Western Blots

by Tanya Schneider

Western blotting is useful in detecting a protein of interest that may be mixed with others (such as in a cell lysate) or verifying the identity of a protein on a gel. In general, a mixture of proteins is resolved using a denaturing acrylamide gel. The separated proteins are then transferred from the gel onto a nitrocellulose or PVDF membrane. The protein of interest is probed by incubating the membrane with a specific antibody. The membrane next is incubated with a secondary antibody that recognizes any bound primary antibody. The secondary antibody generally enables (through a variety of mechanisms) eventual identification of any band on the membrane that was bound by the antibody. This technique is limited by the success of the gel separation, the transfer step and the specificity of the antibody.

I. Running Gels for Western Blots

Some people have success using the Phast system to run and transfer a gel to a membrane. I have found this technique limiting due to the small size of the Phast gel and related small-scale separation of proteins, which tends to result in a messy western blot. I have had much better success and resolution using minigels and transferring them to membrane in a separate buffer chamber. However, both techniques are detailed below.

A. Phast gels

Run a Phast gel as you typically would, following Phast gel protocol. You may want to run duplicate gels - one to transfer and one to stain in order to compare the protein gel with your western blot. Use protein standards that are easy to identify on your gel and on your membrane after transfer (BioRad Kaleidoscope markers are nice as each protein band is a different color on the gel).

B. Minigels (Hoefer Scientific Instruments)

Minigels can be stored (4 ºC) for several weeks wrapped in plastic wrap, so more than one can be cast at once. Use of a running gel with a stacking gel poured on top allows for good separation.

Prepare running gel solution (recipes below), adding APS and TEMED immediately prior to pouring the gel. When using a stacking gel layer, pour the running gel solution to 3 cm below the top of the glass plate. Tap the gel gently against your bench to force bubbles to the surface. Gently introduce 100 µL of water-saturated butanol to create a top layer over the running gel. Allow to polymerize for at least 1 hour.

While some suggest that you pour the stacking gel layer just prior to gel use, I've stored gels with the stacking layer in place for weeks with no problems. Again, mix up the stacking gel layer using the following recipe. Rinse off the butanol layer on top of the running gel with water. Apply stacking gel and add combs to form wells in the stacking layer.

C. Running gel recipes (using 1.5 mm-thick combs - yields 2 gels)

	5%	7.5%	10%	12.5%	15%

Monomer solution (mL)	3.3	5	6.7	8.3	10
4x running buffer (mL)	5	5	5	5	5
10% SDS (mL)	0.2	0.2	0.2	0.2	0.2
Water (mL)	11.4	9.7	8.0	6.4	4.7
10% APS (µL)	100	100	100	100	100
TEMED (µL)	6.7	6.7	6.7	6.7	6.7

Stacking gel recipe (2 gels) 0.44 mL monomer solution 0.83 mL 4x running buffer 33 µL 10% SDS 2.03 mL water 16.7 µL 10% APS 1.7 µL TEMED	Monomer Solution 60 g acrylamide 1.6 g bis-acrylamide water to 200 mL
4X running gel buffer (1.5 M Tris-HCl, pH 8.8) 36.3 g Tris 150 mL water adjust to pH 8.8 with HCl water to 200 mL	4X stacking gel buffer (0.5 M Tris-HCl, pH 6.8) 3.0 g Tris 40 mL water adjust to pH 6.8 with HCl water to 50 mL
Tank buffer 30.28 g Tris 155.13 g glycine 10 g SDS water to 10 L	Water-saturated n-butanol 50 mL n-butanol 5 mL water mix; use top layer to overlay gels

D. Running Minigels

Assemble minigel using tank buffer (see above) as running buffer in upper and lower chambers. Typical run for 2 gels is 1 hour at 40 mA constant current. Tracking dye should run to the bottom of the gel for complete separation. Cooling is optional. Again, you may want to run duplicate gels, one to stain and one to transfer.

II. Transfer of gels

A. Phast gels

1. Cut PVDF membrane to fit size of actual gel (not stacking gel). Rinse membrane in methanol briefly, then soak in Towbin transfer buffer for at least 5 min. at room temp.
2. Apply membrane to top of the phast gel in phast gel chamber, taking care to remove bubbles between membrane and gel. Place the plastic phast gel buffer strip holder (remove buffer strips, though) over membrane.
3. Transfer simply with heat - set phast system to 60 ºC for 30 min to 1 hr.
4. Soak gel and membrane in methanol and separate.

B. Minigels – wet transfer method (Hoefer Transphor TE62)

1. Prepare membrane as described above. Also prepare 6L Towbin transfer buffer and chill prior to use. I pre-chill the buffer to avoid having to hook the chamber up to a cooling bath during the transfer. Pre-wet two sheets of blotting paper and sponges (with chamber) in transfer buffer.
2. Remove stacking gel from minigel. Equilibrate running gel in Towbin transfer buffer for 5-15 minutes (longer time permits gel to resize in new buffer, but may allow proteins under 40 kDa to diffuse out of gel).
3. Assemble transfer stack in the following order, taking care to avoid bubbles between layers (I find that rolling across each layer with a pipette helps) and keeping the layers wet at all times: gray cassette panel goes on bottom, followed by sponge, blotting paper, membrane, gel, another sheet of blotting paper, another sponge. Now you're ready to close up the cassette with the black panel on top (diagram is available in Transphor manual).
4. Orient cassette in chamber with the hinges face up and the black side of the cassette facing the black cathode panel. Tap cassette lightly to remove bubbles. Add buffer as required following fill lines on chamber.
5. Transfer time may depend on size of your proteins. Transfer at 40 V for 2 hours has worked well for me with a range of proteins under 100 kDa.

Towbin transfer buffer
25 mM Tris (18.2 g)
192 mM glycine (86.5g)
1% SDS (6 g)
15% methanol (900 mL)
water to 6 L

The concentration of SDS and methanol can affect transfer. More methanol makes it more difficult to transfer larger proteins. These concentrations have worked fine for me with proteins under 100 kDa.

C. Evaluating transfer efficiency

Your protein markers should be visible on the membrane after transfer if it was reasonably successful. It is also possible to visualize all proteins transferred to the membrane using Ponceau S stain. This is a rapid, reversible stain that allows you a quick check on transfer. Incubate membrane 5-30 minutes with just enough stain to cover membrane. Destain in water until background is white – if you destain longer, the protein bands will disappear as the stain is water-soluble.

Ponceau S stain
1.0 g Ponceau S
50 mL acetic acid
water to 1 L

III. Probing and detection

The remaining steps are the same regardless of what gel/transfer method used. This protocol is a guideline, and many of these recommendations can be optimized for your particular experiment. The length of blocking time, incubation with antibody, whether you incubate the membrane with the antibody in the presence of nonspecific proteins, even the composition of blocking proteins can vary. If you think you have high background after you visualize your blot, try more stringent blocking and/or washing conditions. All steps are carried out at room temperature.

- Block membrane with TBST containing 3% BSA for at least 30 minutes.
- Wash membrane with TBST for 1 minute.
- Dilute primary antibody in TBST as recommended (often 1:100 or 1:1000). Incubate with membrane for 30 minutes. I have included 2.5 % dry milk with my antibody and secondary antibody on occasion to reduce background.
- Wash 3X with TBST for 5 min.
- Incubate with secondary antibody (usually a 1:10,000 dilution of stock antibody) in TBST for 30 minutes. The secondary antibody is chosen based on its ability to recognize the primary antibody (i.e., if the primary antibody was made in rabbit, use of a goat anti-rabbit secondary antibody would be appropriate).
- Wash 3X with TBST.
- Wash 1X with TBS. Remove membrane from buffer so that it's not dripping wet.
- Typical detection involves use of ECF substrate (Pharmacia). This product relies on your secondary antibody being conjugated to alkaline phosphatase (AP). In the presence of AP, a phosphate group is cleaved from the ECF substrate, forming a highly fluorescent product which fluoresces at 540-560 nm. ECF substrate should be reconstituted from the kit, aliquotted, and stored at -20 ºC. Apply just enough ECF substrate to cover the surface of the membrane. Incubate up to 5 minutes or until yellow bands start to appear. Allow membrane to dry a bit and scan on STORM using the blue fluorescence option. Longer incubation can be necessary if you have very little protein, but this can also lead to increased background.

TBST
10 mM Tris-HCl pH 8 (5 mL 1M Tris-HCl pH 8)
150 mM NaCl (4.37 g)
0.1% Tween-20 (0.5 mL)
water to 500 mL

TBS
20 mM Tris-HCl (3.2 mL 1M Tris HCl with 0.8 mL 1M Tris base)
150 mM NaCl (1.76 g)
water to 200 mL