**Immunoblotting**

*Walker Orr, 05/07/2024*

**Buffers**

**RIPA Buffer (Lysis)**

50 mM Tris-HCl, pH 8.0

150 mM NaCl

0.1% Triton X-100

0.5% Sodium deoxycholate

0.1% SDS

Protease inhibitors

**Loading Buffer (Laemmli)**

4% SDS

20% glycerol

0.004% bromophenol blue

0.125 M Tris-HCl

(10% 2-mercaptoethanol “BME”)

Check pH and adjust to pH 6.8 if needed.

Add BME just before use.

**Running Buffer**

25 mM Tris

190 mM Glycine

0.1% SDS

**Transfer Buffer**

25 mM Tris

190 mM Glycine

20% Methanol

For larger proteins: 0.1% SDS.

**Ponceau S staining buffer**

0.2% (w/v) Ponceau S

5% glacial acetic acid

**Tris-buffered saline with Tween-20 (TBST)**

20 mM Tris, pH 7.5

150 mM NaCl

0.1% Tween-20

**Blocking Buffer**

3% bovine serum albumin (BSA) in TBST

**Stripping Buffer**

20 ml 10% SDS

12.5 ml 0.5 M Tris HCl, pH 6.8

67.5 ml milliQ water

0.8 ml 2-mercaptoethanol

These buffers can also be made from commercially-supplied 10x stock solutions.

**Cell Lysis and Protein Quantification**

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| **Harvest Cells** |
| 1. Prepare ice and keep RIPA buffer and protein lo-bind tubes to receive the lysate on ice. 2. Aspirate media from the cell flasks and wash with room temperature PBS. 3. Add RIPA buffer to the cells:    1. T25: 500 μl. 4. Use a cell scraper to detach cells from the side of the flask. Transfer to pre-chilled protein lo-bind tube and keep on ice for 30 minutes. Pre-chill a microcentrifuge to 4C. Flick the tube occasionally to promote lysis. 5. Centrifuge lysate for 15 minutes at max rcf and 4C. |
| **Quantify Total Protein** |
| 1. Use Pierce BCA Protein Assay Kit (Thermo 23225/23227). **Alternatively, use Qubit Protein Broad Range assay.** 2. Directions from the assay are transcribed below: 3. Prepare a dilution series of BSA standards in RIPA buffer:  |  |  |  |  | | --- | --- | --- | --- | | **Standard** | **Vol. of Diluent** | **Vol. and source of BSA** | **Final [BSA] μg/mL** | |  | 0 | 300 μl Stock | 2000 | |  | 125 | 375 μl Stock | 1500 | |  | 325 | 325 μl Stock | 1000 | |  | 175 | 175 μl vial B | 750 | |  | 325 | 325 μl vial C | 500 | |  | 325 | 325 μl vial E | 250 | |  | 325 | 325 μl vial F | 125 | |  | 400 | 100 μl vial G | 25 | |  | 400 | 0 | Blank |  1. If you are concerned about protein concentration exceeding the assay range, prepare 1:10 dilutions of lysate samples. 2. Prepare a volume of working reagent as follows for triplicate measurements of standards and of samples:    1. Calculate the volume of working reagent required: (9 + [# samples]) \* .6 mL) = total volume required.    2. Prepare working reagent by mixing 50 parts reagent A (clear) with 1 part reagent B (blue). When mixing a turbidity will appear and quickly disappear to yield a clear, green working reagent. 3. Add 10 ul of standard or analyte in triplicate to wells in a 96 well plate and note their positions in your lab notebook. Add 200 ul of working reagent and mix well with a multichannel pipette. 4. Cover plate and incubate at 37C for 30 minutes. 5. Cool plate to room temperature. Measure absorbance at 562 on plate reader. |
| **Determining Protein Concentration from A562** |
| 1. Subtract the average measurement of the Blank standard replicates from the 562 nm measurements of all other individual standard and unknown sample replicates. 2. Prepare a standard curve by plotting the average Blank-corrected 562 nm measurement for each BSA standard vs. its concentration in ug/mL. Use the standard curve to determine the protein concentration of each unknown sample.    1. A point-to-point curve is preferable to a linear fit. |
| **Sample Storage** |
| 1. For optimal storage, combine sample 1:1 with 2x Laemmli buffer (remember to add 2-mercaptoethanol “BME” to the Laemmli buffer stock). Boil for 5 minutes at 95C, then centrifuge for 1 minute at 16,000 x g, then (aliquot?) and freeze at -20C. |

**SDS-Page, Transfer, and Immunoblotting**

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| **SDS-PAGE** |
| 1. Load equal amounts of protein (20 ug is a good starting place) in each lane of a mini or midi format SDS-PAGE del, along with molecular weight markers (ladder). Note that the choice of gel percentage will depend on the proteins of interest; a 4-20% gradient gel separates proteins of all sizes very well. |
| 1. Bring the electrophoresis tank to the cold room (this will limit diffusion of smaller proteins in the gel). Run the gel for 5 minutes at 50V. |
| 1. Increase voltage to 100-150 V to finish the run in about an hour. |
| **Transfer (Tank)** |
| 1. For all subsequent steps involving the membrane, it is \*critical\* that all surfaces, implements, etc. be cleaned with DI water. |
| 1. Place the transfer cassette (1), gel (1), filter paper (2), nitrocellulose membrane (1), and grey sponges (2) in 1x transfer buffer for 10-15 minutes. If using PVDF, wet in 100% methanol until the membrane becomes translucent. |
| 1. Make sure the transfer cassette is clear side down. Place a sponge, piece of filter paper, and membrane on the cassette, in that order. Use tweezers when handling the membrane. Then, place the pre-wetted gel on top of the membrane. Place another piece of filter paper on the sandwich. Underwater, roll the sandwich out to remove any air bubbles. Place another sponge on top and close and lock the cassette. Make sure the filter paper and membranes are carefully aligned with the cassette; there isn’t much clearance. |
| 1. Place the transfer cassette with the assembled sandwich in the tank with the **black side of the sandwich facing the black side of the electrophoresis insert.** The SDS-coated (negative) protein in the gel will migrate towards the cathode (red) where it will become immobilized on the membrane. |
| 1. Place an ice pack in the tank. Bring the blotting tank and extra transfer buffer with you to the walk-in cooler (4C). |
| 1. Fill the blotting tank to the fill line with transfer buffer. |
| 1. Transfer overnight (16 hours) at constant current of 10 mA. |
| **Transfer (Semi-Dry)** |
| 1. Prepare 500 mL Trans-Blot Turbo Transfer buffer by mixing 100 mL 5x transfer buffer with 300 mL milliQ water and 100 mL ethanol. |
| 1. Wet and equilibrate membrane and two transfer stacks.    1. Nitrocellulose: immerse in 30 mL 1x transfer buffer for 2-3 minutes.    2. PVDF: immerse in 100% methanol or ethanol until membrane is translucent, then transfer to a gel tray containing 30 mL 1x transfer buffer. Equilibrate membrane for 2-3 minutes.    3. Transfer stacks: immerse 2 stacks (these are supplied separated by blue sheets) in a gel tray containing 50 mL transfer buffer for 2-3 minutes. |
| 1. Place one wetted stack on bottom of cassette. This will serve as the bottom ion reservoir stack. |
| 1. Place wetted membrane on top of the wetted stack in the cassette. |
| 1. Place gel on membrane.    1. Do not equilibrate the gel before transfer.    2. If needed, remove any air bubbles with blot roller.    3. For two mini gels: orient the bottom of each the gel toward the center. |
| 1. Place second wetted transfer stack on top of gel. This will serve as the top ion reservoir stack.    1. Roll assembled sandwich with blot roller to expel trapped air bubbles.    2. Do not add extra transfer buffer to the cassette; saturated transfer stacks provide ample buffer. Once assembled, absorb excess transfer buffer from the cassette with paper towels. |
| 1. Close and lock cassette lid. Insert cassette in the instrument and begin transfer. |
| 1. Recommended settings:  |  |  |  |  |  | | --- | --- | --- | --- | --- | | **Protocol Name** | **MW, kDa** | **Time, min** | **2 Mini Gels or 1 Midi Gel** | **1 Mini Gel** | | STANDARD SD |  |  | Up to 1.0 A; 25 V constant | | | 1.5 MM GEL |  |  | 2.5 A constant;  Up to 25 V | 1.3 A constant;  Up to 25 V | | HIGH MW |  |  | | LOW MW |  |  | | MIXED MW | 5-150 | 7 | | 1 Mini TGX | 5-150 | 3 | NA | 2.5 A constant; up to 25 V | |
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| Acceptable Gel Combinations:   |  |  |  |  |  | | --- | --- | --- | --- | --- | | Combination | OK | OK | Not OK | No OK | | Cassette 1 | 1 mini gel | 2 mini gels or 1 midi gel | 1 mini gel | 2 mini gels or 1 midi gel | | Cassette 2 | 1 mini gel | 2 mini gels or 1 midi gel | 2 mini gels or 1 midi gel | 1 mini gel | |
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| **Antibody Incubation** |
| 1. Rinse blot in water and stain with Ponceau S solution to check transfer quality. Handle the membrane carefully; dispense water on the side of the container rather than letting it hit the blot directly. |
| 1. Rince off the Ponceau S stain with three washes of TBST. |
| 1. Block in 3% BSA in TBST at room temperature for an hour. 5% milk is also acceptable **OR**   Block for 5-10 minutes in Bio-RAD EveryBlot Blocking Buffer. |
| 1. Incubate overnight in the primary antibody solution against the target protein(s) at 4C. Use a 2D shaker at the gentlest possible setting to make sure all parts of the membrane remain covered in liquid. |
| 1. Rinse the blot 3-5 times for 5 minutes in TBST. |
| 1. Incubate in HRP-conjugated secondary antibody solution for 1 hour at room temperature. |
| 1. Rinse blot 3-5 times for 5 minutes with TBST. |
| **Exposure** |
| 1. To make chemiluminescent substrate, mix (immediately before use) 1:1 cytivia ECL Western Blotting Detection Reagents 1 and 2. You need .125 ml solution per cm2 membrane (for a Bio-RAD mini gel membrane [60 cm2], this comes out to around 7.5 ml per blot). |
| 1. Incubate 1 minute, then drain by the edge on a paper towel. |
| 1. Capture the chemiluminescent signals using a CCD camera-based imager. |
| 1. Use ImageJ or other image analysis software to read the band intensity of the target protein. |

**Supplemental Section I: Stripping and Reprobing**

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| 1. Warm stripping buffer to 50C. |
| 1. Add the buffer to the membrane in a container designated for stripping. Incubate at 50C for 45 minutes with some agitation. |
| 1. Rinse the blot under running water for 1 minute. |
| 1. Transfer the membrane to a clean container, wash 5 times for 5 minutes in TBST. You need to remove every last trace of 2-mercaptoethanol, which will denature your detection antibodies. |
| 1. Repeat antibody incubation and exposure steps from the section above. |

**Supplemental Section II: Tested Antibodies and Concentrations**

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| **QVEU Index** | **Primary Antibody** | **Tested Concentration** | **Notes** |
| Ab06 | SCARB2 | 1:2000 |  |
| Ab20 | EXT1 | 1:1000 | Terrible antibody: a lot of nonspecific binding |
| Ab02 | GAPDH | 1:5000 |  |
| Ab24 | 3C | 1:1000 |  |
| Ab25 | 3D | 1:1000 |  |

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| **QVEU Index** | **Secondary Antibody** | **Tested Concentration** | **Notes** |
| S08 | Anti-mouse HRP | 1:10,000 |  |
| S06 | Anti-goat HRP | 1:1000 |  |

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| **QVEU Index** | **Conjugated Primary Antibody** | **Tested Concentration** | **Notes** |
| Ab23 | ALFA Nanobody (Alpaca)-HRP | 1:1000 |  |
| P05 | GAPDH-HRP | 1:5000 |  |