

## Immunoblotting Protocol Chemiluminescent Detection System

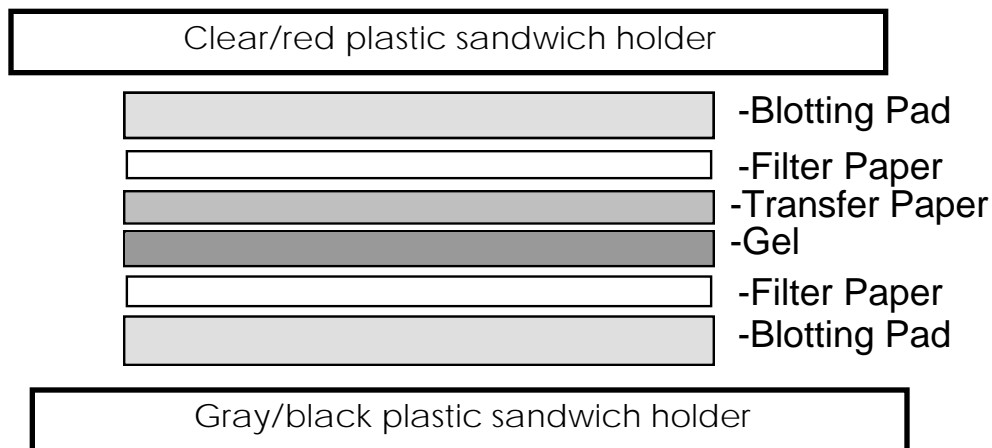
### Step 1. Protein Transfer.

- **Prepare 500 ml transfer buffer.**
- **Prepare the transfer membrane and filter paper.**
  - If using immobilon (PVDF) transfer paper, pre-wet the paper for 30 seconds in methanol, rinse with distilled water and place in a shallow dish with transfer buffer
  - If you are using nitrocellulose just rinse the paper in TTBS for 1 min.
- **Prepare the polyacrlamide gel for transfer.**

Soak the gel in transfer buffer for ten minutes to remove salts that may result in poor transfers.
- **Prepare the blotting pads.**

Soak the pads in transfer buffer until they are saturated. Remove air bubbles by squeezing the pads while immersed in the buffer. Any remaining bubbles will block the transfer of the proteins.
- **Assemble Blot Apparatus**

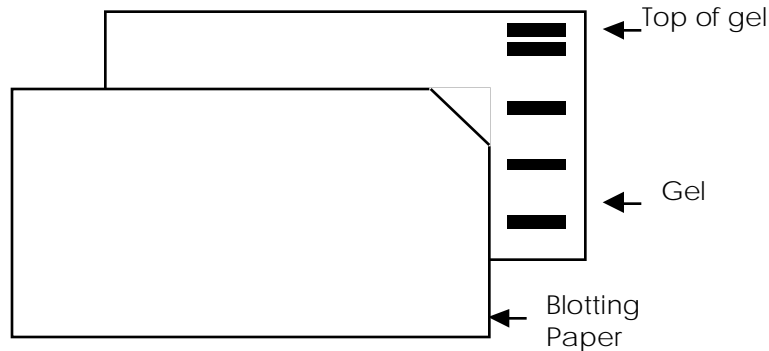
Place 1 blotting pad on the black part of the sandwich holder (**build on black**). Place the filter paper on the gel. Bubbles can be displaced with your fingers. On top of the blotting pad, place one sheet of filter paper followed by the transfer paper, gel and another filter paper.



- **Orientation of Gel.**

Clip a corner of the paper. This will now be the top left corner. When the clipped corner is on the top left that means the protein side of the paper is facing up. Remember that the protein is transferred directly to the paper creating a mirrored image of what is on the gel. To keep the

alignment straight, the gel should be placed with the pre-stained MW standards on the right. Then place the paper with the clipped corner on the RIGHT. Make certain the corner is aligned with the top of the gel. The will result in a left to right correct oriented blotting paper.



- **Transfer Protein**

Close the sandwich holder and place assembly into the gel box. Be certain to align the black with black and clear side with the red side of the transfer apparatus. Fill the inner portion with transfer buffer, place the stir bar into the box and add the ice cooler container. Transfer the protein at 100 V (constant) for 1 hours. **Rule of thumb** = smaller proteins < 50 kDa will be transferred after 0.8 hours larger proteins > 100 kDa will take 1.5 hours). Alternatively the blot can be transferred overnight at 30V in the cold room.

## Step 2 Blotting and Detection

**Blocking** -Block for one hour to 30 min with 10 ml of 5% blocking buffer (TTBS and 5% dry milk [0.5 g dry milk per 10 ml TTBS]) on a rocking platform. This is a good stopping point. Seal it and leave the blot overnight in the cold room.

**Wash** - Decant the blocking buffer and add 10-20 ml of TTBS, wash on rocker for 5 min .

**Primary antibody incubation** - Decant TTBS and add the first antibody solution. Incubate for one hour (room temp) or overnight at 4°C. For RhoA and p-ERK use 1:1000 of the antibody in blocking buffer

**Wash** - Decant the primary antibody buffer (can be saved and reused several times. Depends on each primary. Store at 4°C) and add 10-20 ml of TTBS and wash three times for five minutes on a rocking platform.

**Secondary Antibody Incubation** - The most important part of this step is to make certain you use the correct secondary (2°) antibody. DO NOT INCUBATE FOR MORE THAN 1 HOUR AT ROOM TEMP!!!!!!

- 1) If you will detect with the Santa Cruz chemiluminescence kit, use a HRP (horseradish peroxidase) conjugated antibody.
  - If the 2° antibody is from BioRad use a 1:10,000 dilution (2 µl in 20 ml)
  - If the 2° antibody is from SantaCruz use a 1:20,000 dilution (1 µl in 20 ml of blocking buffer)
- 2) If you are using the BioRad Immunostar kit use the alkaline phosphatase (AP) linked (2°) antibody . Read those destructions for further information.

**Wash** - Decant the secondary antibody buffer and add 10-20 ml of TTBS and wash three to four times for five minutes on a rocking platform.

### Detection

- Get one disposable pipet, a clean blotting dish, 1 ml of Santa Cruz luminol A and 1 ml of B in separate microfuge tubes (add them together just prior to using them) and a forceps.
- Remove the membrane and drain excess liquid without letting the blot totally dry.
- Prepare the saran wrap and two or three pieces of kim wipes
- Pipet the substrate solution into the new dish and incubate the blot for about 5 min. This is important or the background may be very high.
- Remove the membrane, draining the excess liquid from the blot and place it on top of the kim wipes.
- Do not let the blot get dry or the signal will be lost.
- Expose the gel for several different exposures, starting with 1 min, 5 min and a 10 to 15 min exposure.

### Develop the Film

- Prepare both the developer and fixer. If the developer is older than 2 weeks or is very dark, dump it and make a new preparation.
  - 100 ml developer and 400 ml distilled water
  - 125 ml fixer and 375 ml fixer
- Fill a clean tub with water
- The film should be in the developer for 5 min (exactly – use my watch in the center desk drawer). Agitate the film every min or so.
- Rinse in the water bath for 30 sec with agitation.
- Fix the film for 3 min or so.
- Rinse for 2 to 5 min. Do not the time short cut or the film will turn chalky.
- Dry by hanging or leaning (do not lay flat)

### Solutions

	<u>10 X (1 liter)</u>	<u>1X (1 liter)</u>
<b>Transfer Buffer</b>	19.3 g Tris-Base 90.0 g Glycine pH should be 8.1 - 8.4 Do not adjust	100 ml 10 X 100 ml Methanol 800 ml H <sub>2</sub> O
<b>Phosphate buffered saline (1x PBS):</b>	9.1 mM dibasic sodium phosphate, 1.7 mM monobasic sodium phosphate 150 mM NaCl. Adjust pH to 7.4 with NaOH.	
<b>TTBS</b>	10 mM Tris-base, pH 8.0 150 mM NaCl. 0.05% Tween-20	1.21 g / liter 8.76 g / liter 0.5 ml / liter
<b>Blocking Buffer</b>	- 5% Non-fat dry milk in TTBS	2.5 g / 50 ml TTBS