**Broad-Range Gel with and without Western Blot**

1. **1x Urea-Thiourea sample buffer preparation (Total time :4- 5 hrs, hands on time: 4-5 hrs)**
   1. **Materials**
      1. 8M Urea
      2. 2M Thiourea
      3. 0.05M Tris (pH 6.8)
      4. 75mM DTT
      5. AG-501-X8 Resin
      6. 3% SDS
      7. .022mm filter
   2. **Methods**
      1. Weigh 48g urea and 15.2*g* thiourea into a clean 150ml glass beaker; add a stir bar and 40ml of water (do not add too much water: the urea plus thiourea takes up over half the final volume).
      2. Use gloves to avoid contamination and skin contact with thiourea.
      3. Stir gently on a hot plate and avoid temperatures above 40°C (heating urea speeds the formation of cyanate).
      4. Add 10g of resin; stir mixture at room temperature for 15 minutes.
      5. Filter the mixture through filter paper into a 100ml graduated cylinder. The volume should be ~80-90ml.
      6. Carefully rinse the resin with one or two 5ml aliquots of deionized water. Transfer the filtered solution back to a clean 150 ml beaker.
      7. Weigh 0.605g Tris base, and 3g SDS and add to the urea-thiourea solution and stir till dissolved.
      8. Adjust pH to 7.5 carefully; add 37% HCl 100ul at a time (~300uL - 400ul).
      9. Add 1.155g of solid DTT and stir till dissolved.
      10. Continue adjusting pH down to 6.8 using 20ul aliquots of 2M HCl (the buffering capacity rapidly declines as the pH is lowered – use care not to overshoot!). However if you mistakenly overshoot use 2M Tris base to bring pH back to pH 6.8.
      11. Transfer solution back to the 100ml graduated cylinder; add DI water upto 100ml, mix. Filter through a 0.22mm filter (Millipore) to remove any fine particulate material.
      12. Transfer filtered sample buffer to 1ml or 0.5ml Microfuge vials; store at -80°C, thaw as needed just before use.
2. **Muscle sample preparation**
   1. **Materials**
      1. Dry ice
      2. 1x Urea-thiourea buffer
      3. Muscle samples
      4. Homogenizer
      5. 100% glycerol
      6. 1% bromophenol blue stock solution
      7. Bromophenol blue powder (to make 1% 1% bromophenol blue stock solution stock)
   2. **Methods**
      1. 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)
      2. Place the appropriate amount of sample buffer in the Dounce homogenizer (30:1 buffer to tissue, volume (ml)/weight (g)).
      3. Homogenize until no more tissue pieces are seen.
      4. Vortex samples for 30-60 seconds
      5. Spin briefly at 4000 x g for 30 seconds
      6. Take out 10 ul of the supernatant for protein assay (add 90uL water and keep aside on ice)
      7. Take rest of the supernatant out (leaving the pellets undisturbed) and place in new labeled 2.0 mL tubes.
      8. Aliquot samples into 500 uL volumes. Store at –80 ºC until ready to load on gels.
      9. After completing the protein assay, enter the adjusted concentration and homogenized volume into the protein assay spreadsheet. This will give you the appropriate volumes of glycerol, BPB, and UTU buffer to add to the sample.
      10. Heat samples at 60 ºC for 10 minutes. Also heat some glycerol in a separate test tube
      11. Add 30% glycerol to the samples
      12. Add bromophenol blue (1% stock), so that the final concentration will be 0.03%
          1. Make the 1% stock solution by adding 0.1 g of bromophenol blue to 10 mL DI water.
          2. Mix well and you can store it at room temperature.
      13. Vortex until samples are mixed with the glycerol
      14. Centrifuge at 13.200 x g for 10 minutes
      15. Remove supernatant and place in a clean tube
      16. Load on gels or store at -80 ºC
3. **RC-DC Protein Assay**
   1. **Materials**
      1. BSA standard
      2. RC-DC Reagent Kit
      3. Spectrophotometer
   2. **Methods**
      1. Prepare BSA standards (2.0,1.0,0.5,0.25 and 0 mg/ml BSA in water) by serial dilutions.
      2. Prepare samples (1:10 dilution by adding 10ul of sample to 90ul of water)
      3. Pipet 100 ul of standards and samples into dry clean test tubes
      4. Add 500 ul of RC Reagent I into each tube
      5. Vortex, let stand at RT for 1 minute
      6. Add 500 ul of RC Reagent II into each tube and then vortex
      7. Centrifuge at 15.000 g for 4-5 minutes
      8. Carefully aspirate the supernatant without disturbing the pellet
      9. Allow remaining supernatant to drain or evaporate
      10. Add 510 ul of Reagent A’ ( Reagent A’ is made by adding 20 ul of DC Reagent S to each 1 ml of DC Reagent A)
      11. Vortex. Incubate at RT for 5 minutes, or until pellet is dissolved. Vortex before proceeding to next step.
      12. Add 4 ml of DC Reagent B to each tube.
      13. Vortex immediately
      14. Incubate at RT for 15 minutes
      15. Place 200 ul of each standard and sample in triplicates in a 96 well plate.
      16. Use the spectrophotometer in Dr. Esser’s lab by reading the absorbance of the 96 well plate at 750nm using Lowry protein assay.
4. **Loading and Running the Gel Electrophoresis Unit**
   1. **Materials**
      1. Gel
      2. beta-mercaptoethanol
      3. Mini-PROTEAN 3 cell
      4. Bio-rad power supply
      5. 1x TGS buffer
      6. 10xTGS buffer (To make 1xTGS buffer)
      7. standards
   2. To make Standards
      1. Broad range protein standard (*for only broad range gel without western blot)*
         1. 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)

|  |  |
| --- | --- |
| 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
         1. E.g. pipette 5 uL of broad range standard and mix with 100 uL of SDS reducing buffer.
         2. Store sample temperature as other samples (-80 °C)
    1. Peppermint Stick phosphoprotein molecular weight standard
       1. 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.
          1. E.g. pipette 5 uL of standard in 30 uL of urea thiourea sample buffer with 30% glycerol and 0.3% of 1% bromophenol blue.
          2. Store sample temperature as other samples (-80 °C)
  1. **Methods**
     1. Use 4-15% acrylamide gel for a range 20-220 kDa proteins. Use 10% acrylamide gel for 150-40kDa proteins. Use 12% for smaller proteins.
     2. Remove gel from storage pouch and rinse in DI water.
     3. Pull clear tape from bottom of the gel (\*very important or else there will be an error while running gel)
     4. Place gel on one side of the gel cassette and a plate on the other side.
     5. Place gel cassette sandwich into the electrode assembly with the short plate facing inward
     6. Press down on the electrode assembly while closing the two cam levers of the clamping frame
     7. Lower the inner chamber into the mini tank
     8. Fill the inner chamber with ~125 ml of running buffer until the level reaches halfway between the tops of the taller and shorter glass plates of the gel cassettes.
     9. Add~700 ml for 1 or 2 gels of running buffer to the Mini Tank (lower buffer chamber).
     10. Add 200 uL of beta-mercaptoethanol to the inner chamber.
     11. Load the samples into the wells with a Hamilton syringe or a pipette using gel loading tips
     12. Place the Lid on the Mini Tank. Make sure to align the color coded banana plugs and jacks. The correct orientation is made by matching the jacks on the lid with the banana plugs on the electrode assembly.
     13. Insert the electrical leads into a suitable power supply with the proper polarity.
     14. Apply power to the Mini-PROTEAN 3 cell and begin electrophoresis; 200 volts constant is recommended for SDS-PAGE and most native gel applications. Run time is approximately

35 minutes at 200 volts for SDS-PAGE (the blue bands will have reached the bottom of the plate at the black line)

* + 1. After the run, power out the buffer in the sink. Run the sharp edge of the Gel Releaser or a razor blade along each spacer to separate the gel from the spacer.
    2. Remove the gel by floating it off the glass plate by inverting the gel and plate under fixative or transfer solution (depends on if you are proceeding with western blot or not), agitating gently until the gel separates from the plate.
    3. Rinse the Mini-PROTEAN 3 cell electrode assembly, Clamping Frame and Mini Tank with distilled, deionized water after use.

***Note: At this point you can either do a western blot (Follow V, VI,VII) with specific antibody to assess the content of one single protein or do Pro-Q diamond and Sypro ruby stain (follow VIII) to assess content of many proteins.***

1. **Western Blot- Transfer to membrane**
   1. **Materials: Transfer Buffers**
      1. **Anode I (Blotting paper) Add enough deionized water to make 1 L**

|  |  |
| --- | --- |
| **300mM Tris base** | **36.34g Tris** |
| **0.05% SDS** | **2.5 mL 20% SDS** |
| **10% Methanol** | **100mL Methanol** |

* + 1. **Anode II (PVDF membrane) Add enough deionized water to make 1 L**

|  |  |
| --- | --- |
| **20mM Tris base** | **2.42g Tris** |
| **0.05% SDS** | **2.5 mL 20% SDS** |
| **10% Methanol** | **100mL Methanol** |

* + 1. **Cathode Buffer (Gel and 2nd blotting paper) Add enough deionized water to make 1 L**

|  |  |
| --- | --- |
| **25mM Tris base** | **3.03 Tris** |
| **0.05% SDS** | **2.5 mL 20% SDS** |
| **40 mM Caproic Acid** | **5.25g Caproic Acid** |
| **10% Methanol** | **100mL Methanol** |

* 1. **Methods**
     1. Set up 4 containers per gel
     2. Cut membrane to the size of filter and notch one corner being careful not to touch with fingers.
     3. Hydrate membrane in methanol.
     4. Remove the membrane from methanol and place into container marked Anode II buffer. Soak for 15 minutes on shaker
     5. Get out 2 blotting papers and place 1 in Anode 1 buffer and the 2nd in Cathode buffer.
     6. Remove gel cassette from electrophoresis chamber. Crack open both sides. Use spatula to shave off the extra portions of the gel and put a notch in the top left conrer (at about well #1)
     7. Carefully pick up gel and soak gel in Cathode buffer for atleast 5 minutes.
     8. Open the semi-dry transfer cell and stack the contents of the containers in the following order from the bottom to top: 1 blotting paper (Anode 1), membrane (Anode II), gel (cathode buffer), 2nd blotting paper (cathode buffer).
     9. Be sure to align the notches made in the membrane and gel. Use the small roller to roll out any bubbles/creases after putting on the membrane and gel layers to ensure good transfer.
     10. Close lid and set for a constant 0.28 A (0.56 A for 2 gels) for 45 minutes (time will vary according to protein size)
     11. Once complete, gently mark two bands of the ladder on the membrane and label its wavelength with a pencil.

1. **Western Blot-Blocking and Primary Antibody**
   1. **Materials**
      1. **Blocking Buffer**
         1. **PBS (make PBS solution with 5 tablets in 1 L of DI water)**
         2. **Odyssey blocking buffer**
         3. **Mix 1:1 PBS-Odyssey blocking buffer.** 
            1. **Eg:-to make 400 mL mix 200 mL PBS and 200 mL of blocking buffer**
      2. **Primary Antibody**
         1. **Mix 1:1 PBS-Odysssey blocking buffer with 0.2 % tween**
            1. **Eg to make 200mL 199.6 mL of PBS:Blocking buffer and 0.4 mL 100% tween**
      3. **Wash solution**
         1. **Mix PBS and 0.1% Tween**
            1. **Eg to make 1 L use 999 mL of PBS and 1 mL of 100% Tween**
   2. **Methods**
      1. Block membrane in a 1:1 Odyssey blocking buffer and PBS solution
      2. Incubate on shaker for 1 hour at room temperature or overnight at 4 C
      3. Choose the primary antibody and make a 1:1000 (15 uL) dilution in 15 mL (per membrane) of 1:1 Odyssey blocking buffer and PBS 0.2 % tween(solution B). For the most part we use 1:1000 dilutions but it can vary, always check
      4. Incubate on shaker for 2 hours at room temperature or overnight at 4 C. This can be cut down to 1 hour at room temperature if needed.
      5. After incubation, wash membrane 4 times for 5 minutes each time on shaker in PBS+ 0.1% tween
      6. \*\*\* All stages beyond this point should be done in the dark (wrapped in foil)
2. **Western Blot-Secondary Antibody**
   1. **Materials**
      1. **Secondary Antibody**
         1. **Mix 1:1 PBS-Odysssey blocking buffer with 0.2 % tween and 0.01% SDS**
            1. **Eg to make 200mL 199.4 mL of PBS:Blocking buffer and 0.4 mL 100% tween and 200 uL of 10% SDS**
         2. **Wash solution**
            1. **Mix PBS and 0.1% Tween**
   2. **Methods**
      1. Choose the appropriate secondary antibody for the primary antibody that was used. It should be anti-species used (ex. If primary was mouse, secondary shoul be anti-mouse) and the same class (ex.IgG,IgM,etc.)
      2. Prepare 50 mL of solution (BB:PBS + 0.2% Tween+0.01% SDS+antibody) per membrane. Using a 1:7500 dilution for the antibody, you'll need 6.7 uL for 50 mL solution.
      3. Incubate on shaker (wrapper in aluminum foil) for 60 minutes.
      4. After incubation, wash membrane 4 times for 7 minutes each time on shaker in PBS+ 0.1 % tween (solution C). Keep in dark
      5. Rinse the membrane once with PBS. Membrane can remain in PBS until ready to scan (keep in aluminum foil)
3. **Western Blot -Scan** 
   1. **Materials**
      1. Li-Cor odyssey in the core lab physiology room (MS-541) on the 6th floor
      2. Rolling tool
      3. Forcep
      4. Jump drive
   2. **Methods**
      1. Keep image from light
      2. Log on the computer as user physiology (password: physiology)
      3. Double-click the Odyssey V3.0 shortcut on the dsektop to launch the odyssey program.
      4. We will be keeping all our data in the 'Campbell Lab' Click File>Open> Projects, highlight the 'Campbell Lab' folder located at
      5. C:\Users\Physiology\Documents\Licor\Odyssey\Projects and click open
      6. To start a new scan, go to File>Scan>Scan…(or press Alt+F1)
      7. The scanner login dialog box will appear, log in using the username and password 'user'.
      8. Set scan parameters. First, scan membrane on the lowest quality with the desired channel and dimension (usually 10 X 15). Set intensity at 5. Press Start Scan.
      9. Once you obtain the desired results cancel the scan and rescan the membrane on medium quality with the previous set parameters.
      10. When scan is completed, press the save… button (on the same row as the start scan button you just pressed), name the scan with the following format: MMDDYY species muscle antibody gel # (if applicable) name, e.g. 052512 Hum Car cTNI Gel 1 Premi.
      11. Use the analysis name 'original analysis' and click ok.
      12. Before leaving, copy your scans folder (located at C:\Users\Physiology\Documents\Licor\Odyssey\Projects\Campbell Lab, Use shortcut to Projects located on desktop) on the jump drive (F:)
      13. Importing Scan into Odyssey. Open Odyssey and expand the Odyssey Projects folder.
      14. Identify which project you are working with and open it by double clicking on the folder.
      15. Click File>Scan>Import Scan. Select the file that you just saved on the jump drive and click OK.
4. **Western Blot- Ponceau Staining** 
   1. **Materials**
      1. **Ponceau Stain**
   2. **Methods**
      1. After scanning the membrane upstairs, download into Odyssey and make sure the scan worked. Only then begin staining with ponceau.
      2. Pour enough Ponceau S staining solution to cover the membrane and place on the shaker at medium speed for 45 minutes.
      3. Pour out the staining solution and spray with DI water to remove the stain. Continue until most of the red has disappeared on the surrounding area except for on the bands of protein.
      4. Place membrane on a blotting paper in a drawer in order to dry before scanning.
      5. Open Epson scan and verify the mode is set to Full Auto Mode.
      6. Click Customize>File Save Settings.
      7. Label the file exactly how it is labeled in Odyssey with “ponceau” at the end
      8. Select the file to be saved as a Tiff and click OK and then Scan.
      9. Edit the Tiff file in Photoshop. Under the image tab crop the image to only show the membrane and rotate if necessary. Click mode in the image tab and save it as a 16-bit, gray-scale image.
      10. Be sure to save original sane and altered scan (labeled the same with “16 bitat the end) in the correct project folder.
      11. Open Odyssey, go to File and click on Import image.
      12. Label the file exactly how it is labeled in Odyssey with “ponceau” at the end.
      13. Uses the Analysis name write Ponceau.
      14. Use the 800 channel and browse for the 16-bit, gray scale image in the project folder it was saved to, then click OK
      15. Go to alter image display under View. Click linear manual
      16. Go to adjust image curves under View. Select grayscale. Click the boc that says inverse gray scal and adjust the curve.
      17. Follow the Analyzing Scans in Odyssey (for westerns, ponceaus stains, and coomassie stains)protocol for the next step.
5. **Staining with Pro-Q diamond after running gel** Total time: ≥4.15 hours
   1. **Materials**
      1. **Pro-Q diamond**

|  |  |  |
| --- | --- | --- |
| **Steps** | **Reagent** | **Protocol** |
| **1:Fix** | **50% methanol, 10% acetic acid** | **100 mL, 30 min (2 times)** |
| **2:Wash** | **Ultrapure water** | **100 mL, 10 min (3 times)** |
| **3:Stain** | **ProQ Diamond stain** | **60 mL, 60-90 min** |
| **4:Destain** | **Pro-Q Diamon destain solution or 20% acetonitrile, 50 mM sodium acetate, pH 4** | **80-100 mL, 30 min (3 times)** |
| **5: Wash** | **Ultrapure water** | **100 mL, 5 min (2 times)** |

* 1. **Methods**
     1. Use ProQ diamond staining first if you want to visualize phosphorylation of bands followed by Sypro Ruby stain for total proteins.
     2. Slide the notched gel into the gel staining box (11 cm x 11 cm x 3 cm) and add 100 mL 50% methanol, 10% acetic acid. When pouring solutions into the gel box, take care not to pour any solution directly onto the gel or gel might tear.
     3. After 30 minutes, repeat the procedure once. In order to get rid of any solution, turn on the vacuum line connected to the large Erlenmeyer flask that has a stopper attached to a hose that ends with a glass pipette and vacuum out the solution from any corner. Take care not to vacuum the gel by accident.
     4. After the ProQ Diamond stain pipette the stain into the bottle labeled “Stain Waste”, which is located in the cabinet underneath the counter. Do NOT use the vacuum to remove any stain from a gel box. Instead, use a transfer pipette and repeatedly suction the stain out of the gel box until a negligible amount of stain remains.

1. **Scanning with Pro-Q diamond**
   1. **Materials**
      1. **Typhoon scanner on 5th floor or 6th floor core facility**
   2. **Methods**
      1. Check if someone is using the scanner if not write your name in the log
      2. Open the scanner lid and then place your gel in the scanner using plastic
2. **Staining with Sypro Ruby after running gel or after Pro-Q diamond staining** Total time: ≥4.15 hours
   1. **Materials**
      1. **Pro-Q diamond stain**
      2. 200 mL 50% methanol, 10% acetic acid (x2 100 mL)
      3. 500 mL DI H2O (x5 100 mL)
      4. 60 mL ProQ Diamond stain
      5. 240-300 mL ProQ Diamond destain (x3 80-100 mL)
   2. **Methods**
      1. Use ProQ diamond staining first if you want to visualize phosphorylation of bands followed by Sypro Ruby stain for total proteins.
      2. Fix
      3. Slide the notched gel into the gel staining box (11 cm x 11 cm x 3 cm) and add 100 mL 50% methanol, 10% acetic acid. When pouring solutions into the gel box, take care not to pour any solution directly onto the gel. Instead, pour the solution into any corner of the gel box such that the solution level gradually covers the gel.
      4. After 30 minutes, repeat the procedure once. In order to get rid of any solution, turn on the vacuum line connected to the large Erlenmeyer flask that has a stopper attached to a hose that ends with a glass pipette and vacuum out the solution from any corner. Take care not to vacuum the gel by accident.
      5. Wash
      6. After 30 minutes, vacuum the 50% methanol, 10% acetic acid out of the gel box. Add 100 mL DI H2O and vacuum out after 10 minutes. Repeat this process an additional two times.
      7. Stain
      8. With the wash vacuumed out, add 60 mL ProQ Diamond stain to the gel box. After 75 minutes, pipette the stain into the bottle labeled “Stain Waste”, which is located in the cabinet underneath the counter. Do NOT use the vacuum to remove any stain from a gel box. Instead, use a transfer pipette and repeatedly suction the stain out of the gel box until a negligible amount of stain remains.
      9. Destain
      10. After the stain has been removed, add 90 ml ProQ Diamond destain to the gel box. After 30 minutes suction out the destain solution and repeat the process two more times.
      11. Wash
      12. Once the gel has been destained, add 100 ml DI H2O to the gel box and remove it via vacuum suction after 5 minutes. Repeat this process once, but do not remove the DI H2O.
3. **Staining with Sypro Ruby after running gel** Total time: ≥13.6 hours
   1. **Materials**

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| --- | --- | --- |
| **Steps** | **Reagent** | **Protocol** |
| **1:Fix** | **50% methanol, 7% acetic acid** | **100 mL, 30 min (2 times)** |
| **2:Stain** | **Sypro Ruby stain** | **60 mL, 60-90 min** |
| **3:Destain** | **10% methanol, 7% acetic acid** | **80-100 mL, 30 min** |
| **4: Wash** | **Ultrapure water** | **100 mL, 5 min (2 times)** |

* 1. Methods
     1. Fix (Do this fix only if ProQ staining was not done)
     2. Slide the notched gel into the gel staining box (11 c m x 11 cm x 3 cm) and add 100 mL 50% methanol, 7% acetic acid. When pouring solution into the gel box, take care not to pour any solution directly onto the gel.
     3. After 30 minutes, repeat the procedure once. In order to get rid of any solution, turn on the vacuum line connected to the large Erlenmeyer flask that has a stopper attached to a hose that ends with a glass pipette and vacuum out the solution from any corner. Take care not to vacuum the gel by accident.
     4. Stain by adding 60 mL Sypro Ruby stain to the gel box and let it shake in the shaker overnight. After approximately 12 hours have passed, remove the stain from the gel box using a transfer pipette and place in appropriate bottle labeled “Sypro ruby” hazardous waste. Do NOT use the vacuum to remove any stain from the gel box.
     5. Wash for 30 minutes, remove the solution and add 100 mL DI H2O. After 5 minutes, remove the DI H2O and another 100 mL DI H2O to the gel box, but this time do not remove the DI H2O after 5 minutes.