**Sample preparation:**

Frozen human ventricular tissue samples were crushed in a liquid nitrogen cooled mortar and 150 µL extraction buffer (50 mM Tris-HCl pH 6.8, 2% (v/v) SDS, 6% (v/v) glycerol, 1% (v/v) beta-mercapto-ethanol, 0.005% (w/v) bromophenol blue, 4 M urea) was added. The frozen pellet was transferred into a 1.5 mL eppendorf tube and boiled for 3-5 min in order to solubilize the proteins. Samples were cooled down on ice and spun down hard in a table-top centrifuge, and the clear supernatant transferred to a fresh Eppendorf tube to remove debris. 5 µL of each sample were loaded onto a 4%-20% gradient SDS-PAGE and left to run for 45 minutes at 180 mV. Gels were stained with Coomassie to estimate protein content.

**PhostagTM-SDS-PAGE**

Prepare separating Phostag-SDS-PAGE gel according to manufacturer’s instructions (for cTnI 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.

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..

**Western-blot**

Wash gels in transfer buffer (48 mM Tris-HCl pH 9.2, 39 mM glycine, 0.13% (w/v) SDS, 15% (v/v) Methanol) containing 10 mM EDTA for 10 min to complex Mn2+. Wash three more times in transfer buffer without EDTA. Transfer samples onto nitro-cellulose membrane using semi-dry blot for 1 h at 1 mA/cm2 (roughly 50 mA for a mini-gel).

**Transfer Buffer**

1. Dissolve in 500 ml of Di water;
   1. 48 mM Tris-HCl pH 9.2
   2. 39 mM glycine
   3. 0.13% (w/v) SDS 2.6 g (for 2L)
   4. 15% (v/v) Methanol 300 mL (for 2L)
   5. containing 10 mM EDTA (transfer buffer 2 without EDTA)
2. Fill to 1 L with distilled water.

EDTA MW= 292.24 g/mol (so 584.48 mg for 10 mM in 200 mL of solution (or 292.24 mg for 100mL solution))

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.

Primary antibody: mouse anti-cTnI from HyTest (clone 84); diluted 1:5000 in TBS-T containing 5% (w/v) semi-dried milk powder, incubate overnight in the cold-room

Wash membrane three times for 5-10 min with TBS-T

Secondary antibody: Goat anti-mouse HRP-conjugated (FisherScientific); 1:1000 in TBS-T containing 1% (w/v) semi-dried milk powder, incubate for 1h at room temperature

Wash the membrane three times for 10 min with TBS-T, briefly rinse with MilliQ water, add ECL reagent and image membrane.

This protocol will walk you through making Extraction buffer, Sample preparation, protein assay, PhostagTM-SDS-PAGE preparation, running, transferring and Western-blotting/analysis. Adapt the protocol for each protein you are probing for.

**\*\*Buffer protocols at the end of document\*\***

**Sample preparation:**

1. Combine Frozen human ventricular tissue samples with 150 µL extraction buffer
2. Homogenize with beeds
3. The frozen pellet was transferred into a 1.5 mL eppendorf tube and boiled for 3-5 min in order to solubilize the proteins.
4. Samples were cooled down on ice and spun down hard in a table-top centrifuge, and the clear supernatant transferred to a fresh Eppendorf tube to remove debris.
5. **Perform lowry protein assay (per kit instructions) to calculate protein concentrations on extracted supinate.**
6. **After protein assay, add 0.75g bromophenol blue to sample (0.005% (w/v)**
7. **Sample is ready for PhostagTM-SDS-PAGE**

**Protein Assay:**

* 1. Label 5 (2.0 mL) eppendorf tubes for the standards.
     1. Label the tubes as the following: 2.0, 1.0, 0.5, 0.25, and 0.0.
  2. Perform a serial dilution of the 2.0 µg/µL BSA standard in order to prepare the 5 standards.
  3. Add 100 µL BSA to the ‘2.0’ tube.
  4. Add 100 µL DI H2O to the ‘1.0’, ‘0.5’, ‘0.25’, and ‘0.0’ tube.
  5. Add 100 µL BSA to the ‘1.0’ tube and lavage the solution 10 times.
  6. Extract 100 µL from the ‘1.0’ tube, add it to the ‘0.5’ tube, and lavage 10 times.
  7. Extract 100 µL from the ‘0.5’ tube, add it to the ‘0.25’ tube, and lavage 10 times.
  8. Extract 100 µL from the ‘0.25’ and dispose of it in order to conserve all of the tubes volumes at 100 µL.
  9. Add 500 µL RC Reagent I to each standard tube and to each sample tube.
  10. Vortex each tube and let them incubate for 1 minutes at room temperature.
  11. Add 500 µL RC Reagent II to each standard tube and to each sample tube.
  12. Vortex each tube.
  13. Centrifuge standard and sample tubes at 15,000 rpm for 5 minutes.
      1. Be sure to keep the joint of the tube (where the lid attaches) facing upwards when orienting the tubes inside of the centrifuge. This ensures that the pellet will form in a predictable area and will make the subsequent pipetting easier to manage.
  14. Drain tubes and set them aside to dry.
      1. When draining, keep the tube at eye level so as to the monitor the precipitate. You do not want any of the precipitate to get flushed out with the solution. If this occurs, stop immediately and re-centrifuge the sample for a few minutes (same rpm).
      2. Dry the tubes in 30 minute intervals. Keep the tubes upright for the first 30 minutes and then turn them on their sides for the next 30 minutes if the solution has not yet evaporated. After an hour, use a kim wipe to dry the remaining solution. Be careful not to dry any solution that is clearly in contact with a precipitate.
  15. Add 510 µL Reagent A’ to each tube. Vortex each tube, let them incubate for 5 minutes, and then vortex the tubes again.
  16. Acquire an appropriate number of 15 mL falcon tubes and label them as the standards (2.0, 1.0, 0.5, 0.25, and 0.0) and the unknowns.
      1. Add 4 mL Reagent B to each falcon tube.
  17. Add the entirety of each eppendorf tube’s contents (including bubbles) into the falcon tube.
  18. Vortex each falcon tube and allow them to incubate for 15 minutes.
  19. Load the standards and samples in triplicates into a 96-well plate.
      1. Use an insulin syringe to pop any bubbles that appear in the wells.

**Run the protein assay.**

* 1. Turn on the spectrophotometer.
  2. Open up the SoftMax Pro program.
  3. Click on “Assays” and select “Lowry”.
  4. Click on “Template” and enter the standards and unknowns.
  5. Both the standards and the unknowns are measured in mg/mL, but the unknowns have a dilution factor of 10.
  6. Wipe down the 96-well plate with a kim wipe and insert it into the spectrophotometer.
  7. Save the file and also export it as a text file.
  8. Take out the 96-well plate and shut down the spectrophotometer.

**After protein assay, add 0.75g bromophenol blue to sample (0.005% (w/v)**

**Sample is ready for PhostagTM-SDS-PAGE**

**PhostagTM-SDS-PAGE:**

* + - 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.
      5. Spray distilled water on the gel – plate surface [in between the 2 plates] to test for leaking.
         1. Wait a couple of minutes to see if any leaks occur
         2. “Dr. Larry” procedure to get the water out of the gel caster: hold caster firmly and shake to remove water droplets [do not drop!!!]

**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.

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
   2. 2.50 mL 1.5 mol/L Tris/HCl Solution, pH 8.8
   3. 100 µL 5.0 mmol/L Phos-tagTM AAL Solution
   4. 100 µL 10 mmol/L MnCl2 Solution
   5. 100 µL 10% (w/v) SDS Solution
   6. 3.15 mL Distilled Water
   7. 50 µL 10% (w/v) Ammonium Persulfate (APS)
2. Lastly, add 10 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 30 minutes.

-“Dr. Larry” procedure again: hold caster firmly and shake to remove water droplets

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 2 mL**

1. Combine:
   1. 300 µL 30% (w/v) Acrylamide Solution
   2. 2.50 mL 0.50 mol/L Tris/HCl Solution, pH 6.8
   3. 200 µL 10% (w/v) SDS Solution
   4. 1.17 mL Distilled Water
   5. 10 µL 10% (w/v) Ammonium Persulfate (APS)
2. Lastly, add 2 µL of TEMED (tetramethylethylenediamine)
3. Then pipette this gel in between the plates - all the way to the top. (quickly move to creating wells)

D] 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. Till then prepare the sample by spinning it on vortex, adding distilled water and 4X Sample buffer [Laemmli buffer] and incubating at 90 C for 12 minutes. The amount you add will be based on the protein concentration of the sample. Remember you must add an equal volume of protein.

-The sample is heated after being diluted in order to denature the proteins. This ensures that the negatively charged amino acids is neutralized & enabling the protein to move from top to bottom when the electrical charge is applied [traveling from negative to positive on the gel]. Do NOT heat to >100 degrees [the test tube tops will pop off]

-Example: sample heart = [500µg/µl] protein [aim for ~40µg of protein] à 7.5 µl 4X Sample Buffer + 7.5 µl Water + 15µl sample heart [33.33 µg of protein]

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

- “Dr. Larry” procedure again: hold caster firmly and shake to remove water droplets [do not drop!!!] Can place gel caster upside down to rid of water.

E] Adding the buffer on the gels

After the samples have reached the desired temperature, mix them well on the Mini Spin Centrifuge.

-30 seconds, 5.0 ref

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

2. Fill the buffer chamber [bottom to top] with 1X ELECTROBLOT BUFFER [put in fridge to cool].

3. Place the well decal [plastic template] on the glass plate and fill the sample wells.

-Use the P100 pipette from the circular rack and the special elongated loading tips

-Make sure to slowly load the 8 wellsà do not blow the sample everywhere

4. Connect the buffer chamber with the voltage system, at 25mA, constant voltage until the blue protein marker line reaches to the bottom of the gel plates.

-A = Constant, Voltage will auto-adjust to maintain the consistent amps needed

-Can check the time on the voltage system if needed [~60-90 minutes]

**Western-blot**

Wash gels in transfer buffer (48 mM Tris-HCl pH 9.2, 39 mM glycine, 0.13% (w/v) SDS, 15% (v/v) Methanol) containing 10 mM EDTA for 10 min to complex Mn2+. Wash three more times in transfer buffer without EDTA. Transfer samples onto nitro-cellulose membrane using semi-dry blot for 1 h at 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 [plastic template] 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.

H] **Ponceau S Staining procedure**

1. After 3 hours take out the case from transfer chamber, and gently take the gel sheet out and put it in another Tupperware.

2. Soak membranes in 20% methanol for 2 minutes

3. Transfer membranes and rock in Ponceau S staining solution for 5 minutes at room temperature while rocking

- Ponceau S staining solution (100mL)

- 0.5% (w/v) Ponceau (0.5 g)

- 1% (v/v) acetic acid (1 mL)

4. Remove background stain with 5% (v/v) acetic acid to confirm protein transfer (scan the membrane).

5. 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.

6. Block the membrane with TBS-T containing 5% (w/v) semi-dried milk powder for 1h at room temperature.

3. Wash membrane 3x for 5 minutes with TBS-T

-Rock on tilt table

Primary antibody: mouse anti-cTnI from HyTest (clone 84); diluted 1:5000 in TBS-T containing 5% (w/v) semi-dried milk powder, incubate overnight in the cold-room

Wash membrane three times for 5-10 min with TBS-T

Secondary antibody: Goat anti-mouse HRP-conjugated (FisherScientific); 1:1000 in TBS-T containing 1% (w/v) semi-dried milk powder, incubate for 1h at room temperature

Wash the membrane three times for 10 min with TBS-T, briefly rinse with MilliQ water, add ECL reagent and image membrane.

I] Application of antibodies

1. If greater then 40ug of protein loaded dilute antibodies in a 1:500[10 µL] ratio. If less, then 40ug loaded uses 1:250[20ul]. [In 5 ml of blocking buffer.]

-Concentrations are manufacturer specific

2. Make pouches using the freezer bag. Seal it from all sides except one and place the gel sheet on it. Pipette the primary antibodies into the pouch.

3. Rock it for few minutes and keep it in the fridge overnight on a rocker.

REFER TO NNERL antibody sheet

J] Addition of secondary antibodies [IN THE DARK]

1. After the samples have been incubated in the fridge overnight take the samples out of the pouch and wash 3 times in TBS and rock for 5 minutes each time.

2. Put 5 ml of blocking buffer in the centrifuge tube and secondary antibody [anti—rabbit HRP linked]. If greater then 40ug protein loaded use 1:2000 [2.5 µL] if less, then 40ug loaded use 1:1000 [5 µL].

3. Prepare the freezer bag pouches as above and place the gel sheet on it and pipette out the secondary antibodies. Incubate for 1 hour on the rocker.

4. Take the sample out of the pouch and wash the gel sheets with TBS buffer 3 times for 5 minutes each.

- ECL Reagent A 10 ml + ECL Reagent B 10 ml

- 2 minutes on rocker

K] Imaging

1. This will be done on the Kodak Imaging Station IR2000.

2. Put the membrane on plastic sheet and inside the chamber after making sure the room lights are switched off.

**Buffer recipes**

**Extraction buffer (100mL):**

1. Add 40 mL of DI water to a beaker and place on hotplate (Ramsey) with a stir-rod of 250 RPM and a temp 30 degrees C
2. Add in;
   1. 0.788 g Tris (for 50 mM in 100mL solution)
   2. 2g of SDS (to get 2% (w/v) in 100mL solution)
   3. 6 ml of glycerol (to get 6% (v/v) in 100mL solution)
   4. 24.024 of Urea (to get 4M in 100mL solution)
   5. 1 ml of beta-mercapto-ethanol (to get 1% (v/v) in 100mL solution)
3. Add HCl to bring solution to a pH of 6.8 by added 5-100uL at a time. (tris buffer zone is 8ish, so it will be hard to get through this but after the pH is below 8, GO SLOW! – roughly should take 400-500 µL of HCL) – use KOH if you undershoot.
4. Lastly, take the final solution and fill to 100mL and aliquot out into two 50 mL tubes labelled and dated.

**30% (w/v) Acrylamide Solution (30% T, 3.3% C) (unless purchased)**

1. Combine
   1. 29.0 g of acrylamide
   2. 1.0 g of N,N'-methylene-bisacrylamide
2. Make to 100 mL with distilled water. Filter and store at 4˚C in the dark.

**1.5 mol/L Tris/HCl Solution, pH 8.8 (4x solution for resolving gel)**

1. Add 40 mL of DI water to a beaker and place on ‘Ramsey’ with a stir of 250 RPM and a temp 30 degrees C
2. Add in;
   1. 18.2 g of Tris base (FW: 121, pKa = 8.2 at 20˚C)
3. Add HCl to bring solution to a pH of 8.8
4. Fill to 100 mL with distilled water. Store at Room temp.

**0.50 mol/L Tris/HCl Solution, pH 6.8 (4x solution for stacking gel)**

1. Add 40 mL of DI water to a beaker and place on ‘Ramsey’ with a stir of 250 RPM and a temp 30 degrees C
2. Add in;
   1. 6.06 g of Tris base (FW: 121, pKa = 8.2 at 20˚C)
3. Add HCl to bring solution to a pH of 6.0
4. Fill to 100 mL with distilled water. Store at Room temp.

**10% (w/v) SDS Solution**

1. Dissolve 10.0 g SDS in 90 mL of distilled water with stirring
2. Bring to total volume to 100 mL with distilled water. Store at Room temp.

**5.0 mmol/L Phos-tagTM AAL Solution containing 3% (v/v) MeOH**

1. In a plastic tube, dissolve 2 mg of Phos-tagTM AAL-107 into 20 µL of methanol
2. Add in 640 µL distilled water

The viscous oil product, Phos-tagTM AAL-107 (10 mg) placed in a plastic tube is completely dissolved in 0.10 mL methanol. The solution is diluted with 3.2 mL distilled water by pipetting.

*Note: If a trace amount of insoluble material appeared as white fine powder (impurity) in the solution, it can be separated by centrifuging using two 2-mL microtubes.*

*Store the solution in the 2-mL microtubes at 4˚C in the dark. From the supernatant solution,*

*45 mini-slab gels (50 µmol/L Phos-tagTM, 1-mm-thick, 9-cm-wide, 7.7-cm-long) can be prepared.*

**10 mmol/L MnCl2 Solution**

1. Dissolve 64 mg MnCl2 in 50 mL of distilled water.

Note: Do not use the other anion salt, such as Mn(NO3)2 and Mn(CH3COO)2.

**10% (w/v) Ammonium Persulfate Solution (10% APS)**

1. Dissolve 10 mg (NH4)2S2O8 (FW: 228) in 0.10 mL of distilled water.

Note: Freshly prepare prior to use.

**Running Buffer, pH 8.3 (10x solution)**

1. Dissolve;
   1. 15.1 g of Tris base (0.25 mol/L)
   2. 5.0 g of SDS
   3. 72.0 g of glycine (1.92 mol/L)
2. Make to 0.50 L with distilled water. Do not adjust pH with acid or base. Store at 4˚C.

Use: Dilute 50 mL of the 10x solution with 450 mL distilled water.

**Sample Buffer (3x solution)**

1. Dissolve;
   1. 1.5 mg Bromophenol Blue (BPB, a tracking dye)
   2. 0.60 g SDS
   3. 3.0 mL glycerol
   4. 3.9 mL 0.50 mol/L Tris/HCl, pH 6.8
   5. 1.5 mL 2-mercaptoethanol
2. Make to 10 mL with distilled water. Store at –20˚C.

**Acidic Solution for Fixation of Proteins (1 L) for 30-60 minutes**

1. Combine;
   1. 0.10 L of acetic acid
   2. 0.50 L of methanol
   3. 0.40 L of distilled water

**Coomassie Staining Solution (CBB) (0.5 L) for 2-4 hours (or overnight)**

1. Dissolve;
   1. 0.25 g of Coomassie Brilliant Blue (CBB)
   2. 40 mL of methanol
2. Then add;
   1. 10 mL of acetic acid
   2. 50 mL of distilled water

**Washing and De-staining Solution (1 L) until desired background is achieved**

1. Combine;
   1. 10 mL of acetic acid
   2. 50 mL of methanol
   3. 40 mL of distilled water

**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
   2. 2.50 mL 1.5 mol/L Tris/HCl Solution, pH 8.8
   3. 100 µL 5.0 mmol/L Phos-tagTM AAL Solution
   4. 100 µL 10 mmol/L MnCl2 Solution
   5. 100 µL 10% (w/v) SDS Solution
   6. 3.15 mL Distilled Water
   7. 50 µL 10% (w/v) Ammonium Persulfate (APS)
2. Lastly, add 10 µL of TEMED (tetramethylethylenediamine)

**Stacking Gel Solution (e.g., 4.5% (w/v) acrylamide) total 4 mL**

1. Combine:
   1. 600 µL 30% (w/v) Acrylamide Solution
   2. 100 µL 0.50 mol/L Tris/HCl Solution, pH 6.8
   3. 100 µL 10% (w/v) SDS Solution
   4. 2.34 mL Distilled Water
   5. 20 µL 10% (w/v) Ammonium Persulfate (APS)
2. Lastly, add 8 µL of TEMED (tetramethylethylenediamine)

**Mn2+ – Phos-tagTM SDS-PAGE adopts almost the same gel compositions for Laemmli's method, but SDS may be unnecessary as an additive in the resolving and stacking gels.** **In the presence of SDS in the gel, the band of target protein would be rather broad and/or tailing.**

**Most pre-stained molecular weight markers contain**

* 1. **mM EDTA**

**You can add 1 mM MnCl2 to markers to complex the free EDTA**

**Transfer Buffer**

1. Dissolve in 500 ml of Di water;
   1. 48 mM Tris-HCl pH 9.2
   2. 39 mM glycine
   3. 0.13% (w/v) SDS
   4. containing 10 mM EDTA (transfer buffer 2 without EDTA)
2. Fill to 1 L with distilled water.

Stain membrane

**Ponceau**

remove background stain

**5% (v/v) acetic acid**

membrane wash TBS-T

**10 mM Tris-HCl pH 8,**

**150 mM NaCl,**

**0.05% (v/v) Tween-20**

Block the membrane with TBS-T containing

**With 5% (w/v) semi-dried milk powder**

Or

- 0.2% (w/v) Ponceau (0.2 g)

- 3% (v/v) Trichloroacetic acid (TCA)

- 3% (v/v) Sulfosalicylic acid

To a previously unopened bottle containing 500 g of trichloroacetic acid (TCA), add 227 ml of H2O. The resulting solution will contain 100% (w/v) TCA.

Primary antibody: mouse [anti-cTnI from HyTest](https://shop.hytest.fi/product/troponin-i-cardiac-antibody) (clone 84); diluted 1:5000 in TBS-T containing 5% (w/v) semi-dried milk powder, incubate overnight in the cold-room

Wash membrane three times for 5-10 min with TBS-T

Secondary antibody: Goat anti-mouse HRP-conjugated (FisherScientific); 1:1000 in TBS-T containing 1% (w/v) semi-dried milk powder, incubate for 1h at room temperature

Wash the membrane three times for 10 min with TBS-T, briefly rinse with MilliQ water, add ECL reagent and image membrane.

[Stripping Solution](https://labchem-wako.fujifilm.com/europe/product/detail/W01W0119-1637.html)

[PVDF membrane](https://labchem-wako.fujifilm.com/europe/product/detail/W01W0103-2343.html)