**Titin Gel Running Protocol**

**Making Gel Caster: Owl System**

* + - 1. Preparation of gel plates/Gel caster
         1. Put the glass plates together [with a 1 mm white spacer] on a clean surface.
      2. Putting the glass plates on the gel caster unit to create the gel- glass sandwich
         1. Insert the spacers [white 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.

Slightly tighten the caster, then slide the white template in to ensure the spacers are even and spaced out correctly.

Make it tighter, but not too tight

* + - 1. Using a prefilled syringe of Vaseline, coat the bottom gap between the glass plates with Vaseline (this will prevent the acrylamide plug from leaking.
      2. Place parafilm over the grey mats in the caster (this will keep things clean/prevent Vaseline from getting all over the grey mats and caster).
      3. Secure the plates into the caster using the white and black screws by turning them and pushing in and up 180 degrees [don’t over rotate them].
         1. make sure it is tight and secure or else the plug will leak out the bottom).

**Making Titin Gel:**

**Materials:**

* + 30% bis-Acrylamide ([Bio-Rad 1610158](https://www.bio-rad.com/en-us/sku/1610158-30-acrylamide-bis-solution-37-5-1?ID=1610158))
  + [3M Tris, pH 9.3](#Tris)
  + [10% APS](#APS) (ammonium persulfate)
  + TEMED (tetramethyl ethylenediamine)
  + 50 mL of 50% Glycerol (25 mL water + 25 mL Glycerol)
  + 13 μL TEMED ([Sigma 110732](https://www.sigmaaldrich.com/US/en/product/mm/110732))
  + SeaKem Gold agarose (Lonza Group, [Fisher BMA50150](https://www.fishersci.com/shop/products/lonza-seakem-gold-agarose-2/p-2861592))
  + [5X Titin Buffer](#Titin_Buffer)
  + Water saturated Iso-Butanol

**Making the Acrylamide Plug:**

1. Combine in a 15 mL falcon tube:

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| 1.924 | mL | ( | 962.0 | μL \* 2 ) | Deionized water |
| 1.7 | mL | ( | 850.0 | μL \* 2 ) | 50% glycerol |
| 2.12 | mL | ( | 706.7 | μL \* 3 ) | 3 M Tris (pH 9.3) |
| 2.72 | mL | ( | 906.7 | μL \* 3 ) | 30% bis-Acrylamide |
| 24 | μL |  |  |  | 10% APS |

Mix by gently pipetting a few times**.**

1. Add **13 µL of TEMED** (tetramethyl ethylenediamine). Mix by gently pipetting a few times**.**
2. Quickly, add **3 mL** of the Acrylamide plug solution between the glass plates by pipetting it into the top left corner – do so slowly and with one continuous ejection. Don’t eject past the first stop on the pipette (if you do, you risk getting a bubble in the plug)
3. Repeat for second gel
4. Using a transfer pipette, quickly draw up Iso-Butanol (STAY ABOVE THE WATER LINE to ensure you only take Iso-Butanol and add 5 or so mL to the same left top corner. Repeat for both gels.
   * Iso-Butanol serves two purposes: 1. It will level out the plug so it is flat and meets the agarose gel evenly and 2. It will clean out the acrylamide that ran down the left side of the plates.
5. Let the plug polymerize for 30 minutes. While waiting, turn on the oven to 70°C.
6. After the gel is hardened dump the iso-butanol, and wipe down in-between the plates with KimWipes. Ensure the inside is clean and dry.
7. Please the Gel casters, Ladder combs, 5 mL pipette tip, and a funnel into the oven at 72°C. After **60 minutes**, proceed to making the Agarose gel.
   * You can remove the gels from the stands, they’re no longer needed. Then clean off the Vaseline and wax paper. Always keep a clean gel caster.
   * Preheating the glass plate assembly, well comb, and syringe prevents premature agarose gelling when the solution touches the colder surfaces. In addition the plates are less likely to crack during pouring if they are closer to the temperature of the hot agarose.

**1% Agarose gel**

1. Combine in a beaker:

|  |  |  |
| --- | --- | --- |
| 16.0 | mL | 5X Titin Buffer |
| 15.7 | mL | Deionized water |
| 48.3 | mL | 50% glycerol\* |
| 0.8 | g | SeaKem Gold Agarose\*\* |

\*Glycerol is included in the mixture to increase the solution viscosity inside the gel and thus sharpen the protein bands.

\*\*It is essential to use SeaKem Gold agarose for optimal migration of high molecular weight proteins. This type has large pore size and excellent mechanical stability. Other types of Agaroses may be used, but the protein mobility will be significantly reduced.

1. Add stir bar to beaker, and weight / record the weight of the beaker
   * This weight will be used so you can replace the water that lost during heating
2. Place the beaker on the hot plate and stir at 100 rpm / heat to 250 °C
   * Place a small beaker with 50 mL of water next to it. You will use this to replace any lost water.
   * Be careful to watch and make sure the agarose does not boil over
   * Using the stir bar during the heating step eliminates non-hydrated agarose granules in the final gel.
3. Once, the agarose is boiling and becomes transparent – take beaker to scale and add the hot water until the weight returns to pre-heating weight.
4. Place beaker back on hot plate and retrieve the Gel casters, Ladder combs, 5 mL pipette tip, and the funnel from the oven.
5. Pour the agarose into the funnel, through the pipette tip, and into the top left corner of the gel caster. Make sure the pour is continuous to keep bubbles from forming.
6. Once both gels are poured, quickly grab the gel combs and place them into each gel BUT only pushing them down until you have wells that are roughly 1 centimeter deep.
   * Sample combs should extend no longer than 1 cm into agarose; otherwise they may be difficult to remove.
   * It helps to wipe the spilled over agarose in the font. I have found that this keeps the level of agarose from dropping too much and allows you to maintain nice walls to your wells.
7. Let the gels sit for 45 minutes and cool to room temperature. Then place in the fridge until you are ready to run them.
   * Some labs say you can make the gels day prior to running them – I have always made them day of… I paranoid about it.

**Loading Gel & Running Electrophoresis**

1. Grab two buckets. Fill one with dry ice and one with ice.
2. Make your Electrophoresis Buffer (150 mL of [5X Titin Buffer](#Titin_Buffer) + 600 mL of DI water).
3. Take 600 mL of your Electrophoresis Buffer and add 10 mM [2-mercaptoethanol](https://www.mpbio.com/us/0219483425-2-mercaptoethanol-cf)
   * 422 μL of 2-mercaptoethanol in 600 mL equates to 10 mM (assuming 99% purity)
   * Note: The disulfide bond formation of large proteins during electrophoresis also retards their migration and may lead to smearing. Thus inclusion of 2-mercaptoethanol in the upper buffer improves resolution of high molecular weight proteins. Alternatively, protein can be alkylated to prevent disulfide bond formation.
4. Take desired samples out and place in the bucket of dry ice. You will begin thawing the samples only when the gels are ready to load.
5. Take the gels out of the fridge and remove them from the stands/caster
6. Slide each gel/glass plate set up into the gel-electrophoresis chamber with the opening of the well/glass plate facing the inside (middle) of the gel-electrophoresis chamber.
7. Slowly wiggle the comb back and forth to un-suction the comb and pull straight up slowly to remove the comb (don’t move or tilt the comb right or left – you want to maintain the integrity of the well walls).
8. After the comb is removed, fill each side with the Electrophoresis Buffer + 2-mercaptoethanol.
9. Now that the gel is ready to load, place the sample on ice
10. Once samples are thawed, Vortex each one directly prior to loading it into the gel
    * Note: for gels analyzing cardiac tissue, add pre-prepped rat psoas or soleus into the first lane as a loading control and to help show cardiac / skeletal muscle specific titin isoforms
11. After you’re done loading – carefully move the Electrophoresis chamber into the fridge and fill with buffer to the top in the middle, and to the fill line on the outside.
12. Connect the Electrophoresis chamber with the voltage system
    * Make sure you connect positive and negative (red to red / black to black) correctly, if you don’t the proteins will run up not down. This sucks…
13. Run Gel nice and slow for 3 hours at 30 mA (15 mA per gel) in the fridge.

FOR ORIOLE STAIN PROCEED RIGHT TO PROTOCOL

FOR SILVER STAIN ONLY:

1. After running, carefully wash the gels in DI water on the rocker for 5 minutes (repeat twice)
2. Lastly, lay out saranwrap, cut multiple slits, and lay gels out to dry overnight.