**Myosin Heavy Chain Gel Running Protocol**

**Making Gel Caster: Owl System**

* + - 1. Preparation of gel plates/Gel caster
         1. Put the glass plates together [with a 0.75 mm black 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 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))
  + 1.5 M Tris 8.8
  + 0.5 M Tris 6.8
  + [10% w/v APS](#APS) (ammonium persulfate)
  + 10% w/v SDS
  + TEMED (tetramethyl ethylenediamine)
  + 50 mL of 50% Glycerol (25 mL water + 25 mL Glycerol)
  + TEMED ([Sigma 110732](https://www.sigmaaldrich.com/US/en/product/mm/110732))
  + Water saturated Iso-Butanol

**Making the MHC running gel:**

1. Combine in a 50 mL falcon tube (34 mL total):

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| 11.42 | mL | ( | 3.807 | mL \* 3 ) | Deionized water (5.71) |
| 6.80 | mL | ( | 3.4 | mL \* 2 ) | 50% glycerol |
| 8.50 | mL | ( | 4.25 | mL \* 2 ) | 1.5 M Tris (pH 8.8) |
| 6.80 | mL | ( | 3.4 | mL \* 2 ) | 30% bis-Acrylamide |
| 240 | μL |  |  |  | 10% SDS |
| 100 | μL |  |  |  | 10% APS |

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

1. Add **40 µL of TEMED** (tetramethyl ethylenediamine). Mix by gently pipetting a few times**.**
2. Quickly, add **16 mL** of the running gel 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 gel polymerize for **60 minutes**.
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. Place the Gel casters into the fridge overnight.

Day 2

**MHC Stacking gel**

1. Combine in a 15 mL falcon tube (10 mL total):

|  |  |  |
| --- | --- | --- |
| 4.29 | mL | Deionized water |
| 2.0 | mL | 50% glycerol |
| 2.6 | mL | 0.5 M Tris (pH 6.8) |
| 1.0 | mL | 30% bis-Acrylamide |
| 100 | μL | 10% SDS |
| 60 | μL | 10% APS |
| 60 | μL | Stacking Gel Dye |
|  |  |  |

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

1. Add **50 µL of TEMED** (tetramethyl ethylenediamine). Mix by gently pipetting a few times**.**

\*\*Stacking gel dye [0.1% (w/v) bromophenol blue, 1% (w/v) SDS, and 3% (v/v) glycerol] - The inclusion of stacking dye in the stacking gel does not interfere with the gel polymerization or alter the run characteristics of the gels. However, it allows better visualization of the loading wells and the careful deposit of sample in the base of the well.\*\*

1. 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 stacking gel; otherwise they may be difficult to remove.
2. Let the gels sit for 60 minutes at room temperature.

**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 4.5 hours at 32 mA (16 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.