**MEI Protocol**

# Western Blotting

By Qiang Wang

A. Preparation of cell lysates

1. Lyse the cells (confluent 100 mm dishese) with 1 ml lysis buffer on ice for 10 min.
2. Spin at 12,000 rpm in an Eppendorf microfuge for 10 min at 4°C.
3. Transfer the supernatant to a new tube and discard the pellet.
4. Determine the protein concentration (Bradford assay, A280, or BCA)
   (We use the Bradford assay from Pierce.)
5. Take x µl (= y µg protein) and mix with x µl of 2x sample buffer.
6. Boil for 5 min.
7. Cool at RT for 5 min.
8. Flash spin to bring down condensation prior to loading gel.

B. Polyacrylamide gel (Bio-Rad Mini Protein III)

1. Resolving gel: 15 ml (for two gels) of a 10% gel
   5.9 ml water
   5.0 ml 30% acrylamide/bisacrylamide (29:1 mix)
   3.8 ml 1.5 M Tris (pH 8.8)
   150 µl 10% SDS
   150 µl 10% ammonium persulfate
   6 µl TEMED

2. Stacking gel: 6 ml
   4.1 ml water
   1 ml 30% acrylamide/bisacrylamide (29:1 mix)
   0.75 ml 1.0 M Tris (pH 6.8)
   60 µl 10% SDS
   6 µl TEMED
   60 µl 10% ammonium persulfate

C. Preparation of gel

1. Assemble the glass plates and spacers (1.5 mm thick).
2. Pour the running gel to about 1 cm below the wells of the comb (~6 ml).
3. Seal with 1 ml water-saturated 1-butanol.
   (Can stop here and leave gel as is overnight if you want.)

4. When gel has set, pour off the butanol and rinse with deionized water.
5. Pour the stacking gel (~3 ml) and insert the comb immediately.
6. When the stacking gel has set, place in gel rig and immerse in buffer.
7. Prior to running the gel, flush the wells out thoroughly with running buffer.

D. Running the gel

1. After flash spinning the samples, load into the wells.
2. Be sure to use markers.
   We use 5 µl Bio-Rad Prestained Standards Broad Range (#161-0318) directly.
3. Run with constant voltage (80-100 V).
4. Usual running time is about 2-2.5 hr.

E. Membrane transfer

1. Cut a piece of nitrocellulose membrane. (Schleicher&Schuell PROTRAN BA83).
2. Assemble "sandwich" for Bio-Rad's Transblot.
3. Prewet the sponges, filter papers (slightly bigger than gel) in 1x Transferring buffer.
   Sponge - filter paper - gel - membrane - filter paper - sponge
   Make sure gel side facing cathode (Black)
4. For the Mini-Transblot, it's 100 V for 1 hr with the cold pack and prechilled buffer. Bigger proteins might take longer to transfer.

F. Antibodies and detection

1. When finished, immerse membrane in Blocking buffer and block for 60 min at room temperature or overnight at 4°C.
2. Incubate with primary antibody diluted in Blocking buffer for 60 min at room temp or overnight at 4°C.
3. Wash 3 x 10 min with 0.05% Tween 20 in TBS.
4. Incubate with secondary antibody diluted in Blocking buffer for 45 min to 1 hr at room temp.
5. Wash 3 x 10 min with 0.05% Tween 20 in TBS.
6. Detect with Amersham ECL kit (RPN 2106).

G. Stripping blot

1. Rinse blot off with 0.05% Tween 20 in TBS.
2. Add about 5 to 10 ml Stripping buffer.
3. Incubate at 80°C for 20 min.
4. Rinse blot off with 0.05% Tween 20 in TBS.
5. Block for about 1 hr with 5% milk/Tween 20 at room temperature, or overnight at 4°C.

# Buffers for Westerns

Lysis buffer:
0.15 M NaCl
5 mM EDTA, pH 8
1% Triton X100
10 mM Tris-Cl, pH 7.4
Just before using add: 1:1000 5 M DTT
1 mM sodium vandadate
Varies Protease inhibitor
1:100 100 mM PMSF in isopropanol
1:1000 5 M ε–aminocaproic aci

2x sample buffer:
130 mM Tris-Cl, pH8.0
20% (v/v) Glycerol
4.6% (w/v) SDS
0.02% Bromophenol blue
2% DTT

10x Running buffer: 1 L
30.3 g Tris base (= 0.25 M)
144 g Glycine (= 1.92 M)
10 g SDS (= 1%)--add last
Do not adjust the pH!!

10x Transferring buffer: 1 L
30.3 g Tris base (= 0.25 M)
144 g Glycine (= 1.92 M)
pH should be 8.3; do not adjust

To make 2 L of 1x Transferring buffer:
400 ml Methanol
200 ml 10x Transferring buffer
1400 ml water

10x TBS (pH 7.6): 1L
24.2 g Tris Base (200 mM)
80 g Sodium Chloride (137 mM)
Adjust pH to 7.6 with HCl

Blocking buffer:
5% Skim Milk
Make up in TBS
Then add 0.05% Tween 20.
Keep at 4°C to prevent bacterial contamination.

Stripping buffer: (sterile filter solution and keep at 4°C)

0.2 M Glycine, pH 2.5
0.05% Tween 20