ABGENT CUSTOM SERVICES:

Western Blotting Protocol

A. Preparation of cell lysates

1. Collect cells (confluent T-25) by trypsinization and spin.
2. Lyze the pellet with 100 ul lysis buffer on ice for 10 min. For 500,000 cells, lyze with 20 ul.
3. Spin at 14,000 rpm (16,000 g) in an Eppendorf microfuge for 10 min at 4 ℃.
4. Transfer the supernatant to a new tube and discard the pellet.
5. Determine the protein concentration (Bradford assay, A280, or BCA) (We use the Bradford assay from Bio-Rad.)
6. Take x ul (= y ug protein) and mix with x ul of 2x sample buffer.
7. Boil for 5 min and cool at RT for 5 min.
8. Flash spin to bring down condensation prior to loading gel.

B. Polyacrylamide gel (14.5 cm × 16.5 cm)

1. Agarose plug:
   1% agarose dissolved in 1 ×Resolving gel buffer.
   (I make 50 ml, keep melting it as I need it, and re-adding water to maintain agarose conc.)
2. Resolving gel:
   24 ml of a 9% gel
   5.4 ml 40% acrylamide/bisacrylamide (29:1 mix)
   3 ml 8x Resolving gel buffer
   15.6 ml water
   12 ul TEMED
   60 ul 20% ammonium persulfate
3. Stacking gel: 8 ml
   1 ml 40% acrylamide/bisacrylamide (29:1 mix)
   2 ml 4 ×Stacking gel buffer
   5 ml water
   8 ul TEMED
   21.6 ul 20% ammonium persulfate

C. Preparation of gel

1. Assemble the glass plates and spacers (1.5 mm thick).
2. Pour an agarose plug (1-2 mm).
3. Pour the running gel to about 1 cm below the wells of the comb (~20 ml).
4. Seal with 1 ml water-saturated 1-butanol. (Can stop here and leave gel as is overnight if you want.)
5. When gel has set, pour off the butanol and rinse with deionized water.
6. Pour the stacking gel (~5 ml) and insert the comb immediately.
7. When the stacking gel has set, place in gel rig and immerse in buffer.
8. 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 15 ul Bio-Rad Kaleidoscope Prestained Standards #161-0324 directly.
3. Run with constant current (35-37 mA with voltage set at >150 V).
4. Usual running time is about 1.3 hr.

E. Using precast gels (Ready Gels from Bio-Rad)

1. Assemble gel in gel rig.
2. Prepare protein samples (10 ug will suffice).

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3. Use 5 ul of Kaleidoscope standard.
4. Run at 200 V (constant voltage) for 30 min.

F. Preparation of membrane

1. Cut a piece of PVDF membrane (Millipore Immobilon-P #IPVH 000 10).
2. Wet in methanol on a rocker at RT for 5 mins. Remove methanol and add 1x Transfer buffer until ready to use.

G. Membrane transfer

1. Assemble "sandwich" for Bio-Rad's Transblot.
2. Prewet the sponges, filter papers (slightly bigger than gel) in 1 ×Blotting buffer. Sponge - filter paper - gel - membrane - filter paper - sponge
3. Transfer for 1 hr at 15 volts at 4 ℃ on a stir plate. Bigger proteins might take longer to transfer. For the Mini-Transblot, it's 100 V for 1 hr with the cold pack and prechilled buffer.
4. Immerse membrane in Amido-Black stain 5 mins.
5. Destain 4 ×5 mins with destaining buffer.
6. When finished, immerse membrane in Blocking buffer and block for one hour at room temperature.

H. Antibodies and detection

1. Incubate with primary antibody diluted to 2 ug/ml in total volume of 3 mL in Blocking buffer for one hour at room temperature.
2. Wash 4 ×5 min with 0.05% Tween 20 in TBS.
3. Incubate with secondary antibody diluted 1:10,000 (HRPOanti-rabbit) in Blocking buffer for 1 hour at room temp.
4. Wash 4 ×5 min with 0.05% Tween 20 in TBS.
5. Detect with Pierce Chemiluminescent kit (Prod # 34080).

I. Stripping blot

1. Rinse blot off with 0.05% Tween 20 in PBS.
2. Put blot into Kapak bag cut to slightly bigger size than blot.
3. Add about 5 to 10 ml Stripping buffer.
4. Remove as much air as possible and seal bag.
5. Immerse into 80 ℃ water bath and incubate for 20 min.
6. Rinse blot off with 0.05% Tween 20 in PBS.
7. Block for about 1 hr with 5% BSA/Tween 20, or overnight with 3% BSA/Tween 20.

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
1:1000 100 mM PMSF in isopropanol
1:1000 5 M ε−aminocaproic acid

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

8x Resolving gel buffer: 100 ml
0.8 g SDS (add last)
36.3 g Trizma base (= 3 M)
Adjust pH to 8.8 with concentrated HCl

4x Stacking gel buffer: 100 ml
0.4 g SDS (add last)
6.05 g Trizma base (= 0.5 M)
Adjust pH to 6.8

10x Running buffer: 1 L
30.3 g Trizma base (= 0.25 M)
144 g Glycine (= 1.92 M)
10 g SDS (= 1%)--add last

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Do not adjust the pH!!

10x Blotting buffer: 1 L

30.3 g Trizma base (= 0.25 M)
144 g Glycine (= 1.92 M)
pH should be 8.3; do not adjust
To make 2 L of 1x Blotting buffer:
400 ml Methanol
200 ml 10 × Blotting buffer
1400 ml water

Blocking buffer: 0.5 L

3% Bovine serum albumin (Fraction V)
Make up in PBS and sterile filter.
Then add 0.05% Tween 20.
Keep at 4 ℃ to prevent bacterial contamination.

Stripping buffer:

0.5 L (sterile filter solution and keep at 4 ℃)
0.2 M Glycine, pH 2.5
0.05% Tween 20

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