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UNIT 10.7 Electroblothing from Polyacrylamide Gels

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Abstract

Transferring proteins from polyacrylamide gels onto retentive membranes is now primarily used for immunoblotting. A second application that was quite common up to about a decade ago was electroblotting of proteins for N-terminal and internal sequencing using Edman chemistry. This unit contains procedures for electroblotting proteins from polyacrylamide gels onto a variety of membranes, including polyvinylidene difluoride (PVDF) and nitrocellulose. In addition to the commonly used tank or wet transfer system, protocols are provided for electroblotting using semidry and dry systems. This unit also describes procedures for eluting proteins from membranes using detergents or acidic extraction with organic solvents for specialized applications.

Keywords

Electroblotting; Electrotransfer; Protein transfer; SDS-PAGE gel transfer; Polyacrylamide gel transfer; Western blotting

INTRODUCTION

Electroblotting of proteins from polyacrylamide gels onto retentive membranes is usually performed to facilitate procedures leading to protein identification and characterization. It is used primarily for immunoblotting, i.e., western blotting, and was previously extensively used for isolation of proteins prior to N-terminal protein sequencing. This latter application is still useful for specialized applications such as defining the N-terminal boundary of protein fragments or analysis of proteins where the genome is poorly defined. For most other prior applications of Edman sequencing, proteomics analysis using mass spectrometry (Chapters 22–25) is now the preferred approach, particularly when studying species where the genome has been completely sequenced.

Key References

LeGendre, N. and Matsudaira, P. 1988. See above.

Thoroughly reviews electroblotting using PVDF membranes.

Mozdzanowski, J. and Speicher, D.W. 1992. Microsequence analysis of electroblotted proteins. I. Comparison of electroblotting recoveries using different types of PVDF membranes. *Anal. Biochem.* 207:11–18.

Compares electroblotting recoveries using different PVDF membranes.

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In addition to immunoblotting, proteins transferred to membranes can be detected using sensitive stains, such as AuroDye or colloidal gold (*UNIT 10.8*). For applications requiring proteins in solution, the protein of interest can be eluted from the membranes as an alternative to direct elution of the protein of interest from the polyacrylamide gel. The electroblotting methods in this unit require that the proteins have already been separated on a polyacrylamide gel (*UNITS 10.1–10.4*). Including protein markers or prestained standards in gels is useful because they will transfer to the membrane and can be used to indicate the size of proteins after downstream detection. For immunostaining, it is particularly advantageous to use a protein standard that will be detected by the secondary antibody such as Magic Mark XP Western standard (Life Technologies).

This unit contains procedures for electrophoretically transferring proteins onto polyvinylidene difluoride (PVDF; Basic Protocol) or nitrocellulose (Alternate Protocol 2). The choice of membrane type for electrotransfer is dependent on the ultimate application for the blot membrane. High-retention PVDF binds proteins tightly and is well suited for applications such as Edman sequencing (Alternate Protocol 1), whereas lower-retention membranes may be advantageous for subsequent protein extraction and are also preferred for immunoblot analysis because they have low non-specific binding after “blocking”. Most protocols are designed for use with tank transfer setups (Table 10.7.1). Alternate Protocols 3 and 4 present procedures for electroblotting in semidry and dry systems, respectively. These latter methods may yield less quantitative recovery of a broad range of proteins on the target membrane but are generally more rapid and are therefore often preferred for immunostaining applications.

In some cases, stained blots are used only to identify protein band patterns while leaving the gel unmodified for subsequent steps (*UNIT 10.8*). If such minimal protein transfer is desired, contact blotting is a suitable alternative (*UNIT 10.6*). The current unit also describes procedures for eluting proteins from membranes using detergents (Basic Protocol 2) or acidic extraction with organic solvents (Alternate Protocol 5).

Unless otherwise indicated, the following protocols are primarily designed for electrotransferring proteins from SDS gels (*UNITS 10.1–10.4*). As proteins in other types of gels have much lower charge densities and, sometimes, opposing net charges for different proteins, substantial modifications to the protocols must be made for these specialized applications (*UNIT 10.2*).

BASIC PROTOCOL 1: ELECTROBLOTTING ONTO PVDF MEMBRANES

The following procedure is based on electrotransfer from polyacrylamide gels containing 0.2% sodium dodecyl sulfate (SDS) in the gel system (commonly referred to as SDS-PAGE). It uses a Bio-Rad solid plate tank transfer apparatus with 7 cm spacing between the electrode plates, which can accommodate gels up to 14–18 cm in size and requires ~2.5 liters transfer buffer. The choice of PVDF membrane depends on the planned subsequent use: high-retention Sequi-Blot membranes (Bio-Rad) are suitable for protein sequencing, whereas lower-retention Immobilon-P membranes (Millipore) are recommended for low-

background immunodetection or staining and for recovering proteins from membranes (Table 10.7.2).

Materials

1× transfer buffer (see recipe)

Polyacrylamide gel containing proteins of interest (*UNITS 10.1–10.4*)

100% methanol

Powder-free gloves

Electroblotting apparatus: "solid" plate electrode tank transfer system (e.g. Trans-Blot Cell, Bio-Rad)

Glass dishes and trays

Gel support sheet (e.g., porous polyethylene sheet, Curtin Matheson)

PVDF transfer membrane: Immun-blot (Bio-Rad), Immobilon-P (Millipore)

Whatman no. 1 filter paper

Power supply (500 V, 300 mA)

Additional reagents and equipment for staining gels (*UNIT 10.5*)

NOTE: Use powder-free gloves when handling all materials for this procedure and handle membranes with forceps at the edges to avoid potential artifactual staining.

Equilibrate the gel and membrane in transfer buffer

- 1 Prior to blotting, prepare the blotting apparatus by thoroughly rinsing with high-purity water.

The optional porous polyethylene sheet is hydrophobic, and it is difficult to get water or buffer into the pores. Be sure to hydrate or "wet" it properly by spraying water under pressure through the sheet or by submerging the entire polyethylene sheet in methanol and then submerging in high-purity water.

- 2 Fill the transfer tank with 1× transfer buffer. Submerge the gel cassette holder, fiber pads, and polyethylene sheet (after initial hydration as described in step 1) in transfer buffer. Place them inside the tank or submerge in transfer buffer using a separate tray.

The transfer buffer can be left in a covered transfer tank for up to 2 hr prior to use. Longer times should be avoided because the methanol in the buffer will evaporate, significantly changing the buffer composition.

- 3 Prepare the polyacrylamide gel containing proteins of interest by disassembling the gel apparatus, removing the stacking gel with a razor blade, and cutting a small piece from the lower left-hand corner of the gel (near lane 1 for gels loaded left to right; see Fig. 10.7.1) to aid in identifying the lanes in subsequent steps.
- 4 Briefly equilibrate the gel in a glass dish containing ~250 ml of 1× transfer buffer for an appropriate time based on gel thickness (0.5-mm-thick gels for 1 min; 0.75- to 1-mm gels for 5 min; 1.5-mm gels for 15 min).

Longer equilibrations in transfer buffer prior to electrotransfer may extract too much SDS from the gel and hence reduce transfer efficiency. This equilibration step will remove excess SDS and prevent the gel from swelling during transfer. The negatively charged SDS, which initially saturates the proteins, allows proteins to move toward the transfer membrane in an electric field and too little SDS bound to the proteins will decrease the proportion of the proteins that transfer to the membrane. This is particularly a problem for very large proteins because they have low mobility in the gel matrix. On the other hand, too much SDS can decrease binding of protein to PVDF membranes. Therefore, the amount of SDS bound to the protein as well as the amount of free SDS in the gel are critical for efficient transfer yields (see Critical Parameters and Troubleshooting).

- 5 Cut the PVDF transfer membrane about 0.5 to 1 cm larger than the gel. Cut a piece of Whatman no. 1 filter paper ~0.5 cm larger than the PVDF membrane.
- 6 Wet the PVDF membrane in a clean glass dish containing 100% methanol for 5 sec. Transfer membrane to a tray containing transfer buffer.

The PVDF membrane will wet almost immediately with the 100% methanol. The hydrophobic membrane will not wet in either water or transfer buffer alone. After initial wetting in methanol, do not let the membrane dry; if drying occurs, rewet the membrane in 100% methanol.

Prepare the gel/membrane transfer sandwich

- 7 Place the opened gel holder cassette on a clean, flat surface. Place one transfer buffer-wetted fiber pad (see step 2) on the top surface, followed by the gel support sheet.

A sheet of porous polyethylene is preferred as a support because it is rigid and facilitates handling, but filter paper can be used as a simple alternative. If the polyethylene sheet is used, place it with the smooth side up (toward the gel).
- 8 Place the gel face down on the support, so that the cut edge is now on the right-hand side (see Fig. 10.7.1B). Pour 5 to 10 ml transfer buffer on top of the gel.

- 9 Remove wet PVDF membrane from the tray containing transfer buffer (step 6). Eliminate air bubbles on both surfaces of membrane by sliding the membrane across the edge of the glass tray. Resubmerge membrane in transfer buffer for a few seconds and then position membrane above gel, letting its center contact the gel, and slowly lower the membrane from the center outward to force any bubbles to the edge of the gel. Rinse gloved hands with high-purity water and smooth the membrane gently to ensure uniform contact with the gel. Inspect the membrane for any trapped air bubbles.

This step is extremely important. Air bubbles will prevent the transfer of proteins.

- 10 Briefly wet the filter paper with transfer buffer and cover the PVDF membrane. Smooth gently to remove any air bubbles. Place the other fiber pad on top of the membrane and close the holder.

Do not allow any area of the PVDF membrane to dry during assembly, or transfer will not occur in these areas. The transfer sandwich should fit together snugly to provide good contact between the membrane and gel. The complete gel sandwich should look like that in Figure 10.7.2.

Conduct protein electrotransfer

- 11 Slide the assembled transfer cassette into the tank with the gel on the cathode side and the membrane on the anode side.

Proteins in an SDS-polyacrylamide gel have a net negative charge and will migrate toward the anode. For transfer from another type of gel, the orientation of the cassette may need to be reversed.

- 12 Fill the transfer tank with 1× transfer buffer so that the buffer completely covers the electrode panels but does not touch the electrical connectors.

- 13 Connect the power supply and perform the transfer at constant current compatible with complete transfer from the gel. For a Bio-Rad Trans-Blot tank transfer apparatus with solid plate electrodes, use 200 to 250 mA constant current. Transfer 0.5-mm gels for 1 hr, 0.75- to 1-mm gels for 2 hr, and 1.5-mm gels for 3 hr.

Longer times do not appear to increase transfer yield but may lead to overheating of the gel. Transfers can be carried out at constant voltage, as well. The advantage of this approach is that proteins migrate faster out of the gel due to the voltage driving force, but the current may increase during the run. Use caution to prevent overheating and/or unacceptably high current. Because of the amount of heat generated at higher transfer rates, it may be necessary to run the transfer in a cold room or with a cooling core to prevent overheating.

- 14 At the end of the transfer period, turn off the power and disconnect the power supply. Open the transfer sandwich and remove the membrane and gel (Fig.

10.7.1C). Thoroughly rinse the membrane with high-purity water, three times, 5 min each.

The membrane can be stained as described in UNIT 10.8 or used for other detection methods. Stained or unstained membranes can be air dried (for ~30 min) and stored in resealable plastic bags at room temperature for several weeks or at -20°C for a permanent record. Dried membranes can be rehydrated in 50% or 100% methanol and used for subsequent detection procedures.

- 15 To assess the efficiency of transfer, stain the proteins remaining on the gel with Coomassie blue or with more sensitive silver solutions, depending on the amount of protein originally loaded on the gel.

ALTERNATE PROTOCOL 1: ELECTROBLOTTING OF PROTEINS FOR SEQUENCE ANALYSIS

One-dimensional or two-dimensional gel electrophoresis combined with electroblotting to PVDF membranes provides a powerful method to obtain proteins of high purity that can be used directly for N-terminal (Edman) protein sequence analysis or for protein isolation prior to fragmentation and isolation of fragments for Edman sequencing. As noted above, Edman sequencing is now only rarely used because mass spectrometry based methods are more efficient when the goal is to identify a protein of interest, provided that the protein was isolated from a species that has been completely sequenced at the genome level. However, for specialized problems Edman sequencing may be the best or only option and electroblotting the protein of interest from a 1D or 2D gel prior to Edman sequencing is often the best purification method. The major concern is avoiding possible chemical modification of proteins such as artifactual blockage of a free N-terminal. Simple precautions such as casting gels in advance or using commercial pre-cast gels and using a scavenger such as thioglycolic acid help minimize possible side reactions during electrophoresis and electroblotting and are described in this protocol.

NOTE: Use high-quality water such as Milli-Q-purified water or equivalent and high-purity electrophoresis reagents (e.g. Bio-Rad) throughout for best results.

Additional Materials (also see Basic Protocol 1)

Thioglycolate (thioglycolic acid, sodium salt; Sigma)

2× or 6× SDS sample buffer (for discontinuous systems; *UNIT 10.1*)

PVDF transfer membrane: Sequi-Blot (Bio-Rad) or Immobilon-PSQ (Millipore)

Additional reagents and equipment for one- or two-dimensional gel electrophoresis (*UNITS 10.1 & 10.4*) and for staining membranes (*UNIT 10.8*) and gels (*UNIT 10.6*)

- 1 Cast polyacrylamide gel including stacking gel at least 24 hr but not more than 48 hr prior to use. Store the gel at room temperature until needed, being sure to

protect it from dehydration (e.g., store submerged in high-purity water) or purchase high quality precast gels.

For preparing gels choose an acrylamide concentration such that the protein(s) of interest will migrate as a sharp, tight band with an R_f between 0.2 and 0.8. Casting a gel in advance allows complete polymerization, reduces the amount of oxidants and free radicals, and minimizes the possibility of blocking the N terminus or modifying other amino groups of the protein.

- 2 Add thioglycolate to the electrophoresis buffer in the cathode buffer chamber to a final concentration of 0.1 mM (11.4 mg per liter buffer).

Thioglycolate sweeps through the gel during electrophoretic separation and scavenges remaining free radicals and oxidants in the gel.

- 3 Solubilize samples in either 2× or 6× SDS sample buffer (for discontinuous systems; *UNIT 10.1*), containing sucrose or glycerol but not urea. Heat to 37°C for 15 min.

If possible, avoid boiling the sample, as the high temperature increases the risk of chemical modification of the protein. Boiling may be necessary for complete solubilization of some samples, however (e.g., whole viruses).

DTT or 2-ME in the sample buffer is acceptable.

- 4 Conduct gel electrophoresis. After electrophoresis is complete, immediately proceed with electroblotting (see Basic Protocol 1, steps 1 to 14).
- 5 After transfer is complete, rinse the membrane six times for 5 min each with a large volume of water (at least 200 ml each time).

The membrane must be thoroughly rinsed with water immediately after transfer is complete. It is critical that the membrane is not allowed to dry prior to thorough rinsing because even partial drying can prevent effective removal of Tris and glycine.

- 6 Stain the membrane with amido black or Ponceau S to detect proteins. Stain the gel after transfer in order to judge transfer efficiency.

ALTERNATE PROTOCOL 2: ELECTROBLOTTING ONTO NITROCELLULOSE MEMBRANES

The electroblotting procedure for nitrocellulose membranes differs little from that for PVDF membranes. Nitrocellulose is compatible for use with a moderate amount of SDS (up to 0.1%) in the transfer buffer. The delicate membranes must, however, be handled carefully and protected from high concentrations of organic solvents.

Additional Materials (also see Basic Protocol 1)

Nitrocellulose transfer membrane: 0.45- μ m nitrocellulose membrane (Schleicher & Schuell, Bio-Rad)

Additional reagents and equipment for membrane (*UNIT 10.8*) and gel staining (*UNIT 10.6*)

- 1 Prepare the transfer apparatus and gel as described for PVDF membranes (see Basic Protocol 1, steps 1 to 4).
- 2 Cut the nitrocellulose transfer membrane to a size slightly larger than the gel and cut a piece of filter paper slightly larger than the membrane.
- 3 Wet the membrane by slowly introducing the membrane from one corner into a glass dish of 1 $\times$ transfer buffer. Equilibrate the membrane for 15 min.

IMPORTANT NOTE: Never soak the membrane in 100% methanol. During electrotransfer and staining, avoid exposure to high concentrations of organic solvents, which will dissolve the membrane. Up to 20% methanol can be used in the transfer buffer without affecting the membrane.

- 4 Proceed with electrotransfer as above for PVDF membranes (see Basic Protocol 1, steps 7 to 13).
- 5 When transfer is complete, rinse membrane three times for 5 min each with high-purity water; detect proteins on the membrane using an aqueous stain such as Ponceau S if desired.
- 6 Assess efficiency of transfer by staining the gel after transfer as appropriate.

ALTERNATE PROTOCOL 3: PROTEIN ELECTROBLOTTING IN SEMIDRY SYSTEMS

An alternative to the tank transfer system is the semidry transfer system. In this procedure, the gel is stacked horizontally on top of the membrane in the transfer apparatus. Because only a small volume of transfer buffer is used, SDS from the gel is less effectively diluted, which may result in incomplete binding and lower yields, especially with PVDF membranes. For this reason, semidry transfer units are not recommended when reproducible high recoveries of electroblotted proteins are desired (e.g., for subsequent Edman sequence analysis). Some procedures recommend stacking multiple transfer sandwiches to achieve several transfers simultaneously. To prevent unbound protein from migrating through the next gel and onto the membrane in the next transfer stack, porous cellophane sheets or dialysis membranes are placed between adjacent transfer stacks (see Fig. 10.7.3). Semidry electrotransfer requires shorter transfer times than tank transfer because the distance between electrodes is greatly reduced, thereby increasing the field strength.

Additional Materials (also see Basic Protocol 1)

Semidry transfer apparatus (e.g. Trans-Blot SD Semidry Transfer Cell, Bio-Rad)

Mylar mask (optional)

Prepare the transfer sandwiches

- 1 Cut transfer membrane just slightly larger than the gel containing proteins to be transferred, then equilibrate in the appropriate transfer buffer (see Basic Protocol 1, step 6).
- 2 Cut six sheets of filter paper the same size as the transfer membrane and wet thoroughly in transfer buffer.
- 3 Remove gel containing proteins of interest from the electrophoresis unit. Using a razor blade, cut the lower corner of the gel next to the first lane. Equilibrate the gel in transfer buffer (see Basic Protocol 1, step 4).
- 4 Layer a Mylar mask (optional) followed by three sheets of filter paper on the anode of the transfer unit. Smooth each piece of filter paper to avoid trapping air bubbles between the layers.

It is important to remove all air bubbles from the transfer stack as they will block the flow of current through that area of the gel, creating blank spots on the transfer membrane.

- 5 Layer the prepared transfer membrane on top of the filter paper. Gently roll a test tube or Pasteur pipet over the membrane to push out any air bubbles.
- 6 Place the gel on top of the transfer membrane so that the cut edge is on the left-hand side. With a pair of scissors, cut the lower corner of the membrane even with the gel (to aid in realigning gel and blot after final staining). Again, remove any air bubbles (see Basic Protocol 1, step 9).

When assembling this transfer sandwich, the gel is placed on top of the membrane, in contrast to the opposite order for tank type sandwich assembly as in Basic Protocol 1. Therefore, it is unnecessary to turn the gel over to obtain the proper orientation (compare Figures 10.7.1 and 10.7.3).

- 7 Layer the remaining three sheets of filter paper individually on top of the gel, rolling out any air bubbles after each addition.

It is possible to transfer multiple gels simultaneously using semidry blotting. As shown in Figure 10.7.3, a sheet of porous cellophane or dialysis membrane (Bio-Rad or Sartorius), preequilibrated for 5 min in transfer buffer, can be placed between the transfer stacks to prevent proteins from migrating onto an adjacent transfer stack membrane. However, proteins on the gel closest to the anode tend to be transferred more efficiently; thus, transferring multiple gels simultaneously is not recommended for critical applications such as protein sequencing.

Perform the protein electrotransfer

- 8 Attach the cathode unit on top of the transfer sandwich and connect leads to the power supply. Transfer proteins at constant current for no more than 1 hr.

Do not exceed 0.8 mA/cm² gel surface area. If the outside of the unit becomes warm during transfer, the current is too high and should be lowered.
- 9 After the transfer period, turn off the power supply and disconnect the leads. Remove the cathode and uppermost three layers of filter paper. Cut the corner of the transfer membrane above the cut corner of the gel. Alternatively, mark the transfer membrane by tracing the gel with a soft lead pencil.
- 10 Proceed with protein staining or immunodetection, as desired, or dry and store membranes at –20°C for later use (optional).

ALTERNATE PROTOCOL 4: PROTEIN ELECTROBLOTTING IN DRY SYSTEMS

A modern alternative to the tank and semidry transfer systems is the dry transfer system. In this procedure, the polyacrylamide gel is placed in a disposable, preassembled stack that contains a PVDF or nitrocellulose membrane, a proprietary buffer matrix, and copper-coated electrodes. No transfer buffer is used. The stack is placed in a proprietary power apparatus with built-in programs (varying voltage and time) designed to electrotransfer proteins from polyacrylamide gels to various membranes. Dry electrotransfer requires shorter transfer times than both tank and semidry transfers.

Additional Materials (also see Basic Protocol 1)

Dry transfer apparatus (e.g. iBlot 2, Life Technologies)

Proprietary transfer stack kit (e.g. preassembled gel stacks, absorbent pads, and filter paper from Life Technologies)

Prepare the transfer sandwiches

- 1 Open a new preassembled gel stack and remove the top stack. Remove and discard the separating sheet from bottom of the top stack.
- 2 Prepare the polyacrylamide gel by first removing it from the electrophoresis unit. Cut the lower corner of the gel at the first lane using a clean razor blade. This helps in identifying the lanes in later steps. Rinse the gel with high-quality water such as Milli-Q-purified water.
- 3 Place gel on top of the exposed membrane on the bottom stack.

Since the gel is placed on top of the membrane, it is unnecessary to turn the gel over to obtain the proper orientation. Further, depending on the preassembled stack being used and the gel size, multiple gels may be transferred at once.

- 4 Briefly wet the filter paper with high-quality water such as Milli-Q-purified water, and place it on top of gel. Smooth gently with a roller to remove any air bubbles.

This step is critical to achieving high quality transfer. It is important to remove all air bubbles from the transfer stack as they will create blank spots on the transfer membrane.

- 5 Place the top stack on top of filter paper. Be sure to orient the top stack such that the copper electrode faces upward. Once again, smooth gently with a roller to remove any air bubbles.

As before, removal of air bubbles is critical for proper electrotransfer.

Perform the protein electrotransfer

- 1 Place the assembled stack into the dry transfer apparatus such that the orientation of the stack aligns with the electrical contacts in the unit.
- 2 Place an absorbent pad on top of stack such that the metal electrical contact is aligned with the corresponding contact in the dry transfer apparatus.
- 3 Close and lock lid on dry transfer apparatus.
- 4 Power on dry transfer apparatus and select program appropriate for specific experimental conditions.

Variables to consider include type of membrane (PVDF or nitrocellulose), gel area (mini, midi, multiple gels, etc.), and other optimizations based on previous runs. Typically, the dry transfer apparatus will include preset programs that are best to try initially then modify based on empirical results.

- 5 After the transfer program has completed, open the dry transfer apparatus. Discard the absorbent pad, top stack and filter paper. Save and stain gel to evaluate efficiency of electrotransfer. Remove transfer membrane from bottom stack.

Typically, all reagents used in this protocol are disposable and should only be used one time.

- 6 Cut the corner of the transfer membrane above the cut corner of the gel. Alternatively, mark the transfer membrane by tracing the gel with a soft lead pencil.
- 7 The membrane may then be stained or used for immunodetection. The gel may also be stained, dried, and stored for later use.

BASIC PROTOCOL 2: PROTEIN ELUTION FROM PVDF MEMBRANES USING DETERGENTS

Binding affinities of most proteins to PVDF membranes are relatively strong. The most efficient protocol for protein recovery from PVDF membranes requires the use of detergents, which limits the possible use of extracted samples because detergents are often incompatible with subsequent procedures. This protocol is a simple procedure to elute proteins from the membrane into a Triton/SDS solution. A protocol that employs acidic extraction with organic solvents is also described (Alternate Protocol 5).

Materials

Polyacrylamide gel containing proteins of interest

Ponceau S stain (*UNIT 10.8*; optional)

50% methanol (optional)

Triton/SDS elution buffer (see recipe)

PVDF transfer membrane (Immobilon-P, Millipore)

Transfer apparatus: solid plate electrode tank transfer system (e.g., Trans-Blot, Bio-Rad)

Additional reagents and equipment for staining membranes (*UNIT 10.8*)

- 1** Prepare gel and membrane for transfer as for PVDF membranes (see Basic Protocol 1, steps 1 to 6).
- 2** Assemble the gel/membrane transfer sandwich and place in transfer tank (see Basic Protocol 1, steps 7 to 10).
- 3** Conduct electrotransfer (see Basic Protocol 1, steps 11 to 14), using lower field strengths for step 13 (e.g., transfer for 6 hr at 100 mA for 1.5-mm gels).

Temperatures >20°C during the transfer can increase the interaction between protein and matrix and make extraction more difficult. If problems arise in eluting proteins from the membrane, try running the transfer in a cold room overnight at low current (50 mA).
- 4a** Visualize transferred proteins with any membrane-compatible stain (*UNIT 10.8*). If bound stain will interfere with subsequent steps, use one of the following alternative detection methods.
- 4b** Visualize proteins with Ponceau S stain (*UNIT 10.8*). After staining with Ponceau S, wash the membrane with water, place under clear plastic wrap, and mark bands with a soft pencil by outlining the protein band through the plastic. Completely destain the membrane with water (a permanent indentation made by the pencil will be left on the membrane).

- 4c** For visualizing bands after partial drying of the membrane, fill a glass dish with 50% methanol and place the dry membrane on the surface of the solution. Do not submerge the membrane. Identify protein bands (portions of the membrane containing proteins will wet more quickly and dry slower than areas that do not contain protein). Remove the membrane from the surface of the methanol solution and place on a clean glass surface. Cover the membrane with clear plastic wrap and use a pencil to mark the protein bands as described in step 4b.
- 5** Excise the band(s) of interest by carefully cutting the membrane with a clean razor blade or scalpel.
- Do not allow the membrane to dry during this process. Keep the membrane wet (e.g., submerged in water) at all times (unless using the partial drying detection procedure).
- 6** Place the excised membrane band in a 1.5-ml microcentrifuge tube containing 0.2 to 0.5 ml of Triton/SDS elution buffer for every square centimeter of membrane. Centrifuge the sample 10 min at maximum speed in a microcentrifuge at room temperature to release the protein from the membrane.
- 7** Remove supernatant, place it in a new microcentrifuge tube, and centrifuge again to remove any particulate material.

Multiple extractions with the elution mixture aid quantitative recovery. In most cases, three extractions remove 90% to 100% of the protein from the membrane.

ALTERNATE PROTOCOL 5: PROTEIN ELUTION FROM PVDF MEMBRANES USING ACIDIC EXTRACTION WITH ORGANIC SOLVENTS

Blotted proteins are usually of relatively high purity and thus good candidates for additional characterization. If the protein cannot be further characterized on the blot and contamination of samples with large amounts of detergent (Basic Protocol 2) is unacceptable, acidic elution with organic solvents may be an alternative. The use of organic solvents limits this protocol to PVDF membranes, which have high chemical resistance. This protocol may produce highly variable recoveries for different proteins and is best suited for relatively low-molecular-weight proteins and large peptides (<50 kDa).

Additional Materials (also see Basic Protocol 2)

Extraction solution (see recipe)

50% methanol

1:2 (v/v) acetonitrile/formic acid

NOTE: All solutions should be made with high-purity water and can be stored at room temperature for at least 1 month.

- 1 Rinse the desired number of 1.5-ml microcentrifuge tubes (two tubes per protein spot or band) three times with extraction solution, and air dry under an aluminum foil dust cover.

Minimize possible contamination with dust at all times.

- 2 Visualize and mark the protein bands by partial drying of the membrane using 50% methanol (see Basic Protocol 2, step 4c). Use a scalpel or razor blade to cut out the protein bands.
- 3 Place pieces of membrane containing the protein of interest in a clean microcentrifuge tube.

Combining several bands from multiple gels is usually necessary. Use a pair of tweezers cleaned with 100% methanol to transfer the membrane pieces to the microcentrifuge tube. Avoid contaminating the membrane pieces and microcentrifuge tube with airborne dust or residues from fingers.

- 4 Add 200 μ l extraction solution to each microcentrifuge tube.

An alternative mixture of 1:2 acetonitrile/formic acid seems to be as efficient as the extraction solution described above for some proteins.

- 5 Agitate tubes for 48 hr at 4°C.

- 6 Repeat the extraction using 1:2 acetonitrile/formic acid. Combine the extracts.

Multiple extractions (up to three) with the same or different solvent mixtures are often beneficial. The extracts should be combined and kept on ice at all times.

- 7 Lyophilize protein extracts to provide highly purified samples suitable for most subsequent experiments.

REAGENTS AND SOLUTIONS

Note

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2E; for suppliers, see SUPPLIERS APPENDIX.

Extraction solution (40% acetonitrile/1% trifluoroacetic acid)

Add 1.0 ml trifluoroacetic acid to 59 ml high-purity water, then add 40 ml acetonitrile. Store at room temperature. Solution is stable for at least 1 month in a tightly sealed container.

Transfer buffer, 20×

200 mM Tris base

2.0 M glycine

Do not adjust pH of final solution (stable at room temperature for at least 1 month)

(Prepare 1× transfer buffer by mixing 200 ml 20× stock, 400 ml methanol, and water to 4 liters.)

Prepare 1× transfer buffer on the same day of use. Keep at room temperature.

Triton/SDS elution buffer

50 mM Tris-Cl, pH 9.0 (APPENDIX 2E)

2% (w/v) SDS (APPENDIX 2E)

1% (v/v) Triton X-100

Prepare solution on the same day of use. Keep at room temperature.

COMMENTARY

Background Information

The transfer of proteins from polyacrylamide gels onto blot membranes offers many benefits. Transferred proteins can be eluted from the membrane, probed with antibodies (immunoblotting), used for N-terminal protein sequencing, or stained by a variety of highly sensitive techniques. In addition, several manipulations can be performed on the same blot by loading samples in multiple lanes on the gel before electroblotting. Previously, this unit discussed *in situ* protease or chemical cleavage of proteins for further structural analysis (UNIT 11.2 & UNIT 11.5); however, these approaches have been largely replaced by in-solution or in-gel methods due to generally lower yields when using electroblotted samples (UNIT 11.1 & 11.4).

Prestained protein standards are useful guides for cutting the membrane prior to different analysis procedures, such as using one part for immunoblotting and the other for protein staining. Prestained radiolabeled standards are useful for aligning the blot membrane with autoradiograms. Prestained standards will migrate differently on the gel compared with the unmodified standard proteins, usually showing higher molecular weight. For immunoblots, the best standards are those that react with the western blot reagents to yield visible bands directly on the immunoblot.

During electrotransfer, proteins migrate out of gels in an electric field according to the charge on the protein. Most electrotransfers employ a tank transfer apparatus (Fig. 10.7.2) in which the gel/membrane transfer sandwich is mounted in a cassette and placed in a tank of Tris/glycine/methanol transfer buffer (Table 10.7.1). The semidry transfer system (Fig. 10.7.3) is an alternative. The major advantages of the semidry system are its use of substantially less buffer and the shortening of transfer time due to the close positioning of the electrodes, which produces a high field strength with minimal heating. The dry transfer system is another alternative. This approach uses no liquid buffer, rather prepackaged cassettes containing a buffer matrix, and substantially reduces transfer time due to close positioning of the electrodes, high field strength, and high currents.

A variety of transfer membranes, which can effectively bind proteins based on different types of protein-membrane interactions, are commercially available (Table 10.7.2). These include PVDF, nitrocellulose, and nylon membranes as well as a number of derivatized membranes. PVDF membranes bind proteins primarily through hydrophobic interactions and are commonly used for their chemical resistance as well as physical stability. High-affinity PVDF membranes such as Sequi-Blot (Bio-Rad) and Immobilon-PSQ (Millipore) are preferred for blots intended for use in N-terminal protein sequencing, whereas low-retention membranes such as Immun-Blot (Bio-Rad) and Immobilon-P (Millipore) may produce lower background in both immunoblotting and common staining procedures. In addition, low-retention membranes are preferred when proteins will be extracted from the membrane.

Nitrocellulose binds proteins primarily by hydrophilic and/or electrostatic interactions and for this reason is less sensitive than PVDF membranes to the concentration of SDS in both the gel and transfer buffer. Its tolerance to SDS allows relatively good recovery of poorly migrating proteins because higher concentrations of SDS can be left in the gel or added to the transfer buffer without adversely affecting protein binding to the membrane. In addition, nitrocellulose is often used for immunoblots, although PVDF membranes have replaced nitrocellulose for this application in many laboratories. The major limitations of nitrocellulose are its poor binding of low-molecular-weight proteins and peptides, mechanical weakness, and lack of resistance to organic solvents.

PVDF can be derivatized to modify the nature of protein-membrane interactions and to potentially increase the strength of protein binding. Derivatization most commonly involves adding a charge to the membrane so that proteins will be held on the membrane by electrostatic charges as well the usual hydrophobic or hydrophilic interactions. The procedure for electrotransfer onto derivatized membranes is essentially identical to that for PVDF or nitrocellulose membranes described in this unit. Although derivatized membranes are better for binding RNA and DNA, most derivatized membranes have not offered clear advantages for blotting proteins.

Proteins can also be eluted from Immobilon-P membranes, and two procedures for doing so are described in this unit: protein elution using detergents (Basic Protocol 2; Szewczyk and Summers, 1988) and acidic elution with organic solvents (Alternative Protocol 5; D.W.S., unpub. observ.). Although gel electrophoresis combined with subsequent electroblotting is often a powerful procedure for microscale protein isolation, one limitation of this system is that subsequent elution of proteins from PVDF membranes in good yields is often difficult.

Critical Parameters and Troubleshooting

The protocols listed in this unit should be sufficient for the transfer of most proteins. The efficiency of protein transfer is easily assessed by staining both the transfer membrane and the gel after transfer. When difficulty is encountered in the transfer recovery of a specific protein, several factors can be considered to improve transfer efficiency.

A first consideration is the type of gel used to separate proteins for transfer. Higher percentages of acrylamide make the transfer of proteins out of the gel more difficult. Gel thickness also affects transfer: the thicker the gel, the farther the protein has to migrate to

reach the membrane. In general, effective transfer of proteins out of gels up to 1.5 mm thick can be achieved; thicker gels, although having increased protein capacity, offer little advantage owing to reduced removal of proteins from the thicker gel matrix.

In this unit, the transfer buffer described is essentially half-strength Towbin buffer (Towbin et al., 1979). This buffer contains a mixture of Tris and glycine and is the buffer of choice if the electroblotted proteins will be used for sequence analysis. Tris, glycine, and most other low-molecular-weight buffer components do not normally bind to PVDF membranes and can be completely removed by thorough rinsing with high-purity water. However, incomplete rinsing or partial drying of the membrane prior to rinsing can result in high residual levels of these compounds, which could be detrimental for sequence analysis.

The amino groups in the buffer act as scavengers for contaminants that could potentially modify proteins. Several simple precautions noted in Alternate Protocol 1 can be taken to minimize side reactions of proteins during electrophoresis and electroblotting (Speicher, 1989). If potential artifactual chemical modifications are not critical, several other buffers may be used. CAPS buffer (cyclohexylaminopropanesulfonic acid; LeGendre and Matsudaira, 1988) has a high pH (pH 11) and low ionic strength and because of its low conductivity, permits the use of high electric fields. For the transfer of very basic proteins that bind SDS poorly, high pH may prove advantageous in keeping the total net protein charge negative; however, the high pH of the CAPS buffer can also lead to deamination of sensitive asparagine and glutamine side chains. Other types of electrotransfer buffers such as Tris/borate (Vandekerckhove et al., 1985) do not differ significantly in overall performance from the Tris/glycine buffer.

Selection of membrane type is dependent on the ultimate use of the transferred protein. PVDF membranes are often preferred because nitrocellulose membranes are less chemically and mechanically stability. For immunoblots, either nitrocellulose or Immobilon-P (Millipore) are often preferred because they produce a lighter background than high-retention PVDF membranes. Other applications may require specialized membranes. For example, imaging an immunoblot probed with near-infrared fluorescence antibodies with a LI-COR Odyssey scanner greatly benefits from membranes with low auto-fluorescence, including Immun-Blot low fluorescence (Bio-Rad) or Immobilon-FL (Millipore).

It is not uncommon in electroblot procedures to observe less than optimal transfer of proteins. If the membrane is entirely blank and little or no protein is left in the gel after transfer, it is likely that the electrodes were connected in a reversed order or that the membrane was placed on the wrong side of the gel. Blank spots on membranes are caused by air bubbles, which block the flow of current.

Another reason for poor transfer of proteins relates to the amount of SDS in the gel, which initially coats the proteins and provides mobility during electrotransfer. Nitrocellulose membranes are not sensitive to the amount of SDS in the gel and can even tolerate addition of SDS to the transfer buffer to encourage transfer of recalcitrant proteins. PVDF membranes are more sensitive to excess SDS, which can inhibit protein binding to the hydrophobic membrane; nevertheless, in a limited number of cases, addition of SDS to the

transfer buffer may be beneficial. Low-retention PVDF membranes are particularly sensitive to excess SDS concentrations, whereas high-retention PVDF membranes are less so. Methanol is a common component of many transfer buffers because it facilitates dissociation of bound SDS from proteins. Therefore, if proteins transfer from the gel efficiently but do not bind well to PVDF membranes, the methanol concentration can be increased to 20% and/or the gel can be preequilibrated in transfer buffer for 15 to 30 min prior to transfer to reduce the SDS concentration (Mozdzanowski et al., 1992). Conversely, if proteins remain in the gel after transfer, the methanol concentration can be reduced or eliminated, and if necessary a low concentration of SDS (0.005%) can be added to the transfer buffer to facilitate electrotransfer from the gel. In niche cases, it may be beneficial to increase the SDS concentration further. One specific example is the transfer of proteins from polyacrylamide gels containing the small molecule Phos-tag, a divalent cation complex that binds phosphorylated proteins. When using a wet transfer system, the transfer efficiency is increased by the addition of 0.1% SDS to the transfer buffer (Kinoshita-Kikuta et al., 2014).

If proteins have transferred efficiently but the stained membrane has blurred bands or a swirled pattern, it is likely that there was insufficient contact between the gel and membrane during transfer. The transfer sandwich should be held together firmly both to avoid having the gel shift position during transfer and to ensure close contact between the gel and membrane. Another common problem with transfers is the presence of bright white spots within protein bands on the membrane. These are due to air bubbles trapped between the gel and membrane or, in the case of PVDF membranes, possibly due to local drying of the membrane during transfer unit assembly. As indicated in the protocols, it is very important to check for air bubbles before electrotransfer. Air bubbles can be rolled out from the two surfaces by using a test tube, or the membrane can be carefully lifted to release the bubble and replaced without shifting the position of either gel or membrane.

Anticipated Results

Proteins in the middle two-thirds of the gel usually transfer to high-retention PVDF membranes with an average yield of 50% to 80%. The protein pattern on PVDF membranes stained with amido black or Coomassie blue should closely resemble the pattern on duplicate lanes of the gel stained with Coomassie blue. The staining intensity on the blot should be slightly higher than on the gel because the proteins are concentrated on the surface of the blot rather than distributed throughout the thickness of the gel. Proteins below the dye front usually will not be recovered on the membrane due to the presence of very high SDS concentrations in this region of the gel. Proteins in the top 20% of the gel are often incompletely transferred out of the gel due to slow migration within the gel matrix.

Time Considerations

Assembling the transfer unit takes ~30 min. Electroblothing can be completed in 2 to 4 hr in most cases using a tank transfer unit, ~1 hr using a semidry blotting unit, and ~10 min using a dry transfer unit.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 10.7.1.

Aligning the gel for transfer. (A) If the polyacrylamide gel has been loaded from left to right, cut a small piece from the corner of the lower left-hand edge of the gel near the first lane. (B) When preparing the transfer sandwich, turn the gel over so that the cut edge is on the lower right-hand corner of the gel. This will ensure that the transferred proteins will appear in the same order as in the original gel. (C) After transfer, trim the membrane above the cut corner of the gel to mark orientation. Dots indicate where the proteins were before transfer.

Figure 10.7.2.

Electroblotting with a tank transfer unit. The polyacrylamide gel containing the protein(s) to be transferred is placed on the smooth side of the polyethylene sheet (or filter paper sheets) and covered with the PVDF membrane and then a single sheet of filter paper. This stack is sandwiched between two fiber pads and secured in the plastic gel holder cassette. The assembled cassette is then placed in a tank containing transfer buffer. For transfer of negatively charged protein, the membrane is positioned on the anode side of the gel. Charged proteins are transferred electrophoretically from the gel onto the membrane.

Figure 10.7.3.

Electroblotting with a semidry transfer unit. In most cases, the lower electrode is the anode, as shown. Position the Mylar mask (optional) directly over the anode. Layer on three sheets of filter paper that have been wetted in transfer buffer. For negatively charged proteins, place the preequilibrated transfer membrane on top of the filter paper followed by the gel and three additional sheets of wetted filter paper. If multiple gels are to be transferred, separate the transfer sandwiches by inserting a sheet of porous cellophane or dialysis membrane between each stack. Place the cathode on top of the assembled transfer stack(s). Transfer the proteins by applying a maximum current of 0.8 mA/cm^2 gel area.

Table 10.7.1

Examples of Electrotransfer Units

Transfer Unit	Product
Wet	Trans-Blot Cell (Bio-Rad) XCell (Thermo Fisher Scientific) TE 62 Transfer Unit (GE Healthcare Life Sciences)
Semi-Dry	Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad) FisherBiotech Semi-Dry Blotting Apparatus (Thermo Fisher Scientific) TE 70 Semi-Dry Transfer Unit (GE Healthcare Life Sciences)
Dry	iBlot 2 Dry Transfer System (Thermo Fisher Scientific)

Table 10.7.2

Examples of Common Electrophoretic Membranes

Membrane Type	Product
PVDF (high-retention)	Sequi-Blot (Bio-Rad) Immobilon-P ^{8Q} (Millipore) Westran S (Whatman)
PVDF (low-retention)	Immun-Blot (Bio-Rad) Immobilon-P (Millipore) Westran Clear Signal (Whatman)
Nitrocellulose	Nitrocellulose (Bio-Rad) Protran Nitrocellulose (Whatman) Protran Nitrocellulose (Schleicher & Schuell)