# Western Transfer and Immunoblotting

## 1. Purpose

To perform electrophoretic transfer of proteins from pre-cast polyacrylamide gels onto a PVDF membrane to facilitate interrogation of a complex protein mixture by incubation with antibodies and detection with enhanced chemiluminescence.

## 2. Procedure

### 2.1 Western Transfer

As with SDS PAGE procedure, use appropriate buffer system and transfer apparatus throughout;

- NuPAGE 4-12% Bis-Tris gels (see 2.1.1 - Buffer system A - Materials)
- 6% Tris Glycine gels (see 2.1.2 - Buffer system B- Materials)

### 2.1.1 NUPAGE 4-12% Bis-Tris gels western transfer

Chill NuPAGE transfer buffer (Materials) on ice before use. Pre-wet PVDF membrane in methanol for 10 sec and then put into transfer buffer. Soak sponges and blotting paper in transfer buffer. Break open cassette with gel knife and remove gel from cassette using a piece of pre-wet blotting paper to assist gel handling. Assemble transfer stack in Invitrogen Xcell II blot module (Materials), taking care to avoid the introduction of air bubbles between the PVDF membrane and gel.

Invitrogen Xcell II blot assembly: (1 gel per module)

- Cathode
- 2 sponges
- 1 blotting paper
- Gel
- 1 PVDF membrane
- 1 blotting paper
- 4 sponges
- Anode

Slide transfer stack into Xcell SureLock Mini-cell, seal, then and add enough transfer buffer to just cover the sponges within the stack. Do not overfill central chamber as this will lead to excessive heat generation during transfer. Add water to the outside chamber to help diffuse any heat generated during transfer.

Connect Xcell II blot module to power source and transfer at 30 V for 1 h 30 min.

### 2.1.2 6% Tris Glycine gels western transfer

Chill Tris Glycine transfer buffer (Materials) on ice before use. Pre-wet PVDF membrane in methanol for 10 sec and then put into transfer buffer. Soak sponges and blotting paper in transfer buffer. Break open cassette with gel knife and remove gel from cassette using a piece of pre-wet blotting paper to assist gel handling. Assemble transfer stack in Bio-

Rad Mini-Transblot Electrophoretic Transfer cell (Materials), taking care to avoid the introduction of air bubbles between the PVDF membrane and gel.

For each sandwich position the gel nearer the black side of the cassette and position the black side of the cassette closest to the black electrode when inserting into the module (i.e. gel to black – black to black).

Bio-Rad Transfer cell assembly: (2 sandwiches per tank)

- Black side of cassette
- 1 sponge
- 1 blotting paper
- Gel
- 1 PVDF membrane
- 1 blotting paper
- 1 sponge
- Clear side of cassette

If only 1 gel is to be transferred, use spare cassette and sponges to fill up space in the transfer tank to avoid wasting buffer. Put ice block and magnetic stirrer in tank and fill the tank with transfer buffer. Place tank on magnetic stirrer plate. Connect tank to power source and transfer at 100V for 1 h 30 min.

2.2 Blocking, antibody incubations and washes

Remove PVDF membrane from western transfer assembly and put directly into a tray of PBS to prevent membrane from drying out (can re-wet with methanol if necessary).

Insert membrane (6.5cm X 7.5cm) without overlap into roller bottle (Materials).

Add 10 mL 5% milk PBS-T and block for 1-2 hr at RT on rollers (or o/night if necessary).

Dilute $1^{o}$ antibody in 5 mL 1% milk PBS-T and incubate on rollers at $4^{o}$C o/night (or 1 hr at RT if necessary).

Rinse blot quickly in PBS-T then wash blot for 15-20 min in PBS-T (3 x 20 mL).

Dilute $2^{o}$ HRP conjugated antibody in 5 mL 1% milk PBS-T and incubate on rollers at RT 1 hr.

Rinse blot quickly in PBS-T then wash blot for 15-20 min in PBS-T (3 x 20 mL).

Finally, rinse blot in PBS (no Tween) for 5min (1 x 20 mL).

2.3 Chemiluminescent detection

Bring Immobilon western chemiluminescent HRP substrate (Materials) to room temperature before using. Mix equal volumes of each reagent 1:1 in a Falcon tube and protect from light. Require a total volume of 2 mL (i.e. 1mL luminol + 1mL peroxide) per membrane in each roller tube.

Pour off final PBS wash. Add 2 mL of detection reagent to each roller bottle containing 1 membrane. Incubate on rollers for 2 min. Pour off reagent and remove membrane from roller bottle. Drain excess reagent onto tissue paper, then wrap blots in plastic wrap, with the protein side facing down on the smoothest surface and protect from light. Expose membrane to Kodak image station.

2.3 Exposure to Kodak Image Station 4000MM

Adjust settings on Kodak Image Station 4000MM (Materials);

- aperture is 0 for open
- focal plane is 0mm
- excitation filter is set to black
- emission is not filtered

Preview membrane position with lid open and adjust field of view/zoom to fit membrane.

Perform several exposures, typically 10 sec, 1 min and 5 min.

Capture white light exposure (lid open for ambient light, aperture 8, t = 0.05sec) to record position of protein ladder on membrane.

Save captured images as ".bip" files.

## 3. Materials

3.1 Chemical reagents

- Buffer system A - NUPAGE 4-12% Bis Tris gels
    - Invitrogen Xcell II blot module (Invitrogen EI002)
    - To make 1L 1x NuPAGE Transfer buffer with 10% methanol
        - 20x NuPAGE Transfer Buffer (Invitrogen NP0006-1) 50 mL
        - MilliQ-$H_20$ 850 mL
        - Methanol (BDH AnalaR 101586B) 100 mL
        - Chill transfer buffer on ice before use.
    - *Note: Invitrogen recommend transfers in the presence of 20% methanol for transfer of 2 gels in the same module or 10% methanol for 1 gel in module. However, transfer efficiency of the second gel can be poor, so it is recommended that only 1 gel is transferred per Xcell II blot module.*

- Buffer system B - 6% Tris Gly gels
    - Mini Transblot Electrophoretic Transfer Cell ( Bio-Rad 170-3930)
    - To make 1 L 10x Tris Glycine Transfer buffer stock without methanol
        - Tris (Amresco 0826) 14.5g
        - Glycine (Sigma G8898) 72g
        - MilliQ-$H_2O$ to 1L
    - To make 1L 1x Transfer buffer with 20% methanol
        - 10X Tris Glycine Transfer buffer 50 mL
        - MilliQ-$H_20$ 750 mL

    - Methanol (BDH AnalaR 101586B) 200 mL
    - Chill transfer buffer on ice before use

- To prepare 1L of 10x PBS-T
    - 10x PBS 1 L
    - Tween-20 (Sigma) 10 mL

- To prepare 500 mL of 1x PBS-T wash buffer
    - 10x PBS-T 50mL
    - Milli-Q $H_2O$ 450mL

- To prepare 50 mL 5% milk PBS-T blocking buffer
    - Skim milk powder (Marvel) 2.5 g
    - 1x PBS-T 50 mL

- To prepare 50 mL 1% milk PBS-T antibody incubation buffer
    - 5% milk blocking buffer 10 mL
    - 1x PBS-T 40 mL

- Immobilon western chemiluminescent HRP substrate (Millipore WBKLS0500)

- Details of core antibodies used

| **Antibody** | **Supplier** | **Cat. #** | **Species** | **Dilution** |
|---|---|---|---|---|
| anti-PSD95 | Affinity BioReagents | MA1-046 | Mouse | 1:20000 |
| anti-SAP102 | Santa Cruz Biotech. | sc-6925 | Goat | 1:2000 |
| anti-NR1 CT | Upstate | 05-432 | Mouse | 1:1000 |
| anti-NR2A | Upstate | 07-632 | Rabbit | 1:1000 |
| anti-NR2B (BWJHL) | Upstate | 05-920 | Mouse | 1:5000 |
| anti-Chapsyn110 (PSD93) | Neuromab | 75-057 | Mouse | 1:5000 |
| anti-GLUR1 (E-6) | Santa Cruz | sc-13152 | Mouse | 1:200 |
| anti-GLUR2 | Zymed laboratories | 32-0300 | Mouse | 1:1000 |
| Anti-CamKII | Chemicon-Millipore | MAB8699 | Mouse | 1:20000 |
| Anti-Dlg (SAP97) | BD Biosciences | 610875 | Mouse | 1:1000 |
| anti-rabbit HRP | Upstate | 12-348 | Goat | 1:10000 |
| anti-mouse HRP | Upstate | 12-349 | Goat | 1:15000 |
| anti-goat HRP | Abcam | ab6885 | Donkey | 1:30000 |

3.2 Equipment

- PVDF – Hybond-P membrane (GE Healthcare RPN 303F) – cut to 6.5 cm x 7.5 cm
- 3MM CHR paper (Whatman 3030-931) – cut to 7 cm x 8 cm
- Power supply – Power Pac (Bio-Rad)
- Zoom power supply adapters (Invitrogen ZA10001)
- Roller bottles 35 mL (Sarstedt 58.537)
- Push cap for roller bottle (Sarstedt 65.790)
- Plastic wrap – Saran
- Kodak Image Station 4000MM

- Kodak Molecular Imaging Software Version 4.0

## 4. Quality Control

Western blots that demonstrate obvious blotting artifacts such as significant, uneven background or signal fadeout are excluded from further analysis.

## 5. Example Data

## 6. Supporting Information

## 7. Document History

This document was created by Rachel T. Uren on 22 February 2008.