**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 thaw.

- Put gel-plates in buffer chamber with glass side facing out

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

- Mark the top of each well with a marker on each gel-plate

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

2. 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. Take buffer chamber to fridge then fill the rest of the chamber with the remaining Running Buffer

4. Connect the buffer chamber with the voltage system (red on lid goes to red on chamber)

- **Run Gel for 2 hours @ 150 mV constant volt or until color portion is about an inch from the bottom of the gel**

**Western-blot (after electrophoresis)**

1. Before electrophoresis ends:

* Make [transfer buffer](#Transfer_Buffer)
* Cut two nitrocellulose membraned the size of the filter paper. With the shiny side (inside of the roll) facing up, cut the bottom right corner of each membrane
* Put four pieces of filter paper inside the transfer cassettes then in the transfer chamber. Fill the transfer chamber half way with transfer buffer

1. Take running chamber out of fridge and dump out running buffer
2. Take off red clips and slip gel-plates out of the chamber
3. Separate the glass plate from the aluminium plate (keeping the gel on the aluminium plate)
4. Cut the well off at the line between the stacking and running gel using a razor blade
5. Cut the gel just below the color line and then 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).
6. Wash gels in [transfer buffer + 10 mM EDTA](#Transfer_Buffer) (to complex the Mn2+) for 10 minutes
7. Meanwhile, cut the PDVF membrane roughly bigger than the gels and soak in methanol for 20 minutes then in [transfer buffer](#Transfer_Buffer)
8. Wash the gels and membranes in transfer buffer without EDTA for 10 minutes
9. 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. Nitrocellulose membrane (shiny side of membrane facing down on the gel so the cut corners of the gel and the membrane line up)
   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)
10. Take running chamber to fridge and fill to max line with transfer buffer
11. Put lid on transfer chamber with black probe at the back of the chamber and red probe at the front of the chamber
12. Transfer samples onto membrane using the wet transfer chamber for 1.5 hours @ 50mA constant amp.

**Blocking**

1. Before transfer has finished, make TBS-Tween + Milk
2. Save 40 mL of TBS-Tween + Milk for antibodies later on
3. Remove transfer chamber from fridge and take out cassette
4. Carefully remove the membrane from the gel and place membranes in light sensitive black boxes with TBS-Tween and rock for 5 minutes
5. Pour out TBS-Tween and pour in TBS-Tween + Milk to boxes with membranes
6. Block the membranes for 1.5 hours.

**Primary Antibody**

1. After blocking, wash membraned in TBST 3 times for 5 minutes each
2. During the last wash, make primary antibody dilution using 10mL of TBST + Milk for each membrane
3. After washing membranes, put primary antibody in box with membranes and rock overnight in fridge.

**Secondary Antibody**

1. Take membranes out of fridge from overnight rocking and pour out primary antibody
2. Wash membranes in TBST 3 times for 5 minutes each
3. **Turn off lights to make secondary antibody dilution** using 10mL of TBST + Milk for each membrane
4. Pour out last wash of TBST and pour in secondary antibody dilution to black light sensitive boxes
5. Places boxes with membranes on rocker under tin foil and let rock for 1 hour

**Imaging – All imaging must be done with lights off**

1. Wash membranes after secondary antibodies in TBST 3 times for 5 minutes each
2. If a HRP-Conjugated secondary antibody was used, use ECL signalling reagent to develop by combining 3mL of each reagent and placing membrane in mixture for 60 seconds before imaging
   * Take membrane out of reagent and place face down on chemidoc in room 531
   * Use chemiluminescent blot application to image membranes
3. If a Alexa Fluor conjugated secondary antibody was used, take membranes out of TBST and place between two pieces of filter paper and in a dark box to let dry for 10 minutes
   * Take membrane out of filter paper and place face down on chemidoc in room 518
   * Image with wavelength indicated on secondary antibody

\*\*After imaging you may want to perform a Ponceau total protein stain on the membranes. See Ponceau Stain Protocol for more information\*\*