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JOHNS HOPKINS MEDICINE

Hackam Lab
Westerns Blots

PREPARE FIRST

1. **Lysis Buffer Cocktail**

   - Lysis buffer (add 40 µl/well, if using a 6-well plate)
   - Protease inhibitor cocktail (10x)
   - Phosphotase inhibitor (40x)

2. **SDS Page Gel**

   - Cast gel using a 1.5 millimeter glass casting set. Remember to wet the rubber membrane the glass sits on.

   - Make gel according to this recipe
     - Deionized water
     - 30% acrylimide gel mix
     - 1.5 M tris (pH 8.8)
     - 10% SDS
     - 10% ammonium persulfate
     - TEMED ***Note: When this is added the liquid starts to solidify, so move quickly***

   - Add mixture to glass set with a disposable pipette. Leave an inch from the top. Fill the rest with 70% ethanol to make a nice line.

   - Let stand for 30 minutes. After 30 minutes, make stacking gel. Let stand for 20 to 30 minutes.

HARVESTING CELLS

- Wash with cold PBS x 2.
- **Be sure to aspirate all the PBS.**
- Add 40 µl of lysis buffer cocktail to each well.
- Scrape cells.
- Place in 1.5 milliliter tubes (shorthand-labeled).
- Spin down at 10,000 r.p.m. for 10 minutes at 4°C.
- Transfer supernatant to a prechilled, well-labeled tube (cell type, treatment, date, etc.).

PREPARING TISSUE LYSATES

- Homogenize tissue.
- Spin down at top speed for 15 minutes at 4°C.

BCA TEST FOR PROTEIN DETERMINATION

- Follow template on wall using deionized water, BSA (bovine serum albumin), DI water (18 µl) and 2µl of unknown sample.
- Do samples in duplicate. Once all the standards and samples are loaded, mix copper sulfate and BCA (200 µl copper sulfate to 9800 µl BCA). Add 200 µl to each well and incubate for 20 minutes. Then measure on plate reader.

SETTING UP RUNNING APPARATUS AND TRANSFER APPARATUS

1. **Running SDS page Gel**
   - Insert glass with gel and dam on the electrical apparatus.
   - Fill inner chamber with 1x running buffer.
   - Fill outer chamber with old running buffer (about three-quarters of the way).
   - Remove comb and load protein.

   - After measuring plate, take amount of sample required for 30 µg of protein and add equal amount of 2x sample buffer.
   - Add 1x running buffer up to a total of 15 µl.
   - Mix and then heatshock for 3 minutes at 95°C
   - Load 8 µl of ladder.
   - Load samples and run at 60V until samples get through the stacking gel, then increase to 100 V for 1 to 2 hours.

2. **Transfer**
   - When run is finished, cut membranes and transfer pads (two thick transfer pads and one membrane; which membrane depends on the molecular weight of protein).
   - Soak pads in transfer buffer and soak membrane in Methonal (Methonal for 5 seconds).
   - Wash membrane in DI water for 1 minute, then soak in transfer buffer for 3 to 5 minutes.
   - Place glass with gel into transfer buffer and split the glass.
   - Cut off the stacking gel.
   - Place the thick pad on the transfer apparatus and roll out the air bubbles.
   - Place on membrane and repeat the roll step.
   - Place on gel, roll and place on thick pad, and finish with final roll. Run for 45 minutes at 25 V.

- After transfer is done, place membrane in Ponceu S (a red liquid) and rock for 5 minutes.
- Remove Ponceu S and block with 1% TBS-Tween-BSA for 45 minutes on the shaker. {confirm spelling of Ponceu S. Also, is it a brand or generic name?}

PRIMARY ANTIBODY

- When blocking is done, place membrane in plastic pouch.
- Before sealing, add 4 ml of 1% TBS-T and add the appropriate amount of antibody (1:1000 dilution equals 4 µl antibody, 4 mL TBS-T).
- Probe for primary overnight at 4°C placed on rocker.

SECONDARY ANTIBODY

- Next day, remove membrane from pouch.
- Wash with PBS tween 3 x 10 minutes each wash.
- After last wash, add 10 milliliters of fish tween and 2 µl of secondary antibody (1:5000).
- Place on rocker for 1 hour at room temperature.

- Wash secondary with PBS tween (3 x 5 minutes) and add HRP.
- Mix Supersignal with Super enhancer at 1:1 (TV 1 ml) and add to the top of the membrane (the protein side of the membrane).
- Let sit for maximum 1 minute.
- Develop film.
- When finished with film, wash blot with PBS tween, wrap in plastic wrap and place in fridge.

STRIPPING AND RE-PROBING

- Wash membrane in DW water briefly.
- Then wash membrane in PBS-tween for 20 minutes and soak in stripping buffer for 20 minutes (can strip for up to 2 hours when removing abundant antibody from previous probe).
- Wash 2 minutes with PBS-tween to remove stripping buffer.

Proceed to probing with primary antibody.