

Western Protocol

Gel preparation using Mini-PROTEAN 3 Cell Assembly (BIO-RAD):

1. Place a short plate on top of the spacer plate
2. Slide the two plates into the casting frame, keeping the short plate facing front. Insure both plates are flush at the bottom on a level surface.
3. Lock the pressure cams to secure the glass plates.
4. Engage the spring loaded lever and place the gel cassette assembly on the gray casting stand gasket. Insure the horizontal ribs on the back of the casting frame are flush against the face of the casting stand and the glass plates are perpendicular to the level surface. The lever pushes the Spacer Plate down against the gray rubber gaskets.
5. Place comb in between glass plates. Only put teeth in between glass. Mark 1 cm below where teeth are on outside of glass plate.
6. Pour separating gel into 50 ml beaker: see recipes below
7. Take up 7 mls of gel using 5 ml pipet. Put pipet on edge of glass and hold at a 90 degree angle and begin filling to mark made on glass plate.
8. Add 500ul of H₂O. Let sit for 45 minutes.
9. When gel is polymerized, invert apparatus and empty H₂O. Blot with Whatman.
10. **Make stacking gel:**
 - 1.6 mls of 30% Acrylamide/0.8% Bis
 - 3 mls upper buffer
 - 7.2 mls Di H₂O
 - 60uls 10% APS
 - 12uls Temed
11. Pour in stacking gel. Place comb into gel. Let sit for 45 minutes.
12. Remove the gel cassette sandwich from the casting frame and place it into the electrode assembly with the short plate facing inward.
13. Slide the gel cassette sandwich and electrode assembly into the clamping frame.
14. Press down the electrode assembly while closing the two cam levers of the clamping frame.
15. Lower into the mini tank and add 400mls of 1x Running Buffer.

Sample Preparation:

1. Dilute sample if necessary in dilution buffer below (or RIPA)
2. For whole cell preps, typically use 50-100ug of protein per lane (20-50ug for nuclear)
3. For each sample, add desired amount of protein to a micro centrifuge tube followed by 6x SDS Sample buffer or 2x Laemmli (which can be purchased from Bio-RAD) Note: BME should be added fresh to the loading buffer
4. For the protein markers, (Rainbow-Amersham) add 5ul of markers to tube and 1ul of 6x sample buffer or 5ul of 2x Laemmli buffer. (Note: if you want to be able to see the marker while the gel is running, load 7-8ul instead of 5ul.
5. Incubate at 95-100°C for 3 minutes.
6. Quick spin the sample
7. Load on gel
8. Note: All sample volumes can be different as long as the total protein amounts are the same.

Running gel:

1. Once gel is polymerized, remove gel sandwich from casting stand and insert into inner cooling core of running rig.
2. Fill inner core with 1X running buffer. Fill outer chamber with 1X running buffer until half of the screws are covered. (make 400ml for mini rig, 500ml/gel for Criterion)
3. Blow out wells. Load samples. Run at 75 volts for 1-2 hours or longer depending on the amount of separation that you need.
4. Before gel is done running, get out 2-3 pyrex dishes and fill with transfer buffer.
5. Soak 2 filter papers and 2 fiber pads per gel for at least 5 minutes.
6. Soak membrane in transfer buffer for at least 5 minutes.
7. Stop electrophoresis.

Setting up Transfer sandwich:

1. Remove gel. Separate plates. Use spacers to separate. For Criterion rig, use lid to help pop open the cassette. Cut off stacking gel/wells with razor or spacers.
2. Put plates into transfer buffer and nudge gel off plate with spatula into the transfer buffer
3. Soak gel for 10 minutes (this is necessary to ensure that the gel does not shrink during the transfer and cause a blurred signal)
4. Open sandwich with black side down and stack in this order: fiber pad, filter paper, gel, membrane, filter paper, fiber pad. Make sure to roll out bubbles after gel, membrane and second filter paper.
5. Close and put into transfer chamber. Black to back. Put in frozen block. Fill transfer chamber with transfer buffer. Can use the buffer that you had used for the pre-soak above.
6. Transfer at 30V overnight in the cold room (amps should not exceed .04)
OR at 100V for 1-2 hours on bench changing ice pack half way through if needed.

Washes and Detection:

1. Open sandwich. Put membrane in clean container (pipet tip box for example).
2. Rinse membrane with dH₂O 2-4x.
3. Block with 50mls 1 hr RT (Blocking buffer: 5% non-fat dry milk in TBS-T)
4. Wash with TBS-T 3x10min at RT.
5. Add primary antibody and incubate for 1 hour at RT or overnight at 4C.
6. Wash in TBS-T 3x10 minutes at RT.
7. Add secondary antibody at 1:5000 in TBST with 5% milk and incubate for 1 hour at RT. (Some antibodies may work better with 1:3000 or without the milk. Check with lab members for prior conditions that have been used for your specific antibody.)
8. Wash in TBS-T 3 x 10min at RT.
9. Drip dry the membrane onto a paper towel and lie flat on a piece of Saran Wrap with the protein side facing up.
10. Mix 3 mls of ECL Reagent 1 with 3 mls of Reagent 2 into a 15ml conical tube and pour onto the membrane. Let incubate for 1 minute.
11. Dump solution off of membrane and drip dry on paper towel.
12. Cover membrane with saran wrap. Expose to film for short time immediately for 1 minute, 5 minute etc depending on strength of signal. A few different exposures will likely be needed.

Membrane:

Nitrocellulose

MidSci: catalog number 10-402-480

For 7.5% gel—

5mls 30% Acrylamide/.8% Bis

5mls 4X Lower buffer

10mls Di H₂O MQ H₂O

100uls 10% APS

20uls Temed

For 10% gel—

6.7mls 30% Acrylamide/.8% Bis

5mls 4X Lower buffer

8.3mls Di H₂O MQ H₂O

100uls 10% APS

20uls Temed

Dilution buffer

5ml of 15 mM EGTA and .3 mM DTT

375 ul 20% SDS

120ul 100mM PMSF

1.8ul 10mg/ml leupeptin

6x SDS.Sample Buffer (for SDS-PAGE) Current Protocol Mol. Biol. pg. 10-2A-28

7 ml 4 x Tris.Cl/SDS, pH 6.8
3.8 g (3 ml) glycerol (30% final)
1 g SDS (10% final) (weigh in hood)
0.93 g DTT (0.6M) or 5% β ME (.5ml)
1.2 mg bromphenol blue (0.012%)
H₂O to 10 ml
Store in 0.5 ml aliquots at -20

4 x Tris-Cl/SDS, pH 6.8 (0.5 M Tris-Cl with 0.4% SDS)

6.05 g Tris base in 40 ml H₂O
Adjust pH to 6.8 with 1N HCl
Add H₂O to 100 ml total
Filter 0.45 μ m filter
Add 0.4 g SDS (weigh in hood)
Store up to 1 month

2X Sample Buffer (store in +4)

1 ml of .5 M Tris-HCl pH 6.8 (use upper buffer)
1 ml of 10% SDS (weigh in hood)
1 ml of glycerol
250 μ l .025-.05% Bromophenol blue
0.1 ml B-mercaptoethanol

4x Lower Buffer: 1.5MTris-HCl, 0.4% SDS pH 8.8

191.7g Tris base
4g SDS (weigh in hood)
750ml H₂O
Adjust pH to 8.8 with conc HCl
QS to 1L with H₂O
Stable at RT

Upper Buffer: 0.5MTris-HCl, 0.4% SDS pH 6.8

60.6g Tris base
4g SDS (weigh in hood)
750ml H₂O
Adjust pH to 6.8 with conc HCl
QS to 1L with H₂O
Stable at RT

10x Running Buffer: 0.25M TRis-HCl, 1.9M Glycine, 1% SDS pH 8.6

15.15g Tris base

71.3g Glycine

5g SDS (weigh in hood)

375ml H₂O

Adjust pH to 8.6 with conc HCl

QS to 500ml with H₂O

Stable at RT

Transfer Buffer: 25mM Tris-HCl, 192mM Glycine, 20% (v/v)methanol, pH 8.3

6.06g Tris base

28.8g glycine

400ml methanol (HPLC grade)

Make to 2L with H₂O

Check pH with pH paper after chilled, should be about 8.3

10x TBS

24.08g Tris base

80g NaCl

13ml 6M HCl, pH should be 7.6

QS to 1L

Before use, dilute to 1x and add 1ml of Tween-20 to 1L of 1X TBS

-This will be called 1x TBS-T