1. Urea Thiourea Sample Buffer
   1. Materials
      1. 8M Urea: Sigma, U6504-500G
      2. 2M Thiourea: Sigma, 88810-100G
      3. 0.05M Tris: Fisher, BP152-1
      4. 75mM DTT: Fluka, 43819-5G
      5. 3% SDS: Biorad,161-0301
      6. Rexyn 300: Biorad, R276-500
      7. 0.22 µm filter
   2. Sample Buffer Preparation (for 100 mL volume)
      1. Use gloves when making this solution
      2. Weigh 48 grams of urea and 15.2 g of thiourea into a clean 150 ml glass beaker. Add 40ml of water and stir gently on a hot plate until the solution is at room temperature and all the solids are dissolved. Careful not to heat above 40 ºC
      3. Add 10 grams of mixed bed resin, stir mixture at RT for 15 minutes
      4. Filter mixture through a filter paper into a 100 ml graduated cylinder. Carefully rinse the resin with two 5 ml aliquots of DI water. Transfer the filtered solution back to a clean beaker
      5. Weigh 0.605g of Tris base and 3g of SDS, add to the solution and stir until its dissolved. Adjust pH down to 7.5 using 12 M HCl. (~ 300-400 ul)
      6. Add 1.155g of solid DTT and stir it until its dissolved
      7. Adjust pH down to 6.8 using 2M HCl. Use 10-20 ul. If you mistakenly go past 6.8, correct the pH up using 2 M Tris base.
      8. Transfer solution to graduated cylinder. Add DI water to top it off to 100ml. Mix. Filter through a 0.22 µm filter to remove any fine particulate matter.
      9. Make 2 ml aliquots and freeze at -80 ºC, thaw amount as needed just before use.
2. Muscle Sample Preparation
   1. Materials
      1. Sample tissue
      2. Heating block
      3. Dounce Homogenizer
      4. Urea thiourea buffer
   2. Method
      1. Obtain samples and label 2.0 mL Eppendorf tubes.
      2. For each sample, label 2 Eppendorf tubes.
         1. Label one tube with the surgery date, tissue type, and the word “sample”.
         2. For the other, label with only the surgery date and tissue type.
      3. Weight out sample (0.015-0.02 g).
         1. Nest the tissue into the bottom of the Dounce Homogenizer.
      4. Add an appropriate amount of urea thiourea buffer to Dounce Homogenizer.
      5. Homogenize sample and transfer to clean 2.0 mL Eppendorf tube.
         1. Be careful not to cause too many bubbles while homogenizing.
      6. Homogenization is complete once the solution is clear of identifiable tissue. Note: white tissue fragments, which appear insoluble, are collagen and should not be considered viable tissue.
      7. Pipette the solution into the “sample” tube for the appropriate date. Make sure that everything in the homogenizer (including bubbles and homogenized tissue fragments) is transferred to the Eppendorf tube.
      8. Vortex the sample (30-60 seconds) and centrifuge it at 4000 rpm for 30 seconds.
      9. Add 10 µL of the homogenized supernatant to the other Eppendorf tube (the one **without** the “sample” label) with 90 µL DI H2O.
         1. These tubes will be the unknowns for the protein assay.
      10. Heat the sample for 10 minutes at 60° C.
      11. Cool the sample (in ice) for 5 minutes.
      12. Keep the sample in the -20° C freezer.
3. 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.
4. 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.
5. Sample Normalization according to Protein Concentration
   1. Determine the amount of volume available in the thawed sample tubes.
      1. Use a pipette to determine how much solution is still available.
      2. Enter this value into the “Homogenized Volume Used” cell on the protein assay spreadsheet (attached to the wiki page).
   2. According to the protein assay spreadsheet, add the appropriate amount of 30% glycerol, bromophenol blue, and UTU buffer to each tube in order to adjust each concentration to 1 µg/µL.
      1. Use the adjusted concentration (from the protein assay) as the value for the “[protein] after assay” cell on the protein assay spreadsheet.
      2. The spreadsheet should now contain all the necessary volumes of 30% glycerol, BPB, and UTU in order to perform the correct dilutions.
      3. It is possible that a dilution will require a higher volume than 2.0 mL. In this case, use a larger 5 mL cryogenic vial. Make sure that all the homogenized tissue that has settled to the bottom is transferred into the cryogenic vial. **If this step is necessary**, aliquot the solution evenly into 2.0 mL eppendorf tubes, as the 5 mL cryogenic vials will not fit into the centrifuge.
      4. Vortex each tube.
      5. Centrifuge the tubes at 13,200 rpm for10 minutes.
      6. Divide up each tube into 500 µL aliquots.
      7. Keep samples frozen in the -80°C freezer until it is time to load them.
6. Agarose Gel Preparation
7. Gel Sandwich Assembly
8. Materials

* High vacuum grease: Dow Corning 2966K52
* 70% ethanol
* 2 glass plates (10 cm x 10.5 cm): Hoefer, Inc. SE262P-5
* 2 notched ceramic plates (10 cm x 10.5 cm): Hoefer, Inc. SE262GN-5
* 4 T spacers (12 cm x 1.5 mm): Hoefer, Inc. SE2819T-2-1.5
* 2 Teflon combs, 10 well (1.5 mm): Hoefer, Inc. SE511-10-1.5
* 2 casting clamp assemblies: Hoefer, Inc. SE249
* 4 cams (black): Hoefer, Inc. part of SE245
* 4 clips (small): Hoefer, Inc. part of SE245
* Dual gel caster: Hoefer, Inc. SE245

1. Method
2. Cleaning Gel Sandwich Plates

Both the glass and notched ceramic plate should be cleaned, prior to gel sandwich assembly, using 70% ethanol. Spray the solution on a Kim wipe and clean off any residue.

1. Application of Vacuum Grease

Place one ceramic plate (with the notched part facing up) on top of a clear glass plate. Take care to correctly align the glass plate with the notched ceramic plate, as it is easy to place the spacers in the wrong orientation onto the glass plate.

Apply vacuum grease to both sides of a T spacer and position it onto one side of the glass plate. Do the same for the other side. Take care not to accidentally get vacuum grease on the clean glass surface. Wipe off any residual grease.

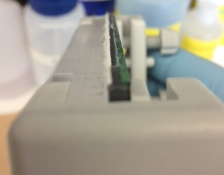
1. Final Assembly

To seal the two plates, place the notched ceramic plate about half way on top of the glass plate and then slide the notched ceramic plate forward until the top of the sandwich (the notched end) is completely aligned. Grease will have come off in the process, so wipe off any residual grease from the top of the gel sandwich.

**At this time, turn on the oven. The final temperature should be approximately 40° C.**

1. Dual Gel Caster

Slide each completed gel sandwich into a casting clamp assembly with the ceramic plate facing the block. Insert the gel sandwich until the bottom portion (with the grease) is approximately 3 mm from the casting clamp assembly. Tighten the screws on the casting clamp assembly until each gel sandwich is secured. Apply vacuum grease to the bottom of the sandwich (see picture below).

Wipe down the dual gel caster assembly with 100% methanol and place the casting clamp assembly into the dual gel caster. Push down on the side of the casting clamp assembly in order to fit a cam into a hole on each side of the caster.

Finally, place a small clip onto each side of the exposed (notched) side of the gel sandwich.

1. Test Seal

To test the seal, pipette a small amount of 70% ethanol into the space between the gel plates. If there are no leaks, then the acrylamide plug can be poured.

1. Acrylamide Plug
2. Materials

* 1.014 mL DI H2O
* 1.700 mL 50% glycerol: Fisher Scientific G33-1
* 2.120 mL 3 M Tris base (pH 9.3)
  + 72.684 g Trise Base: Fisher Scientific BP1521
  + 200 mL DI H2O
    - add 150 mL DI H2O
    - add Tris Base
    - pH to 9.3 with HCl (37%)
    - dilute solution to 200 mL
  + 5-6 mL HCl, 37%: Sigma 77996EM
* 3.630 mL 30% acrylamide
  + 30.0 g acrylamide: Sigma-Aldrich A3553-500G
  + 0.8 g DATD: Sigma-Aldrich 156868-25G
* 24 μL 10% APS: Sigma A3678-25G
* 13 μL TEMED: Bio-Rad 161-0700

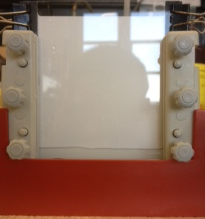
1. Methods
2. 10% APS

Acquire one 2 mL eppendorf tube and label it “10% APS”. Place a small weigh boat (or piece of wax paper) onto the scale and tare it. Once the scale reads zero grams, measure out 0.1 g APS, which can be found in the blue desiccator. Put the APS into the eppendorf tube. Using the 5 mL pipette, rinse off the weight boat (or wax paper) with 1 mL of DI H2O.

1. Final product

Acquire one 15 mL falcon tube, label it “acrylamide plug”, and add into it the following reagents: 1.014 mL DI H2O, 1.700 mL 50% glycerol, 2.120 mL 3 M Tris base (pH 9.3), and 3.630 mL 30% acrylamide. The addition of the next two reagents is time-sensitive and should be done last. Add 24 μL 10% APS and then quickly add 13 μL TEMED. Mix the solution by inverting the falcon tube. Check for bubbles in the solution before proceeding.

1. Pouring the Plug

Empty the ethanol from the gel sandwich cavity into the sink. Using a large transfer pipet, pour the acrylamide plug into the now empty gel sandwich cavity by placing the pipet tip at the side of the gel sandwich near the casting clamp assembly. **Pour the plug until the level reaches about half way through the first screw of the casting clamp assembly.** Place the transfer pipet back into the falcon tube; this will be used to check the polymerization of the plug. Check the falcon tube after **30 minutes**. If the acrylamide has not polymerized yet, then wait an additional 15 minutes. Otherwise, start the process over with new gel plates.

If the acrylamide plug has polymerized, place the gel sandwich into the 40° C oven along with a 5 mL pipette tip and the two Teflon combs. Allow these items to incubate for 45 minutes.

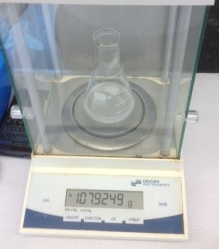
1. Agarose Titin Gel
2. Materials

* 0.24 g Seakem gold agarose: Lonza 50152
* 18 mL 50% glycerol: Fisher Scientific G33-1
* 6 mL 5x titin buffer
  + 30.2850 g Tris Base: Fisher Scientific BP1521
  + 144.1344 g Glycine: MP 4808831
  + 25 mL 10% SDS: Bio-Rad 161-0416
* 6 mL DI H2O

1. Methods

Weigh out 0.24 grams of Seakem gold agarose and add it to a 125 mL Erlenmeyer flask. Using the 5 mL pipette, rinse off the weight boat (or wax paper) with 1 mL DI H2O. Next, add the following reagents into the Erlenmeyer flask: 18 mL 50% glycerol, 6 mL 5x titin buffer, and 5 mL DI H2O.

1. Heating Agarose

**Record the mass of the Erlenmeyer flask and its contents.** **Heat up approximately 5 mL DI H2O in a small beaker along with the agarose Erlenmeyer flask**. Enter 90 seconds into the microwave and place both containers into the microwave. Watch the gel solution carefully, as it will quickly boil over. If this happens, the process of making the agarose gel must be started over.

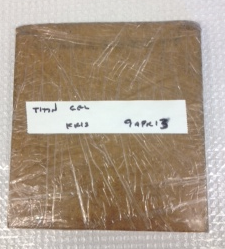
Once the gel solution begins to bubble, stop the microwave and swirl the solution. The glassware gets hot quickly, so it is recommended to wear an insulated glove when swirling the gel solution. After 90 seconds, weigh the Erlenmeyer flask again. Using a 5 mL pipette, add the microwaved DI H2O to the gel solution until the final mass is approximately the same as the original mass.

1. Pouring the Gel

Take the gel sandwich, as well as the combs and the 5 mL pipette tip, out of the oven and **turn the oven off**. Using the pipette tip as a funnel, pour the agarose gel into the gel sandwich cavity. The speed and consistency in which the gel is poured will have a direct effect on the outcome of the gel; therefore, make sure that the gel is poured at a steady pace while maintaining an even level.

Once the gel is poured, ensure that no bubbles remain. Place the combs on top of the gel sandwich thereby sealing the gel sandwich. Wipe off any residual gel with a Kim wipe. Let the gel solidify at room temperature for approximately **30 minutes**.

1. Storing the Gel

The residual gel in the bottom of the Erlenmeyer flask can be used as check for the polymerization of the gel. If after thirty minutes the gel has yet to solidify, wait an additional fifteen minutes. Otherwise, start the entire process over.

If the gel has solidified, dismantle the dual gel caster by removing the cams, as well as the clips. Remove the gel sandwich from the casting clamp assembly without disturbing the combs. Overlap two paper towels and spray them with DI H2O. Place the gel sandwich into the middle of the wet paper towels and wrap the gel. Ensure that the entirety of the paper towels is saturated, as this will prevent desiccation of the gels. Next, wrap the gel in cling wrap, label it (plus name and date), and store the gel in the refrigerator.

1. Titin Buffer Preparations
2. 5x Titin Buffer
3. Materials.

* 30.2850 g Tris-base: Fisher Scientific G33-1
* 144.1344 g Glycine: MP 4808831
* 25 mL 10% SDS: Bio-Rad 161-0416

1. Methods

Acquire a 1000 mL bottle, a 1000 mL beaker, 1000 mL volumetric flask, and a large magnetic stirrer. Add to the 1000 mL beaker approximately 700 mL DI H2O and the large magnetic stirrer. Weight the appropriate amount of reagents in a weigh boat and subsequently add them to the beaker in the order given above. After adding each reagent, wash off the weigh boat with DI H2O into the 1000 mL beaker in order to ensure that all the reagent has made it into the solution.

Next, pour the 1000 mL beaker solution into the volumetric flask. Make sure to use a funnel in order to avoid spilling any solution. Rinse the empty beaker with DI H2O into the funnel. As a final precaution, rinse the funnel and magnetic spinner with DI H2O and then diluter the solution up to the line, which will yield 1000 mL of solution. Pour the solution in the 1000 mL bottle and label it with “5x titin buffer”, your name, and the date.

1. 1x Titin Buffer
2. Materials

* 200 mL 5x titin buffer
* 800 mL DI H2O

1. Methods

Acquire a 1000 mL graduated cylinder and a 1000 mL bottle. Pour 800 mL DI H2O into the graduated cylinder and then add 200 mL 5x titin buffer. Pour the final solution into the 1000 mL bottle and label it with “1x titin buffer”, your name, and the date.

1. Gel Electrophoresis System
2. Gel Electrophoresis Unit Set-Up
3. Materials

* Small titin gel
* Notched ceramic plate (10 cm x 10.5 cm): Hoefer, Inc. SE262GN-5
* Cooling Unit (SE 260 Mighty Small II, basic): Hoefer, Inc. SE260B
* Clamps (red)
* 1x titin buffer
* 2-mercaptoethanol: MP 4806443
* Hamilton syringe: Hamilton 80300

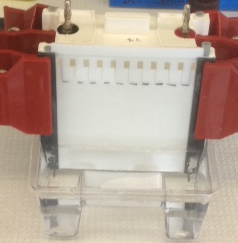
1. Methods
2. Constructing the Cooling Unit

Begin by assembling the cooling unit. The white gel running unit that will hold the gel sandwich should fit into the middle of the clear housing (it should click when it fits properly). Next, unwrap the stored gel from the cling wrap and paper towels. At this point, examine the gel for any structural defects. There can be no air bubbles or space between the gel and the plug, as well as between the gel and the plate. If any such space is present, the gels cannot be used. It is important to note that this portion of the protocol specifically refers to the use of a single gel. The protocol prior to this section refers to the making of two separate gels. However, this protocol only allows for the use of one gel per cooling unit at a time.

1. Readying the Gel for Loading

Place the gel onto one side of the white gel running unit and secure it into place with two red clamps. On the opposite side of the white gel running unit, secure a single notched ceramic plate using the other two red clamps.

**Fill the cavity between the gel and the white apparatus (the upper chamber) with 1x titin buffer** until the fluid level has covered the space between the teeth of the comb. Next, **add 200 μL 2-mercaptoethanol to the upper chamber**. **Fill the clear housing (lower chamber) with 1x titin buffer** until the fluid level has covered the notch present on both sides.

Next, use the Hamilton syringe to loosen the comb from the gel. This step requires great care, as the comb has become embedded in the polymerized gel. The trick is to create a space with the syringe needle between the comb and gel, which should loosen the comb. In addition, the comb can be wiggled out vertically from the gel by lifting up on either side in a sequential manner. Eventually, the comb should slide out of the gel.

**Set the comb aside and mark the end of each lane with a marker, so there is a landmark for loading the samples**. Flush out each individual lane with upper chamber solution (30 µL) using a 200 µL gel-loading tip. In addition, make sure that no residual gel material has obstructed the lanes, as this will have an adverse effect on the way the sample will load.

If at any time the comb causes the gel to dissociate from the acrylamide plug, or if the gel is pulled out along with the comb, the entire gel must be tossed and the ENTIRE process started over. It is not difficult to pull the gel out with the comb, which is why removing the comb takes such great care to execute.

1. Loading the Samples
2. Materials

* 200 µL gel-loading pipette tips: USA Scientific 1022-0600
* Ice
* Dry ice
* Loading samples

1. Methods
2. Determining the Appropriate Loading Volume

After the protein assay is performed and a concentration of 1 µg/µL is obtained for each sample, a volume of 3 µL is enough to achieve bands that can be visualized before saturation is reached.

1. Loading

Using the 200 µL gel-loading pipette tips, load 3 µL of each sample into the appropriate lanes.

Do NOT load important samples into lanes 1 and 10, as these lanes tend to droop down during electrophoretic. Therefore, samples loaded into lanes 1 and 10 will later make quantification difficult.

1. Running the Gel
2. Materials

* Power source (300 V, model 302): VWR 93000-744
* Refrigerator
* Spatula (No. 320, 45 mm): Nasuka 420J2 (HRC54)
* Gel staining box (11 cm x 11 cm x 3 cm): Owl Separation Systems GSB-3

1. Methods
2. Connecting the Gel to the Power Supply

Place the entire cooling unit-loaded gel running unit into the refrigerator. Cap the cooling unit with the clear cover connected to the power cords. Connect both the red and black power leads to their respective terminal on the power supply. Turn the power supply on and set it at a constant voltage; a preferred starting voltage is 40 V. The gel should run for approximately 7 hours, but set the timer to 14 hours (the maximum amount) in to allow for any necessary changes.

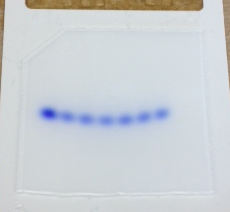
Keep an accurate record of the time and voltage of each gel in order to optimize the procedure. Normally, gels will run in two variable 3-hour intervals in which the voltage is gradually increased. The following is the preferred running time and voltage for a given small titin gel:

|  |  |
| --- | --- |
| Hour | Constant Voltage |
| 1-3 | 40 V |
| 3-6 | 60 V |
| If necessary… | 70 V |

The blue dye should reach the acrylamide plug, which is usually at about the level of the lower chamber solution.

1. Removing the Gel

Once the gel has completed its run, turn off the power supply and uncover the cooling unit. Remove the cooling unit from the refrigerator. Pour both the upper and lower chamber solutions in the sink and run water down the drain. Dismantle the cooling unit by removing the clamps from the gel.

Using the spatula, disassemble the gel sandwich by wedging the spatula end in between the plates and twisting. Take the glass plate off slowly and let the gel rest on the ceramic plate.

Now cut the excess gel where the combs are located. Cut a notch into the upper, left corner of the gel; this will be used as a direction marker during the scanning process. Cut off the acrylamide plug using the spatula end. Slide the gel into a clean gel staining box and secure the lid.

1. Staining

**Use ProQ Diamond staining first if you want to visualize phosphorylation followed by Sypro Ruby stain for total proteins.**

1. ProQ Diamond (Total time: ≥3.5 hours)
2. Materials

* 200 mL 50% methanol, 10% acetic acid (x2 100 mL)
  + 500 mL methanol: Fisher Scientific A412-4
  + 100 mL acetic acid: Fisher Scientific A38-212
  + 400 mL DI H2O
* 500 mL DI H2O (x5 100 mL)
* 60 mL ProQ Diamond stain: Invitrogen P-33300
* 240-300 mL ProQ Diamond destain: Invitrogen P-33310 or you can make your own:
  + 200 mL Acetonitrile: Sigma, 110086-4L
  + 800 mL 50 mM Sodium Acetate, pH 4.0
    - 500 mL DI H2O
    - 4.1015 g Sodium Acetate Trihydrate: Fisher, S209-500
    - pH to 4.0 using glacial acetic acid (7.885 mL)
    - dilute solution up to 1 L

1. Methods
2. Fix: 2x30 minutes

* 100 mL (ProQ Fixative Solution) 50% methanol, 10% acetic acid

1. Wash I: 2x10 minutes

* 100 mL DI H2O

1. Stain: 75 minutes

* 60 mL ProQ Diamond stain

1. Destain: 2x30 minutes

* 90 ml ProQ Diamond destain

1. Wash II: 2x5 minutes

* 100 mL DI H2O

When pouring any solution into a gel box, be careful not to pour directly onto the gel. Instead, pour the solution into the corner of the box. Also, make sure that each solution is disposed of properly. For example, all solutions used for staining process, except for the actual ProQ stain, can be aspirated. However, the stain must be pipetted out manually and place in a separate, marked waste container. Finally, be sure to not touch the gel with your hands, as the stain is very sensitive and will pick-up your fingerprints (even while wearing gloves!), which will make quantification harder.

1. Sypro Ruby (Total time: ≥13.5 hours)
2. Materials

* 200 mL 50% methanol, 7% acetic acid
* 60 mL Sypro Ruby Stain: Invitrogen S-12000
* 100 mL 10% methanol, 7% acetic acid

1. Methods
2. Fix: 2x30 minutes

* 100 mL (Sypro Fixative Solution) 50% methanol, 7% acetic acid

1. Stain: >12 hours

* 60 mL Sypro Ruby stain

1. Destain: 30 minutes

* Transfer to clean container.
* 100 mL (Sypro Destain Solution) 10% methanol, 7% acetic acid

1. Wash: 2x5 minutes

* 100 mL DI H2O