**PhosTag Gel Making Protocol**

**Making Gel Caster:**

* + - 1. Preparation of gel plates/Gel caster
         1. Put the glass plates and Aluminum backing plates [with a 0.75 mm spacer] on a clean surface [like a paper towel].
         2. Spray Clean with 100% methanol and Kim-wipe. [PS: they are expensive so don’t break.] – Clean – sweeky clean!!!
      2. Putting the gel plates on the gel caster unit to create the gel-plate sandwich
         1. Insert the spacers [black ones] on the sides of the plates [1 for each side, so 2 for 1 pair of plates]. This must be done carefully until the spacers fixed in place.
         2. Insert the gel-plates pair in the gel stand vertically with the glass surface facing you.

Make sure all edges are flush or it will leak.

Make it tight, but not too tight

* + - 1. Screw the plates with the little grey screws, not too tight but just enough to hold the gel.
      2. The gel seal [contained in a small white tube] is used on the corners [edges which face opposite the open surface]. [GELSEAL 5GR TUBE or Vaseline]
         1. Put some gel across the bottom of the glass and aluminum
      3. Parafilm is used as a suspender, and a neatly cut parafilm strip is doubly folded and kept on the surface of the gel caster unit.
      4. Put the gel stand in the gel caster and use large black screws clamps on the sides, by turning them and pushing in and up 180 degrees [don’t over rotate them].
         1. Put red clamps on the top of the gel plate and aluminum backing plate.

**Making the PhostagTM-SDS-PAGE:**

\*\*Prepare separating Phostag-SDS-PAGE gel according to manufacturer’s instructions. For this protocol, we’ll be making 2 – 12% gels for cTnI.

Preparation of the gel

1. We need to prepare a running gel and a stacking gel.

-Separating Gel: aka running gel, slightly basic [pH = 8.8] with a [higher acrylamide], has narrow pores in the gel

-Stacking Gel: slightly acidic [pH = 6.8] with a lower [acrylamide], has a porous structure

-Smaller proteins travel more easily and rapidly whereas larger proteins will be “stuck” at the top of the gel

the separating gel contained 12% (w/v) acrylamide, 50 uM Phostag-SDS-PAGE, 100 uM MnCl2, buffers according to Laemmli recipe, APS and TEMED). Let gels polymerize at room temperature for at least 1h. Pour stacking gel (Laemmli recipe without Phostag reagent) on top, add combs and let polymerize for 1h. \*\*make APS fresh weekly\*\*

For roughly equal loading adjust sample volume according to standard SDS-PAGE. Most pre-stained molecular weight markers contain 0.1 mM EDTA which might distort protein bands. You can add 1 mM MnCl2 to markers to complex the free EDTA. Run ventricular samples for 20 min at 20 mA/gel followed by 140-160 mV for at least 2h..

Preparation of RUNNING GEL

**Resolving Gel Solution (10 mL: 12% (w/v) acrylamide & 50 µmol/L Phos-tagTM AAL)**

1. Combine:
   1. 4.00 mL [30% (w/v) Acrylamide Solution](#Acrylamide_solution)
   2. 2.50 mL [1.5 mol/L Tris/HCl Solution, pH 8.8](#TrisHCL)
   3. 100 µL [5.0 mmol/L Phos-tagTM AAL Solution](#PhosTagSoL)
   4. 100 µL [10 mmol/L MnCl2 Solution](#MnCl)
   5. 100 µL [10% (w/v) SDS Solution](#SDS)
   6. 3.15 mL Distilled Water
   7. 50 µL [10% (w/v) Ammonium Persulfate (APS)](#APS)
2. Lastly, add 8 µL of TEMED (tetramethylethylenediamine)
3. MIX WELL.
4. Squirt this mixture using a pipette in between the gel surface till little more than 3/4th of the plate surface is filled. [Level with the center of the top screw on the gel stand.]
5. If the gel parallax appears to be wavy, it should be flattened using water saturated iso-butanol [2-methylbutane] or “Water Saturated Iso-butanol” on shelf.

-This is a slippery substance, use caution while using pipette

-Fill to top of the gel plate.

Wait for the gel to harden up. Takes approximately 1 hour at room temp.

6. After the gel is hardened dump the iso-butanol, and wash 3x with distilled water

**Stacking Gel Solution (e.g., 4.5% (w/v) acrylamide) total 3.2 mL**

1. Combine:
   1. 600 µL [30% (w/v) Acrylamide Solution](#Acrylamide_solution)
   2. 100 µL [0.50 mol/L Tris/HCl Solution, pH 6.8](#Tris)
   3. 100 µL [10% (w/v) SDS Solution](#SDS)
   4. 2.34 mL Distilled Water
   5. 20 µL [10% (w/v) Ammonium Persulfate (APS)](#APS)
2. Lastly, add 8 µL of TEMED (tetramethylethylenediamine)
3. Then pipette this gel in between the plates - all the way to the top. (quickly move to creating wells)

Creating the gel WELLS

1. This is done using the small white 10 well comb.

2. Push the comb in between the gel plates neatly.

-Angle the comb and use even pressure to slide it into place [do not break the glass]

-The first well on either side of the gel is not used and will be filled with 1X Sample Buffer [8 Total wells possible for sample/caster]

3. Wait for the gel to harden up. Takes approximately 1/2 hour at room temp.

4. Pull the combs from gel casters as slowly as possible. Wash it with distilled water three times.

- hold caster firmly and shake to remove water droplets [do not drop!!!] Can place gel caster upside down to rid of water.