**PhosTag SDS PAGE and Western Blot Protocol**

**Loading Gel & Running Electrophoresis**

1. After the wells of the stacking gel have been rinsed and samples desired to analyse have been set on ice to thraw;

- Clip the gel-plates to buffer chamber with the red clamps [longer side facing you].

- Fill the buffer chamber [bottom to top] with [Running Buffer](#Running_Buffer) [put in fridge to cool].

2. Place the well decal [plastic template] on the glass plate and fill the sample wells. Use the P10 pipette

- Leave lane 1 blank

- Load 5 ul Ladder into lane 2 (4µL of ladder + 1 µL of [10 mmol/L MnCl2 Solution](#MnCl))

- Load controls into the next lane(s)

- Load samples in the rest of the lanes

3. Connect the buffer chamber with the voltage system,

- **Run Gel nice and slow at 20 min at 20 mA per gel followed by 140-160 mV for at least 2 hour (or until protein has reached bottom).**

**Western-blot (after electrophoresis)**

1. Separate the glass plate from the aluminium plate (keeping the gel on the aluminium plate)
2. Cut the well off at the line between the stacking and running gel
3. Cut the bottom left corner off the get (I do this so I have a reference to which is side is the front of the gel in case it flips during washes.
4. Wash gels in [transfer buffer + 10 mM EDTA](#Transfer_Buffer) (to complex the Mn2+) for 10 minutes
5. Meanwhile, cut the PDVF membrane roughly bigger than the gels and soak in methanol for 20 minutes then in [transfer buffer](#Transfer_Buffer)
6. Wash the gels three more times in transfer buffer without EDTA.
7. Make a sandwich of the gel for transfer in this order;
   1. Black side of casket
   2. Foam sheet
   3. Filter paper
   4. Gel
   5. PDVF membrane (cut to match gel perfectly – including bottom left corner)
   6. Filter paper
   7. Foam sheet
   8. Grey side of casket
   9. Place casket (clips side down) with the Black side of casket faceing the back (where the negative (black) probe will be)
8. Transfer samples onto PDVF membrane using the wet transfer chamber for 2-4 hours at 140-160 mV or 1 mA/cm2 (roughly 50 mA for a mini-gel).

Stain membrane with Ponceau and remove background stain with 5% (v/v) acetic acid to confirm protein transfer (scan the membrane). Wash the membrane with TBS-T (10 mM Tris-HCl pH 8, 150 mM NaCl, 0.05% (v/v) Tween-20) to remove Ponceau stain. Block the membrane with TBS-T containing 5% (w/v) semi-dried milk powder for 1h at room temperature.

F] Transferring proteins [causing proteins to move out of the gel and onto the membrane]

1. Cut the nitrocellulose gel to fit the well decal Cut off the stacking gel where the wells are located so it is a square gel. Place the membrane in the Tupperware box. Pour transfer buffer over it.

-Label “TBL” for Top Back Left on the membrane

-Label “1” and “10” on the other side

Now take the glass plates apart gently and lift the spacer out. Using the space cut the BOTTOM LEFT portion of the gel.

2. Lift the gel sheet up and place it delicately in the transfer buffer solution [in the Tupperware case] and rock for few minutes.

3. Rock the membranes and the gel in transfer buffer for 20 minutes.

4. Take the western blot kit buffer case, dip the sponge in transfer buffer, followed by dipping 2 pieces of filter paper in the transfer buffer

5. Place the filter paper on the sponge.

6. Lift the gel and place it on the paper [close to black side]. Avoid air bubbles. Put the membrane on it, followed by 2 pieces of paper and another sponge.

-Sandwich Cassette = Sponge, Filter Paper, Filter Paper, Gel, Membrane, Filter Paper, Filter Paper, Sponge

7. Lock the case and place it vertically in the blotting chamber.

-place the hinges of the cassette facing upwards

-Black to Black & Red to Red

-Gel to the black and Membrane to the red electrodes

-Place the black part of the cassette to the black electrodes

-Place the grey part of the cassette to the red electrodes

G] Blotting the proteins

1. Fill the chamber with transfer buffer and put the case [containing sponge, membrane, and gels sheet].

2. Put a stir bar in the bottom of the chamber so the transfer buffer is mixing while the machine runs. Set the stir bar to 3 and a half.

3. Leave the chamber connected at 50 mA for 2 and half – 3 hours.