Pullen Lab Protocol for Western Blotting with Bio-Rad Equipment

Note: this protocol uses the Transblot Turbo "dry" blotter. For Bio-Rad tank blotting, see protocol 3b in this series.

Materials:

- Container with ice
- Protein samples
- 4X Laemmli Buffer with β-mercaptoethanol (stored -20°C)
- Appropriate protein ladder (typically the Li-Cor chemiluminescent ladder, stored -20°C))
- Appropriate polyacrylamide gel (typically 4%-20% TGX mini, stored 4°C)
- Tris-Glycine running buffer (diluted from 10X stock)
- Bio-Rad tank and gel cassette/clamp
- Buffer Dam (if running an odd number of gels)
- 10µL and 20µL micropipettes with tips
- Vortex mixer
- 1.5mL microcentrifuge tubes
- Single-speed minicentrifuge
- Heat block
- Gel opening tool
- 2D rocker
- Blot container
- Blotting packs (typically nitrocellulose, stored 4°C)
- Roller
- Appropriate blot buffers: TBS, TBS-T
- BSA, Cohn Fraction V (stored 4°C)
- Nonfat Dry Milk
- Ponceau S Stain
- Transblot Turbo equipment
- Desired primary antibody
- Appropriate secondary antibody (typically, HRP-conjugated from Li-Cor, stored 4°C)
- ECL detection reagent
- Serological Pipettes
- Pipettor
- ECL detection equipment

PART I: PREPARING SAMPLES

Before you start this, you should calculate the volume of sample required to load 50µg of total protein. If you do cannot have 50µg in 15µL or less for every sample, then you should set loading to the amount you can load in 15µL with your most dilute sample.

1. Turn on the heat block and set for 90°C. Thaw the following and keep on ice:
    a. Samples
    b. 4X Laemmli (with β-ME)
    c. Ladder
2. Into fresh 1.5mL microcentrifuge tubes, aliquot the volume of each sample necessary for 50µg of total protein.
3. Add to the aliquots MilliQ H_2O up to 15µL.
    a. Ex.: if you aliquoted 8µL of sample, then add to it 7µL of water.
4. To each sample add 5µL 4X Laemmli.
5. Briefly vortex then centrifuge in the minicentrifuge.
6. Place mixture tubes on the 90°C heat block for 10min.
7. Place heated tubes on ice, turn off the heat block. After a couple minutes of cooling, vortex and centrifuge again, then keep on ice.
8. These prepared samples can be stored at -20°C, however this is not recommended.

PART II: SDS-PAGE

1. Assemble the gel tank according to the manufacturer's instructions. **Caution**: make sure the seal tape on the bottom of the gel is removed.
2. Fill the tank and inner chamber with 1X Tris-Glycine RUNNING Buffer. The amount you use will depend on the number of gels, and the tank has lines indicating this.
    a. Make sure the inner chamber is filled just above the top of the gel wells.

3. Load 5µL of ladder into one lane.
4. Load 20µL of each sample into their own respective wells – change tips!
5. Make sure all electrical lead colors match up (including the gel clamp), place the top on the tank, and plug it into the power supply.
6. Turn on the power supply, and set for the appropriate CONSTANT VOLTAGE. This value will depend on your application, but it maxes out at 300V (a 10-15min run). An instruction card is kept with the power supply providing additional details on this.
7. Press run.
8. Stop the run when the blue dye front has reach the bottom of the gel.
9. You MUST proceed to blotting immediately.

PART III: BLOTTING

This is a "dry" blot, meaning that no transfer buffer should be added.

1. Retrieve a blot pack and the gel opening tool. Open one of the transblot electrode module
2. Carefully open the gel cast with the gel opening tool.
3. Open the blotting pack, then place the "BOTTOM" stack (including membrane) into the transblot electrode.
4. Gently roll out any bubbles in the stack.
5. Carefully place the gel over the membrane.
6. Place the "TOP" stack over the gel.
7. Gently roll out any bubbles.
8. Lock into place the transblot electrode top.
9. Place the electrode module into the device.
10. Turn on the device and select the appropriate program. Typically this will be "Turbo" → "Mini TGX". Then press the button to run the appropriate electrode module.
11. When the transfer is done (3-7min), immediately move the membrane to a clean container with 1X TBS.
12. Quick rinse the blot 3 times with 1X TBS to remove the transfer buffer.
13. OPTIONAL: check the blot with Ponceau S stain:
    a. Dump the 1X TBS.
    b. Add enough Ponceau S to the container to cover the blot.
    c. Rock vigorously on the 2D rocker (*e.g.* setting 35)
    d. Bands will appear pink wherever there is protein.
    e. To remove the stain dump the excess, quick rinse 3 times with 1X TBS, and then do at least two longer rinses (3-5min) on the rocker, vigorously, or until no more pink staining is evident.
14. The blot can be stored 4°C in 1X TBS. **CAUTION**: do not store blots in TBS-T.

PART IV: PROBING

Always consult the antibody datasheets before starting this.

1. Prepare an appropriate blocking buffer. This will depend on the primary antibody. Typically, we use *Cell Signaling Technologies* primaries, and they work best in a 5% BSA (w/v) 1X TBS-T solution.
2. Dump any residual TBS from the blot container and pipette an appropriate volume of blocking reagent over the blot – this should be enough to just barely cover the blot, usually 10mL for our containers.
3. Incubate at room temperature for 1 hour, with gentle agitation on a 2D rocker (*e.g.* setting 10). **CAUTION**: make sure there are no "dry spots" on the blot when it rocks.
4. Add primary antibody directly to the blocking buffer according to its datasheet (for *Cell Signaling* Abs this is usually 1:1000, so 10µL).
5. Incubate in primary overnight with gentle agitation at 4°C (move the 2D rocker to the cold room).
6. Dump the primary:blocking solution and quick rinse with 1X TBS-T.
7. Rinse at least 3 more times in 1X TBS-T for 5min each.

8. Prepare secondary antibody solution. This again depends on the source, so check the datasheet, but we typically use 5% milk (w/v) 1X TBS-T with secondary antibody diluted 1:5000. **CAUTION:** make sure you are using the correct secondary (*e.g.* anti-mouse secondary for mouse primary antibodies).
9. Dump any residual TBS-T and cover the blot with secondary antibody solution. Incubate at room temperature on the 2D rocker for at least 1 hour.
10. Rinse the blot as in steps 6 & 7 immediately above.
11. Prepare ECL detection reagents as directed by the manufacturer's protocol (we typically use the Li-Cor WesternSure kit, and instructions are provided with it).
12. Expose the blot to ECL detection reagents, and scan the blot using the Li-Cor cDigit device.

*the blot can be stored at 4°C in 1X TBS after any rinsing step, but antibodies will degrade over time.