# Western Blotting For Protein Analysis

## Part 1: Laemmli Gel Electrophoresis Using Mini-PROTEAN II Electrophoresis Cell

Note: Powder-free gloves should be worn throughout the entire procedure.

### A. Preparing the Glass Plate/Clamp Assembly

Equipment needed per gel:
- One longer outer glass plate
- One shorter inner plate
- Two spacers
- One clamp assembly
- One casting stand with silicone gasket
- One comb (10 or 15 well)

Note: Ensure that glass plates, spacers, comb and casting stand gasket are cleaned with deionized water and 100% ethanol and allowed to dry.

1. Lay the longer glass plate down first, then place two spacers along the short edges of the rectangular plate. Next, place the shorter glass plate on top of the spacers so that the bottom ends of the spacers and the glass plates are aligned.

2. Loosen the four screws on the clamp assembly and stand it up so that the screws are facing away from you. Grasp the glass plate sandwich with the longer plate facing away from you, and gently slide it into the clamp assembly along the front face of the acrylic plate with the longer glass plate against the acrylic plate. Tighten the two top screws of the clamp assembly.

3. Place the glass plate/clamp assembly into the alignment area of the casting stand so that the screws face away from you. Loosen the two top screws and allow the plates and spacers to settle against the casting stand base. Ensure that the spacers are vertical and against the clamps. Gently tighten all four screws.

4. Remove the glass plate/clamp assembly from the casting stand and check to see that the bottom ends of the spacers and glass plates are flush. Note: This step is critical to ensuring that the gel will not leak out.

5. Place a cleaned silicone gasket on top of the red foam pad on the casting stand. If you are casting two gels first use the foam pad opposite the alignment area.

6. Place the glass plate/clamp assembly on the silicone gasket with the screws facing away from you and the bottom edge of the acrylic plate against the wall of the casting slot. Snap the acrylic plate underneath the overhang of the casting slot by pressing down on the top of the clamps. The glass plate/clamp assembly is now ready for the gel to be cast.

## B. Casting the Gel

Laemmli discontinuous gels consist of an upper or stacking gel and a lower or resolving gel. The stacking gel acts to concentrate the protein samples resulting in better band resolution in the resolving or separating gel. For most purposes the acrylamide concentration of the stacking gel will be 4% while that of the resolving gel will range from 5-20% depending on the size of the protein to be analyzed.

### Stacking Gel Preparation - 4.0% gel, 0.125 M Tris, pH 6.8

| | |
|---|---|
| Deionized water | 6.1 mL |
| 0.5M Tris-Hcl, pH 6.8 | 2.5 mL |
| 10% SDS | 100 µL |
| Acrylamide/bis 29:1 (30%) | 1.33 mL |
| 10% ammonium persulfate | 50 µL |
| TEMED | 10 µL |
| Total | 10 mL |

### Resolving Gel Preparation - 0.375 M Tris, pH 8.8

| | 5% | 7.5% | 12% | 15% | 20% |
|---|---|---|---|---|---|
| Deionized water | 5.68 mL | 4.85 mL | 3.35 mL | 2.35 mL | 0.68 mL |
| 1.5M Tris-Hcl pH8.8 | 2.5 mL | 2.5 mL | 2.5 mL | 2.5 mL | 2.5 mL |
| 10% SDS | 100 µL | 100 µL | 100 µL | 100 µL | 100 µL |
| Acrylamide/Bis 29:1 (30%) | 1.67 mL | 2.5 mL | 4.0 mL | 5.0 mL | 6.67 mL |
| 10% Ammonium persulfate | 50 µL | 50 µL | 50 µL | 50 µL | 50 µL |
| TEMED | 5 µL | 5 µL | 5 µL | 5 µL | 5 µL |
| TOTAL | 10 mL | 10 mL | 10 mL | 10 mL | 10 mL |

**Note:** 10 mL is sufficient for two gels.

1. In 15 mL centrifuge tubes combine all the solutions except the ammonium persulfate (APS) and the TEMED.

2. Place a comb completely into the assembled gel sandwich. With a marker pen, place a mark on the glass plate 1 cm below the teeth of the comb. This is the level to which the resolving gel is poured. Remove the comb.
3. Add the APS and TEMED to the resolving gel solution and mix gently. Draw the gel solution up into a 10 mL pipet and gently pour it down the side of one of the spacers. Immediately overlay the gel solution with deionized water using a syringe and needle. This must be done slowly and evenly in order to prevent mixing with the gel solution.

4. Allow the gel to polymerize for 45 minutes to 1 hour. Pour off the water overlay. Add the APS and TEMED to the stacking gel solution and mix gently. Dry the area above the resolving gel with Whatman paper and pour the stacking gel as before until it begins to overflow. Insert the appropriate comb to the point where the T portion of the comb rests on the top of the spacers.

5. Allow the gel to polymerize for 30 -45 minutes. Remove the comb by pulling it straight up slowly and gently. Rinse the wells 2 or 3X with deionized water.

## C. Assembling the Buffer Chamber

1. Release the glass plate/clamp assembly from the casting stand.

2. Lay the electrode assembly flat on the bench. With the glass plates of the gel sandwich facing the electrode (and the clamp screws facing out), carefully slide the clamp assembly wedges underneath the locator slots on the electrode assembly. Snap the clamp assembly onto the electrode by pressing at the bottom at the bottom of the clamp assembly until the electrode assembly latch engages each side of the clamp assembly.

3. Turn the electrode assembly over and repeat with a second glass plate/clamp assembly. If you are running only one gel then you must install a blank or dummy gel. This can be made by placing a short and long plate without spacers in the clamp assembly and aligning them on the casting stand as before. Tighten the clamp screws and insert into the electrode assembly.

## D. Loading the samples

### 5X Running Buffer, pH 8.3

| | |
|---|---|
| Tris base | 15 g/L |
| Glycine | 72 g/L |
| SDS | 3 g/L |

in deionized water

Store at 4 °C. Dilute 200 mL 5X stock with 800 mL deionized water for one electrophoresis run.

1. Lower the electrode assembly into the tank. Prepare 1 L of running buffer and fill the inner chamber and the outer chamber until the level reaches halfway between the short and long plates. Remove any air bubbles from the bottom of the gel by swirling a 10 mL pipet so that there is good electrical contact.

2. Using a gel loading pipet tip load the samples, being careful to avoid overflow of the sample into adjoining wells and air bubbles in the sample that can cause the sample to flow upwards and into adjacent wells.

## E. Running the Gel

1. Place the lid on the electrode matching the colours of the plugs on the lid with the jacks on the electrode. Attach the leads to the power supply.

2. Set the power supply to 200 volts, constant voltage. The usual run time is 45 minutes. The current should be approximately 60 mA per gel at the beginning of the run and will drop to approximately 30 mA per gel by the end of the run.

## F. Removing the Gel

1. After the electrophoresis is complete, turn off the power supply and disconnect the electrical leads.

2. Remove the lid and pull the electrode assembly out of the tank. Pour out the buffer and lay the assembly down.Remove the clamp assembly by pushing down on both sides of the electrode latch and up on the clamps until the clamp assembly is released. Slide the clamp assembly away from the electrode.

3. Loosen all four screws of the clamp assembly and remove the glass plate sandwich from it.

4. Gently twist one of the spacers so that the glass plates are separated with the gel adhering to one of the plates. With one of the spacers, cut off the bottom right-hand corner of the gel in order to orient it. Also cut off the stacking gel. With a wet, gloved finger loosen the gel from the plate and allow it to fall into a bath of transfer buffer to equilibrate for 15 minutes.

# Part II: SDS-PAGE Protein Transfer (Western)

## A. Preparation for Blotting

1. Prepare transfer buffer.

| | |
|---|---|
| Tris base | 3.03 g |
| Glycine | 14.4 g |
| SDS (optional) | 1.0 g |
| Methanol | 200 mL |
| Deionized water | bring up to 1 litre |

Refrigerate. Buffer should be at 4 °C at the start of transfer.

2. Cut membrane to the dimensions of the gel and wet it by slowly sliding it into transfer buffer and allowing it to soak for 15-30 minutes. Cut two pieces of thick filter paper per gel to size and soak in transfer buffer along with two fibre pads.

Note: For most immunodetection protocols the membrane can be either nitrocellulose (Protran) or PVDF. PVDF must be pre-wet in methanol, then water before equilibration in transfer buffer. Check with package for instructions.

3. Prepare the plastic cooling units by filling with water and freezing ahead of time.

## B. Assembling the Gel Holder Cassette

1. Open the gel holder cassette by sliding and lifting the latch. Place it in a shallow plastic nalgene box with the gray panel down and the clear panel resting on an angle against the wall of the box.

2. Place a pre-soaked fibre pad on the gray panel of the cassette holder. Centre on the cassette. Place a piece of pre-soaked filter paper on top of the fibre pad. Pour 2-3 mL of transfer buffer on top of the filter paper. Place the equilibrated gel on top of the filter paper, centered in the cassette, with the notched corner in the lower right side.

3. Flood the surface of the gel with transfer buffer and lower the pre-wetted membrane on top of the gel. Using a wet, gloved figure chase out any air bubbles that may be trapped between the gel and the membrane and apply pressure over the entire area of the gel to ensure a good contact between the gel and the membrane.

4. Flood the surface of the membrane with transfer buffer. Complete the sandwich by placing a piece of pre-soaked filter paper on top of the membrane and placing a pre-soaked fibre pad on top of the filter paper.

5. Close the cassette. Hold it firmly so the sandwich will not move and close the latch. Place the casette in the in the electrode assembly so that the grey panel of the cassette holder is facing the black electrode panel. Place the electrode assembly into the tank so that the terminals are in the centre of the tank.

6. If two gels are to be transferred repeat steps 1-5.

7. Centre the buffer tank on top of a non-heating magnetic stirrer. Place a 1" stir bar in the bottom of the tank. If the transfer will be at high voltage (>30 volts) place a frozen cooling unit in the tank. Fill the tank with transfer buffer to just above the level of the top row of circles on the gel cassette holder. Put the lid in place, matching the colour of the electrode leads to the appropriate pins on the electrode assembly. Plug the leads into a power supply.

### C. Running Conditions

1. Standard conditions for the transfer are: 100 volts for 1-2 hours with the cooling unit changed every 45 minutes or 30 volts overnight without a cooling unit.

### D. Removing the Membrane

1. When the run is finished stop the power supply and turn it off. Remove the lid from the tank and lift the electrode assembly out of the tank. Pull out the cassette and open the latch. Separate the sandwhich. If PVDF was used follow the manufacturer's instructions for drying the membrane. If nitrocellulose was used do not allow it to dry out. Either wrap it wet in Saran wrap and store at 4 °C or place directly in TBST until beginning immunodetection.

## Part III: Immunodetection

### A. Preparation for Immunodetection

1. Prepare Tris-buffered saline- Tween 0.1% (TBST).

| | |
|---|---|
| Tris base | 2.42 g |
| Sodium chloride | 8.0 g |
| Deionized water | bring to 1.0 L |
| Tween 20 | 1.0 mL |

Store at room temperature or 4 °C.

2. Prepare blocking solution

-5% blocking reagent from kit or 5% skim milk powder in TBST. It is stable for 1 hour at room temperature and 24 hours at 4 °C.

## B. Immunodetection

1. Block membrane for 1 hour in blocking solution on an orbital shaker.

2. Incubate in primary antibody diluted in blocking solution on an orbital shaker or in a sealed bag on a nutator for 1 hour at room temperature or longer at 4 °C.

3. Wash in TBST. Standard procedure is to quickly rinse 3X and then wash 3X for 5 minutes in a large volume (~ 50 mL).

4. Incubate in secondary antibody diluted in blocking solution on an orbital shaker or in a sealed bag on a nutator for 1 hour at room temperature or longer at 4 °C.

5. Wash in TBST as in step 3 and proceed to detection step.

Note: Dilution and incubation times vary from antibody to antibody. Check with supplier for optimized conditions or perform a dilution/incubation time test. For HRP-labelled secondary antibodies from the NEB Phototype-HRP kit the recommended dilution is 1:1000. This kit includes a biotinylated protein molecular weight standard so if this was run on the gel also probe with the anti-biotin HRP-labelled antibody during the secondary antibody incubation.

6. For the NEB Phototype-HRP kit prepare the LumiGlo substrate. Mix 0.5 mL solution A with 0.5 mL solution B and bring up to 10 mL with deionized water. Incubate the membrane in the LumiGlo substrate for 1 minute with gentle agitation. Drain off excess substrate and wrap in Saran wrap. Do not let the membrane dry.