Chapter 11

Multiple Immunoblots by Passive Diffusion of Proteins from a Single SDS-PAGE Gel

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Abstract

Western blotting enables the detection and characterization of proteins of low abundance. Sodium dodecyl sulfate (SDS) polyacrylamide gel-separated proteins are normally transferred electrophoretically to nitrocellulose or polyvinylidene difluoride membranes. Here we describe the transfer proteins [Ro 60 (or SSA) autoantigen, 220 and 240 kDa spectrin antigens, and prestained molecular weight standards] by diffusion from SDS polyacrylamide gels at 37 °C. Up to 12 immunoblots can be obtained from a single gel by this method.

Key words Non-electrophoretic transfer, Immunoblots, Bidirectional transfer, Nitrocellulose membrane, Autoantibodies, Autoantigens

1 Introduction

Western blotting, involving electrophoretic transfer of proteins to a microporous membrane support with subsequent immunodetection [1], has made a tremendous impact in the field of immunology. Proteins separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) are transferred electrophoretically to mainly nitrocellulose or polyvinylidene difluoride [1–4]. Protein transfer from gels to membranes has been achieved in three ways: (a) simple diffusion [3], (b) vacuum-assisted solvent flow [5, 6], and (c) electrophoretic elution [1, 7, 8]. Only electrophoretic elution has been used widely owing to a variety of reasons, including efficiency, simplicity, and length of transfer. Bidirectional transfer procedure was demonstrated in 1980, when Smith and Summers [9] transferred DNA and RNA from polyacrylamide gels to nitrocellulose in 36 h to obtain two blots. Here we demonstrate that proteins can be efficiently transferred non-electrophoretically from SDS-PAGE gels to nitrocellulose membranes. A similar procedure has been used in 1982 [10] to transfer proteins from thin (0.5 mm)

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native isoelectric focusing gels to nitrocellulose membranes to obtain two blots in 1 h. Diffusion-mediated transfer of immunoglobulins from one side of a native gel after isoelectric focusing to an antigen-coated nitrocellulose sheet has been achieved in a similar fashion [11, 12]. We were able to transfer and immunologically detect a 60,000 molecular weight autoantigen (Ro 60) (Fig. 2) as well as the spectrin antigens (molecular weight >200,000) (Fig. 3) using this method. When prestained molecular weight standards were transferred, only markers up to 118,000 molecular weight could be visualized (these proteins were not visualized immunologically) (Figs. 2 and 3).

Thus, we have obtained up to 12 immunoblots from a single gel using multiple antigens (high-, intermediate-, and low-molecular-weight proteins) and multiple sera. Subsequently several investigators have also obtained similar results and also in a quantitative manner (*see* Chapters 9 and 10).

2 Materials

Prepare all solutions using ultrapure water (prepared by purifying deionized water, to attain a sensitivity of 18 MΩ-cm at 25 °C) and analytical grade reagents. Prepare and store all reagents at room temperature (unless indicated otherwise). Diligently follow all waste disposal regulations when disposing waste materials. We do not add sodium azide to reagents.

2.1 SDS Polyacrylamide Gel

1. Resolving gel buffer: 1.5 M Tris–HCl, pH 8.8. Add about 100 mL water to a 1 L graduated cylinder or a glass beaker (*see* **Note 1**). Weigh 181.7 g Tris–HCl and transfer to the cylinder. Add water to a volume of 900 mL. Mix and adjust pH with HCl (*see* **Note 2**). Make up to 1 L with water. Store at 4 °C.
2. Stacking gel buffer: 0.5 M Tris–HCl, pH 6.8. Weigh 60.6 g Tris–HCl and prepare a 1 L solution as in previous step. Store at 4 °C.
3. Thirty percent acrylamide/Bis solution (29.2:0.8) acrylamide:bis: Weigh 29.2 g of acrylamide monomer and 0.8 g Bis (cross-linker) and transfer to a 100 mL graduated cylinder containing about 40 mL of water. Add a spatula of AG 501-X8 (D) mixed-resin beads and mix for about 30 min. Make up to 100 mL with water and filter through a 0.45 μm Corning filter (*see* **Note 3**). Store at 4 °C, in a bottle wrapped with aluminum foil (*see* **Note 4**).
4. Ammonium persulfate: 10 % solution in water (*see* **Note 5**).
5. *N*,*N*,*N*,*N*′-Tetramethyl-ethylenediamine: Store at 4 °C (*see* **Note 6**).

6. SDS-PAGE running buffer: 0.025 M Tris–HCl, pH 8.3, 0.192 M glycine, 0.1 % SDS (*see* **Note 7**).
7. SDS lysis buffer (5×): 0.3 M Tris–HCl (pH 6.8), 10 % SDS, 25 % β-mercaptoethanol, 0.1 % bromophenol blue, 45 % glycerol. Leave one aliquot at 4 °C for current use and store the remaining aliquots at −20 °C (*see* **Note 8**).
8. Bromophenol blue (BPB) solution: Dissolve 0.1 g BPB in 100 mL water.

2.2 Immunoblotting

1. Nitrocellulose membranes.
2. Western blot transfer buffer: 0.025 M Tris–HCl, 0.192 M glycine, 20 % methanol (*see* **Note 9**).
3. Tris-buffered saline (TBS; 10×): 1.5 M NaCl, 0.1 M Tris–HCl, pH 7.4.
4. TBS containing 0.05 % Tween-20 (TBST).
5. Blocking solution: 5 % milk in TBS (*see* **Note 10**). Store at 4 °C.
6. Diluent solution: 5 % milk in TBST (*see* **Note 10**). Store at 4 °C.
7. Mini PROTEAN® 3 System glass plates.
8. Medium binder clips (1¼ in.).
9. Plastic container.
10. Wypall X-60-reinforced paper.
11. Nitro blue tetrazolium (NBT)/5-bromo-4-chloro-3-indolyl phosphate (BCIP): Dissolve 1 g NBT in 20 mL of 70 % dimethylformamide (DMF). Dissolve 1 g BCIP in 20 mL of 100 % DMF. Add 33 μL of BCIP and 66 μL of NBT to 10 mL of alkaline phosphatase buffer just before adding to membrane. Alternatively, use 1-Step™ NBT/BCIP ready-made mix.

2.3 Antigens and Conjugates

1. Purified red blood cell spectrin, anti-spectrin polyclonal antibody, anti-hemoglobin antibody.
2. BenchMark prestained molecular weight standards.
3. Purified bovine Ro 60: Purify Ro 60 as reported [13, 14] or purchase from Immunovision, Springdale, AK, USA.
4. Prepare human erythrocyte membranes according to Dodge et al. [15].

3 Methods

Carry out all procedures at room temperature unless otherwise specified.

3.1 10 % Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

1. Mix 2.5 mL of resolving buffer, 3.33 mL of acrylamide mixture, and 4 mL water in a 50 mL conical flask. Degas with helium for 10 min. Add 100 μL of SDS, 80 μL of ammonium persulfate, and 10 μL of TEMED and cast gel within a 7.25 cm × 10 cm × 1.5 mm gel cassette. Allow space for stacking gel and gently overlay with isobutanol or water (*see* **Note 11**).
2. Prepare the stacking gel by mixing 1.25 mL of resolving buffer, 0.67 mL of acrylamide mixture, and 3 mL water in a 50 mL conical flask. Degas with helium for 10 min. Add 100 μL of SDS, 40 μL of ammonium persulfate, and 5 μL of TEMED. Insert a 10-well gel comb immediately without introducing air bubbles.
3. Heat aliquots of bovine Ro 60, RBC membranes, and human spectrin antigens at 95 °C for 5 min. Do not add lysis buffer to the prestained protein standard or subject it to heat. Centrifuge the heated samples at 3,000 × *g* for 30 s to bring down the condensate. Load increasing amounts of Ro antigen (1–4 μg) on one gel and same amounts of spectrin (3 μg/lane) or RBC membrane antigens on two other gels along with protein standards (10 μL/well-2 μg/marker/lane). Add protein standards in every other lane (alternating with spectrin) in the gel with spectrin. Electrophorese at 15 mA until the sample has entered the gel and then continue at 25 mA till the dye front (from the BPB dye in the samples) reached the bottom of the gel (*see* **Note 12**).
4. Following electrophoresis, pry the gel plates open with the use of a spatula. The gel remains on one of the glass plates. Rinse the gel with water and transfer carefully to a container with western blot transfer buffer.
5. Cut a nitrocellulose membrane to the size of the gel and immerse in methanol. Rinse twice in distilled water and once with transfer buffer.

3.2 Non-electrophoretic Transfer

1. Immediately following SDS-PAGE, when the dye front reaches the end of the gel, turn off the power supply. Separate the gel plates with the help of a spatula or a similar tool. Remove the stacking gel.
2. Rinse the gel (still supported by the bottom glass plate) carefully with deionized water to remove traces of SDS-PAGE running buffer.
3. Excise the gel with spectrin antigen such that there is one lane with the protein markers and one with the spectrin antigen.
4. Leave the gels to air-dry for 5–10 min (*see* **Note 13**).
5. Gently lay one nitrocellulose membrane, cut to the shape of the gel, on top of the gel (*see* **Note 14**).

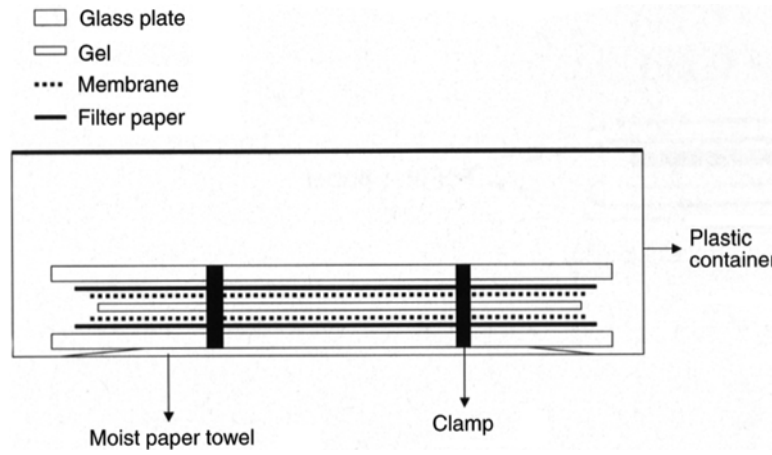

Fig. 1 Gel-membrane assembly for the non-electrophoretic transfer of proteins from SDS-PAGE gels to nitrocellulose membranes to obtain up to 12 blots. The polyacrylamide gel is sandwiched between two membranes, filter paper, and glass plates and incubated at 37 °C for varying periods of time to obtain up to 12 blots (reproduced from ref. 2 with permission from Elsevier)

6. Gently lift the gel-membrane sandwich from the glass plate and place it on a Whatman No. 3 filter (place membrane side directly on the filter paper and the exposed gel side on top) cut to the size of the gel.
7. Place a second nitrocellulose membrane, cut to the shape of the gel, on top of the gel, followed by a Whatman No. 3 filter paper cut similarly (*see* **Note 14**).
8. Place the nitrocellulose-gel-filter paper sandwich between two mini-PROTEAN® 3 System glass plates and secure with clamps.
9. Place this assembly in a pre-warmed humidified plastic container (Fig. 1) and incubate at 37 °C for 30 min (*see* **Note 15**). Remove the membranes for immunoblotting (*see* **Note 16**) (Fig. 2).
10. Repeat this procedure with another set of nitrocellulose membranes and incubate the assembly at 37 °C for 2 h to obtain two more blots from the same gel (*see* **Note 17**).
11. Use the same gel further to obtain blots by repeating this procedure to obtain a total of 12 blots. Incubate gel with the respective membranes for a period of 3 or 4 h to obtain the third and fourth sets of blots. Obtain the fifth and sixth sets of blots (Fig. 3) by incubating with the respective membranes for a period of 9 h or 36 h, respectively (*see* **Note 18**).
12. Cut excess membrane to smoothen edges and also cut the spectrin-containing membrane into individual lanes (*see* **Note 19**).
13. Block the membranes with blocking solution for 1 h.

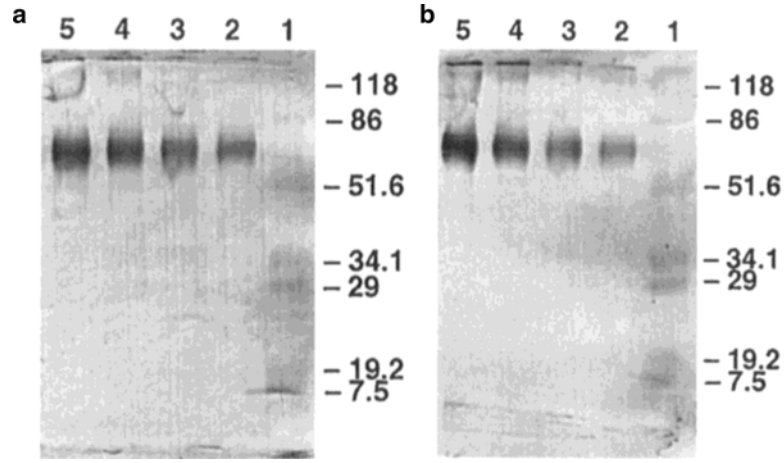

Fig. 2 Ro 60 immunoblots obtained following non-electrophoretic transfer for 30 min or 2 h. (**a**) One of the two Ro 60 blots obtained by the first incubation of nitrocellulose membranes on either side of the gel at 37 °C for 30 min. (**b**) A blot from the second set (blots 3 and 4) obtained from the same gel following incubation at 37 °C for 2 h (reproduced from ref. 6 with permission from Elsevier)

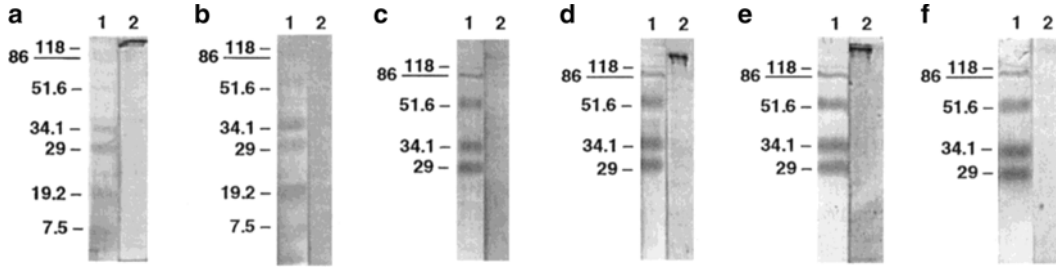

Fig. 3 Immunoblots obtained using spectrin as antigen. *Lane 1* in (**a**)–(**f**) shows prestained SDS-PAGE molecular weight standards. *Lane 2* shows spectrin probed with either preimmune (**b**, **c**, **f**) or anti-spectrin rabbit sera (**a**, **d**, **e**). Panel (**a**) shows one of the first two blots obtained and has been probed with anti-spectrin. Panel (**b**) shows the second of the two blots of the first set obtained from the reverse side of the gel and was probed with preimmune sera. Panels (**c**) and (**d**) show the fifth set (blots 9 and 10) of immunoblots probed with preimmune and anti-spectrin sera, respectively. Panels (**e**) and (**f**) show the sixth set (blots 11 and 12) of blots probed with anti-spectrin and preimmune sera, respectively (reproduced from ref. 6 with permission from Elsevier)

14. Add appropriate anti-sera to the membranes (anti-spectrin, anti-Ro 60, or control sera) and incubate for 2 h.
15. Rinse membrane strips with deionized water 2–3 times (*see* **Note 20**).
16. Wash 5× with TBST, 5 min each time.
17. Add anti-human IgG or anti-rabbit IgG alkaline phosphatase conjugate (1:5,000 dilution, diluted in diluent) and incubate for 1 h.
18. Wash as in **step 14**.

19. Add 500 μL of NBT/5-bromo-4-chloro-3-indolyl phosphate substrate and let bands develop.
20. Rinse 2–3 times with deionized water (*see* **Note 20**).
21. Wash with TBST and arrange strips on paperboard inserts (*see* **Note 21**).

4 Notes

1. Having water at the bottom of the cylinder helps to dissolve the Tris relatively easily, allowing the magnetic stir bar to go to work immediately. If using a glass beaker, the Tris can be dissolved faster if the water is warmed to about 37 °C. However, the downside is that care should be taken to bring the solution to room temperature before adjusting pH.
2. Concentrated HCl (12 N) can be used at first to narrow the gap from the starting pH to the required pH. From then on it would be better to use a series of HCl (e.g., 6 and 1 N) with lower ionic strengths to avoid a sudden drop in pH below the required pH.
3. Wear a mask when weighing acrylamide. To avoid exposing acrylamide to co-workers, cover the weigh boat containing the weighed acrylamide with another weigh boat (similar size to the original weigh boat containing the weighed acrylamide) when transporting it to the fume hood. Transfer the weighed acrylamide to the cylinder inside the fume hood and mix on a stirrer placed inside the hood. Unpolymerized acrylamide is a neurotoxin and care should be exercised to avoid skin contact. Mixed resin AG 501-X8 (D) (anion- and cation-exchange resin) is used when acrylamide solution is made, since it removes charged ions (e.g., free radicals) and allows longer storage. Some investigators store the prepared acrylamide along with this resin in the refrigerator. However, we filter them out before storage. The used mixed resin should be disposed as hazardous waste. Manufacturer's warning states that this resin is explosive when mixed with oxidizing substances. The resin contains a dye that changes from blue-green to gold when the exchange capacity is exhausted.
4. The acrylamide solution can be stored at 4 °C for 1 month. Acrylamide hydrolyzes to acrylic acid and ammonia. The acrylamide mixture, buffer, and water can be prepared in large batches, frozen in aliquots (for greater day-to-day reproducibility), and used indefinitely (*see* ref. 16). Remove the required amount, bring to room temperature, and add the other ingredients for polymerization. However, in our laboratory we make the acrylamide solution fresh about every month when we cast our own gels.

5. We find that it is best to prepare this fresh each time.
6. We find that storing at 4 °C reduces its pungent smell.
7. Simple method of preparing running buffer: Prepare 10× native buffer (0.25 M Tris–HCl, 1.92 M glycine). Weigh 30.3 g Tris–HCl and 144 g glycine, mix, and make it to 1 L with water. Dilute 100 mL of 10× native buffer to 990 mL with water and add 10 mL of 10 % SDS. Care should be taken to add SDS solution last, since it makes bubbles.
8. SDS precipitates at 4 °C. Therefore, the lysis buffer needs to be warmed prior to use.
9. Dilute 100 mL of 10× native buffer to 800 mL with water and add 200 mL of methanol. Avoid adding methanol directly to the 10× buffer, since it precipitates its ingredients. Even in such a scenario the precipitate can be redissolved by the addition of 800 mL water.
10. Add 100 mL of 10× TBS to a 1 L graduated cylinder and make it to about 800 mL with water. Transfer 50 g skim milk powder into the cylinder and stir until dissolved. Make to 1 L with water. Separate 500 mL as the blocking solution. To the remaining 500 mL add 250 μL of Tween-20 (cut end of blue tip to aspirate Tween-20 easily), dissolve, and use it as the diluent.
11. The gel cassette was sealed at the base using 1 % agarose. Overlay the resolving gel with water for gels having acrylamide concentration lower than 8 % and use isobutanol (or isobutanol saturated with water) for gels of 10 % or greater (*see* ref. 17). This overlay prevents contact with atmospheric oxygen (which inhibits acrylamide polymerization) in addition to helping to level the resolving gel solution.
12. Centrifuging the samples prior to the run helps remove insoluble debris, which could produce streaks in the protein lanes (revealed when stained with Coomassie blue). Add a drop of 0.1 % BPB to the upper chamber buffer. This helps to form a much stronger dye front during the electrophoretic run.
13. Membrane contact with the gel is much better when the gel is not moist. Therefore it is important to dry the gel for 5–10 min. The membrane will now stick well to the gel and gel will peel off the bottom glass plate by just lifting the membrane.
14. Hold the two top corners of the membranes with each hand. Lower the bottom part of the membrane first on the lower part of the gel and gently release the membrane little by little to lay the complete membrane on the gel. This will prevent trapping of bubbles in between the gel and the membrane. A 10 mL pipette was used to roll out air bubbles from the

gel-membrane sandwich prior to placing in transfer cassette. In the case of the gel with spectrin, cut the membrane to fit the two lanes of the gel.

15. The humid chamber consisted of a closed plastic container with a moist Terri Wipes paper towel at the bottom. The container must be big enough to contain the nitrocellulose-gel-filter paper assembly encased within the glass plates.
16. The second set of two blots was also obtained following incubation with the gel for 1 h (*see* Fig. 3b, c).
17. While removing the nitrocellulose membranes from the gel for immunoblotting, it would be common to find that the gel comes up stuck to one of the two membranes. To remove this membrane from the gel, place a fresh, dry nitrocellulose membrane on top of the gel and gently lift the gel. The gel becomes stuck to this fresh membrane, thus releasing the other membrane.
18. Gel dries, in spite of placing in humid chamber, when incubated for longer time periods (36 h). Therefore it is best to use the blots obtained after 12-h incubation with the membrane.
19. Cut a tiny wedge from the bottom left side of the marker lane and the main membrane sheet for orientation purposes. Also, in the case of the membranes with spectrin (*see* Subheading 3.1, **step 3**), excise the spectrin lane from the protein marker lane after matching each spectrin lane with its specific protein marker lane with pencil marks.
20. Rinsing the membrane strips with deionized water 2–3 times will help remove a bulk of the nonspecific antibodies and help reduce the amount of TBST used subsequently and also reduce the number of washes. This wash helps to reduce nonspecific binding of NBT/BCIP to the strip. The water, owing to its low ionic strength compared to TBST, will be able to remove contaminants much better than TBST. Water is much cheaper compared to TBST, in terms of money and labor. Other investigators have found no reduction in detection of specific signals due to washing with water [18].
21. We use paperboards placed in between stacks of ELISA plates in packages of ELISA plates (Costar, Cambridge, MA, USA) for this purpose.

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