Digesting sample in lysis buffer by solubilizing the frozen sample in 1mL lysis buffer. The solution should be homogenized for 30 second (create a foggy solution with no chunks) then centrifuged for 15 minutes at 13,000 x g, where the supernatant and pellet were separated. Save the supernatant as “digested sample” for gels.

Prepare the digested sample by spinning it on vortex, adding distilled water and 4X Sample buffer and incubating at 90 C for 12 minutes (or 95 for 5 minutes). The amount you add will be based on the protein concentration of the sample. Remember you must add an equal volume of protein. (I.e., if sample A is 50ug/ul and B is 25ug/ul, then A needs to be diluted with DI water to a 25ug/ul concentration)

-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 4****X Sample Buffer*** *+ 7.5 µl Water + 15µl sample heart [33.33 µg of protein]*

Lysis Buffer Procedure

Combine;

0.4g of CHAPS

100uL Tris pH 7.5,

1 cOmplete protease inhibitor cocktail tablet

and 9.9 mL ddH2O

5x Sample Buffer Procedure

Place a 150 ml beaker on the balance and tare to zero

Weigh 50gm of glycerol into the beaker

Add 70 ml of DI water

Add stir bar

Add 3.78g of Trizma base

Allow all components to dissolve

pH to 6.8 with HCL

Add 30 ml of DI water.

Add 10 gm SDS and stir until dissolved

Add 25 ml 0.04% bromophenol blue; mix well.

Note to make **4x Sample buffer** combine 800 ul 5x Sample buffer [above] with 200 ul of beta mercaptoethanol

1.5 M Tris, pH 7.5 (lysis buffer)

For 1 L

Dissolve 181.65 g Tris base in around 800 mL of ddH2O

Adjust the pH to 8.8 with concentrated HCl

Bring up the volume to 1 L with ddH2O  
Note: Make sure to let the solution cool down to room temperature before making the final pH adjustment.

A] Preparation of gel plates

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!!!

B] 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

3. Screw the plates with the little grey screws, not too tight but just enough to hold the gel.

4. 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]

-Put some gel across the bottom of the glass and aluminum

5. 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.

6. 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].

-Put red clamps on the top of the gel plate and aluminum backing plate.

7. Spray distilled water on the gel – plate surface [in between the 2 plates] to test for leaking.

-Wait a couple of minutes to see if any leaks occur

- “Dr. Larry” procedure to get the water out of the gel caster: hold caster firmly and shake to remove water droplets [do not drop!!!]

C] Preparation of the gel

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

-Running Gel: aka separating 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

Preparation of RUNNING GEL

1. Take a small beaker for making the gel.

2. Pipette 3.3 ml distilled water.

3. Put 4ml of Acrylamide mix [Bis 30 % acrylamide brown bottle].

4. Add 2.5 ml Tris 8.8.

5. Pipette 100µl of 10% Sodium dodecyl sulfate [SDS].

6. Pipette 100µl of 10% Ammonium persulfate solution [APS]à small test tube.

7. Pipette 4 µl of TEMED.

8. MIX WELL.

9. 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.]

10. 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.

11. Wait for the gel to harden up. Takes approximately 15-20 minutes.

-“Dr. Larry” procedure again: hold caster firmly and shake to remove water droplets [do not drop!!! Or beak the plate (AUSTIN!!!)]

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

Preparation of STACKING GEL

Get a small beaker for making the stacking gel.

1. Pipette 3.4 ml of distilled water.

2. Pipette 830 µl of acrylamide mix.

3. Pipette 630 µl of Tris 6.8pH.

4. Pipette 50 µl of SDS.

5. Pipette 50 µl of 10% APS.

6. Pipette 5 µl of TEMED.

Then pipette this gel in between the plates - all the way to the top.

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. 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]

1. **Muscle sample preparation**
2. **Materials**
3. Dry ice
4. 1x Urea-thiourea buffer
5. Muscle samples
6. Homogenizer
7. 100% glycerol
8. 1% bromophenol blue stock solution
9. Bromophenol blue powder (to make 1% 1% bromophenol blue stock solution stock)
10. **Methods**
11. Take tissue out of -80ºC and place on dry ice. Take out only the vial that you’ll be working with and weigh the tissue (~20 mg)
12. Place the appropriate amount of sample buffer in the Dounce homogenizer (30:1 buffer to tissue, volume (ml)/weight (g)).
13. Homogenize until no more tissue pieces are seen.
14. Vortex samples for 30-60 seconds
15. Spin briefly at 4000 x g for 30 seconds
16. Take out 10 ul of the supernatant for protein assay (add 90uL water and keep aside on ice)
17. Take rest of the supernatant out (leaving the pellets undisturbed) and place in new labeled 2.0 mL tubes.
18. Heat samples at 60 ºC for 10 minutes. Also heat some glycerol in a separate test tube
19. Add 30% glycerol to the samples
20. Add bromophenol blue (1% stock), so that the final concentration will be 0.03%
21. Make the 1% stock solution by adding 0.1 g  of bromophenol blue to 10 mL DI water.
22. Mix well and you can store it at room temperature.
23. Vortex until samples are mixed with the glycerol
24. Centrifuge at 13.200 x g  for 10 minutes
25. Remove supernatant and place in a clean tube
26. Load on gels or store at -80 ºC

1. **RC-DC Protein Assay**

1. **Loading and Running the Gel Electrophoresis Unit**
2. **Materials**
3. Gel
4. beta-mercaptoethanol
5. Mini-PROTEAN 3 cell
6. Bio-rad power supply
7. 1x TGS buffer
8. 10xTGS buffer (To make 1xTGS buffer)
9. standards
10. To make Standards
11. Broad range protein standard (*for only broad range gel without western blot)*
12. Stock sample buffer (store at room temperature)

|  |  |
| --- | --- |
| Distilled water | 4.8 mL |
| 0.5M Tris-HCL pH6.8 | 1.2 mL |
| Glycerol | 1.0 mL |
| 10% (w/v) SDS | 2.0 mL |
| 0.1% (w/v) Bromophenol blue | 0.5 mL |
| Total volume | 1. mL |

1. SDS Reducing buffer (store at room temperature)  **4x**

|  |  |
| --- | --- |
| b-mercaptoethanol | 25 uL |
| Stock Sample Buffer | **475 uL** |
| Total volume | **500 uL** |

1. Dilute standards 1:20 in SDS reducing sample buffer. Heat for 5 minutes at 95 °C. Cool and load 5 uL/well
2. E.g. pipette 5 uL of broad range standard and mix with 100 uL of SDS reducing buffer.
3. Store sample temperature as other samples (-80 °C)
4. Peppermint Stick phosphoprotein molecular weight standard
5. Mix 1 uL of protein standards with 6 uL of SDS gel-loading buffer, heat at 95 °C for 4 minutes and pipet into the well reserved for the standard.
6. E.g. pipette 5 uL of standard in 30 uL of urea thioura sample buffer with 30% glycerol and 0.3% of 1% bromophenol blue.
7. Store sample temperature as other samples (-80 °C)