa and few were larger than 90 kDa (Fig. 3A). Nephrosin is expected to produce a proteolytic zone within the 20–30 kDa region. A simultaneous immunoblotting analysis revealed that nephrosin was present mostly in head kidney, kidney and spleen extracts (Fig. 3B). Very low levels of nephrosin were also detected in gill, intestine and liver extracts. Therefore,

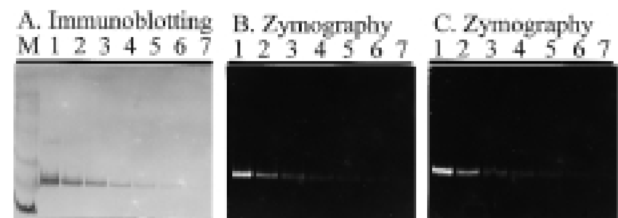

Figure 2. Simultaneous immunoblotting analysis and proteolytic zymography assay of purified carp nephrosin. Ten ng of carp nephrosin (lane 1) and its 2 × serial diluents (lanes 2–7) were applied to SDS-PAGE in a gel containing 0.2% gelatin. (A) After electrophoresis, the proteins were blotted by diffusion blotting and immunodetected by an anti-nephrosin antiserum. (B) The remaining gel was washed with 2% Triton X-100, incubated in 20 mM Tris-HCl, pH 8.0, containing 0.1 mM $ZnCl_2$ and stained with CBB R-250. (C) Another gel containing 0.2% gelatin was directly processed for the proteolytic zymography assay without blotting procedure. Dilutions as in Fig. 1.

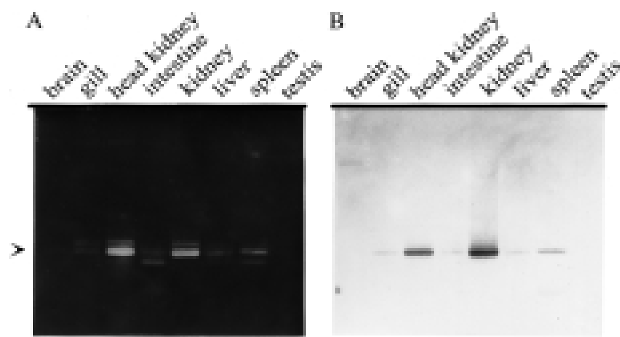

Figure 3. Simultaneous immunoblotting analysis for nephrosin and proteolytic zymography assay with carp tissue extracts. Four μg of carp tissue extracts was analyzed by SDS-PAGE in a gel containing 0.2% gelatin. (A) After electrophoresis, the proteins were blotted by diffusion blotting and immunodetected by an anti-nephrosin antiserum. (B) The remaining gel was treated as described in Fig. 2 for the proteolytic zymography assay. The arrowhead indicates the position of nephrosin.

we can identify the proteolytic zone indicated by the arrowhead on Fig. 3B as nephrosin by comparing the zymogram and the immunoblot.

Previously, we have demonstrated that the FPIII element in the promoter region of the rat pregnancy-specific glycoprotein gene, *rnCGM3,* is able to bind RBPJκ and NFκB in EMSA [20]. We tested here whether diffusion blotting can be used to couple immunoblotting analysis with EMSA for the interaction between FPIII and the NFκB subunit, p50. We transfected 293 cells with a p50 expression construct, pRcCMVp50, and performed EMSA with the labeled FPIII fragment and the p50-enriched nuclear extracts. As shown in Fig. 4A, two protein-DNA complexes were observed (lane 2 in Fig. 4A). The interaction is specific because both radioactive complexes disappeared with the addition of unlabeled FPIII fragment (lane 3 in Fig. 4A). The lower complex consists of RBPJκ and FPIII because the RBPjκ-specific m8 oligonucleotide can compete with the radiolabeled probe (lane 4 in Fig. 4A). RBPJκ is a ubiquitous nuclear factor [21] and therefore is present in our nuclear extract preparation. On the other hand, the upper complex consists of p50 and FPIII because the NFκB oligonucleotide competed with the radiolabeled probe (lane 5 in Fig. 4A). This notion is further substantiated by the fact that a p50-specific antibody could supershift the upper complex (lane 6 in Fig. 4A). These reactions are reproducible and can be repeated under identical conditions (lanes 7–11 in Fig. 4A).

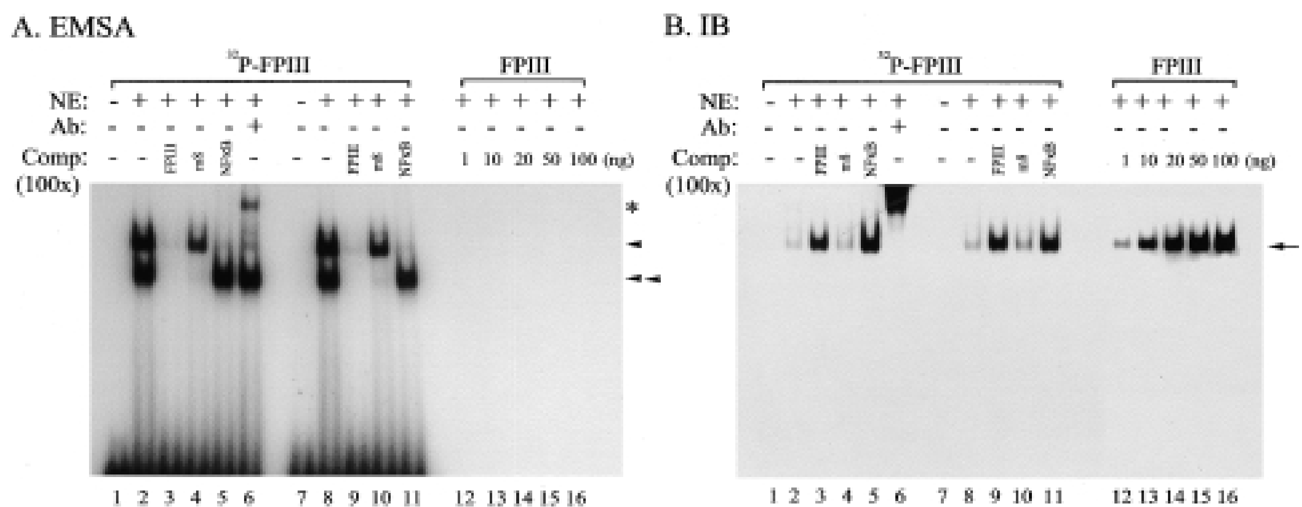

Figure 4. Simultaneous immunoblotting analysis and EMSA. (A) Autoradiography for EMSA using labeled and unlabeled FPIII fragments and the p50-enriched nuclear extracts. Twelve μg of p50-enriched nuclear extracts were incubated with radioactively labeled (lanes 1–11) or unlabeled (lanes 12–16) FPIII fragments in EMSA. The p50-FPIII and RBPJκ-FPIII complexes formed are indicated by the single arrowhead and the double arrowhead, respectively. The asterisk indicates the supershifted p50-FPIII complex. Lanes 7–11 are duplicates of reactions for lanes 1–5. Data were from an overnight exposure at –70°C. (B) Immunoblotting analysis for the FPIII-p50 complex in EMSA as described in (A). The arrow indicates the p50-FPIII complex. Data were from a 10 s exposure for the ECL reaction at room temperature. Diffusion blotting and immunoblotting analysis were performed as described in Sections 2.2 and 2.3. IB, immunoblot; NE, nuclear extracts; Ab, antibody; Comp, competitor; the absence (–) or the presence (+) of the indicated unlabeled oligonucleotide at 100-fold (100 ×) excess.

EMSA with the unlabeled FPIII fragment were performed with increasing amounts of the FPIII fragment and a fixed amount of the p50-enriched nuclear extracts. Complexes with the unlabeled FPIII fragment did not show any signals by autoradiography (lanes 12–16 in Fig. 4A).

A protein blot was generated from the gel by diffusion blotting and the p50–FPIII complex was detected with a p50-specific antibody. The amount of radioactive DNA probe transferred onto the PVDF membrane was not high enough to produce signals if the blot was exposed directly to the film. However, specific signals were detected with a p50-specific antibody and an AP-conjugated second antibody. Weaker signals were observed in complexes with the labeled FPIII probe (lanes 2 and 4 in Fig. 4B) whereas much stronger signals were observed in complexes with the labeled probe and a specific unlabeled fragment in combination (lanes 3 and 5 in Fig. 4B). Under the assay conditions, the limited amount of labeled FPIII probe (0.5 ng) did not cause saturation of all binding proteins and addition of the unlabeled FPIII and NFκB fragments at a 100-fold molar excess greatly increased the amounts of complexes formed. The resulting complexes, although exhibiting no signals in EMSA (Fig. 4A), can be detected by the immunoblotting analysis (Fig. 4B). Immunoblotting analysis also complemented the supershift data in the EMSA (lane 6 in Fig. 4B). The intense signal of the supershifted complex arises mostly from the recognition of our second antibody used in the immunoblotting analysis with the p50 antibody used in the EMSA. Complexes of unlabeled FPIII fragment and p50 were detected by the immunoblotting analysis in a dose-dependent manner with increasing amounts of the FPIII DNA fragment (lanes 12–16 in Fig. 4B). The signal intensity increased with the addition of the DNA fragment in the range of 1–100 ng. The data of Fig. 4B alone can be regarded as a novel version of EMSA which requires no radioactive labeling for detection. In addition, the specificity of the binding can also be verified by competition of binding by structurally similar probes; FPIII, m8 and NFκB (lanes 2–5 in Fig. 4B). Altogether, the data indicate that simultaneous immunoblotting analysis of the DNA binding proteins with EMSA can be easily achieved by the diffusion blotting procedures.

4 Discussion

In this report, we have demonstrated several applications of diffusion blotting. They are (i) multiple blotting, (ii) combined immunoblotting analysis with gel staining, (iii) combined immunoblotting analysis with proteolytic zymography, and (iv) combined immunoblotting analysis with EMSA from a single gel. Diffusion blotting has been the method of choice in transferring proteins from ultrathin gels or gels bound on a plastic support and in multiple blotting from a single gel [4–8, 10]. The efficiency of protein transfer by diffusion blotting was evaluated using immunodetection reaction in this study. Diffusion blotting from a 0.75 mm thick gel for 1 h gave a transfer efficiency of 50% compared to electroblotting and a second blotting yielded an efficiency of 25%. Thus, several blots can be made from a single gel at the cost of decreased transfer efficiency. Actually up to ten [8] or twelve blots [10] can be generated from a 0.5 mm thick gel by diffusion blotting transfer. Using ^{14}C-labeled proteins, diffusion blotting from a 0.5 mm thick gel for 3 min gave a transfer efficiency of 10% and about 30% after 30 min as compared to electroblotting [8]. However, the loss of transfer efficiency in diffusion blotting can exchange for combined immunoblotting analysis with another biochemical technique on a single gel. Significant amounts of proteins remain in the gel after diffusion blotting and thus the remaining gel can be used for other applications such as Coomassie blue staining, silver staining (Fig. 1 D), zymography incubation (Figs. 2 and 3) or exposure to X-ray film (Fig. 4).

In proteolytic zymography assay, several positive signals are frequently observed, which usually requires an additional immunoblotting analysis in another gel to examine a particular protease of interest. To our knowledge, we are the first to analyze a particular protease by immunoblotting analysis and proteolytic zymography simultaneously in the same polyacrylamide gel. We observed a 50% loss of signal intensity in immunoblotting analysis and essentially no loss of signal intensity in gelatinolytic zymography when these two assays were performed in the same gel.

EMSA is widely used to study the interaction between DNA and protein [12, 13]. In this assay, a DNA fragment is radioactively labeled as a probe, incubated with the protein of interest and resolved by electrophoresis. Formation of the protein-DNA complexes usually reduces the electrophoretic mobility of DNA as revealed by autoradiography. DNA probes can also be labeled nonradioactively by incorporation of biotin- or digoxigenin-conjugated dUTP into the DNA. The retarded protein-DNA complexes are then detected with avidin or anti-digoxigenin antibody conjugated with a reporter enzyme. In this report, we demonstrated that EMSA with an unlabeled DNA fragment is achieved by diffusion blotting onto a filter membrane and immunodetection with an antibody specific for the protein of interest. The detection of protein-DNA complexes is further enhanced by using the more sensitive chemiluminescent reactions. Therefore, a DNA fragment without any labeling reaction or treatment can be directly used in our assay system.

A method of simultaneous immunoblotting analysis with EMSA has been reported as "shift-Western blotting" [22]. In this method, a nitrocellulose filter and an anion-exchange membrane are stacked for electroblotting of proteins and the radioactive DNA, respectively, following native gel electrophoresis. The protein-DNA complex was detected by autoradiography of the DNA blot and immunoblotting analysis of the protein blot [22–25]. In this report, we have demonstrated that simple diffusion blotting can also carry out simultaneous immunoblotting analysis with EMSA. The method is particularly powerful in identification of individual components of protein-DNA complexes containing multiple transcription factors because multiple replicas can be made for immunoblotting analysis of each individual transcription factor. Moreover, our method is easier to perform because it does not require two different membranes for electroblotting. In conclusion, multiple blotting, combined immunoblotting analysis with gel staining, combined immunoblotting analysis with proteolytic zymography and combined immunoblotting analysis with EMSA from a single gel can be easily done by simple diffusion blotting.

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