ADMEbio
for proteomic research

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Western Blotting (WB) Protocol

I. Materials and Reagents:

1. Lysis buffer:
   - 0.15M NaCl
   - 5mM EDTA, pH 8;
   - 1% Triton X100;
   - 10mM Tris-Cl, pH 7.4
   - Just before use add:
   - 5M DTT 1:1,000;
   - 100mM PMSF in isopropanol 1:1,000;
   - 5M ε–aminocaproic acid 1:1,000

2. 5X Sample Buffer:
   - 325 mM Tris-HCl (pH 6.8)
   - 10% (w/v) SDS
   - 0.05% Bromophenol blue
   - 50% (v/v)glycerol
   - 5ml of 0.5M DTT (pH8.0)

3. 1.5M Tris-HCl Resolving Gel Buffer: 500ml
   91g Trizma base and adjust pH to 8.8 with 12N HCl

4. 1.0M Tris-HCl Stacking Gel Buffer: 500ml 60.55g Trizma base and adjust pH to 6.8

5. Resolving Gel: 10ml of a 12% gel
   - 4ml 30% acrylamide/bisacrylamide (29:1 mix)
   - 2.5ml Resolving Gel buffer
   - 100µl 10% SDS
   - 3.3ml water
   - 4µl TEMED
   - 100µl 10% ammonium persulfate

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6. Stacking Gel: 10ml
   - 1.4 ml 30% acrylamide/bisacrylamide (29:1mix)
   - 2.5ml Stacking Gel buffer
   - 100µl 10% SDS
   - 6ml water
   - 10µl TEMED
   - 100µl 10% ammonium persulfate

7. 10x Running Buffer: 1L
   - 30.3g Trizma base (= 0.25M)
   - 144g Glycine (= 1.92M) 10g;
   - add SDS (= 1%) finally.
   - Do not need to adjust the pH.
   - 10x transfer Buffer: 1L
   - 30.3g Trizma base (= 0.25M)
   - 144g Glycine (= 1.92M)
   - pH should be 8.3; do not need to adjust pH.
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   - To make 2L of 1x transfer buffer:
   - 400ml Methanol and 200ml 10X TBS buffer
   - Blocking Buffer: 0.5L
   - 3-5% nonfat milk in TBST (0.05%Tween 20) or 5% BSA in TBST (0.05%Tween 20)
   - Make up in TBS and sterile filter. Then add Tween 20 to final 0.05%.
     Keep at 4°C to prevent bacterial contamination.

8. Stripping Buffer: 0.5L (sterile filter solution and keep at 4°C)
   - 0.2M Glycine, pH 2.5, 0.05% Tween 20

II. Experimental Procedure:

1. Preparation of cell lysates:
   - Collect cells (confluent 10cm2 cell culture dish) by trypsinization or scrape and then spin at 3000rpm for 5 minutes at 4°C.
   - Abandon the supernatant, wash the cells twice with PBS, spin down (12,000rpm for 1 minute at 4°C) and collect cells on ice.
   - Split the cells with appropriate amount of Cell Lysis Buffer on ice for 30 minutes, and sonicate on ice for 10-30 seconds three to six times or until there's no cell clusters.
   - Spin at 12,000 rpm for 20-30 minutes at 4°C.
   - Transfer the supernatant to a new tube on ice and discard the pellet.
   - Measure the protein concentration (Bradford assay, A280, or BCA).
   - Add x µl (x=volume of the cell lysates/4) 5X sample buffer.
   - Boil for 5-10 minutes and cool at RT/on ice for 5 minutes.
   - Spin at 12,000rpm for 10 seconds to bring down condensation prior to loading gel.

2. Preparation of gel:
   - Assemble the plastic plates and spacers (1.0mm thick).
   - Pour the running gel to approximately 1cm below the wells of the comb (~6ml).

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- Seal with 1ml water-saturated 1-butanol. (Either proceed or stop here and store gel overnight.)
- After gel has set, pour of butanol and rinse with deionized water.
- Pour the Stacking Gel (~2 ml) and insert the comb immediately.
- When the Stacking Gel has set, place in gel rig and immerse in buffer.
- Prior to running the gel, flush the wells thoroughly with running buffer.

3. Running the gel:

- After flash spinning the samples, load samples into the wells.
- Run with constant voltage (120V). Typical running time is about 1.5hr.

4. Preparation of membrane:

- Cut a piece of PVDF membrane (Millipore Immobilon-P#IPVH 000 10).
- Wet in methanol on a rocker at RT for 5 minutes. Remove methanol and add 1X transfer buffer until ready to use.

5. Membrane transfer:

- Assemble "sandwich" using Bio-Rad's Transblot or equivalent.
- Wash gel with 1X transfer buffer at RT for 5 minutes.
- Wet the sponges, filter papers (slightly bigger than gel) in 1X transfer buffer.
- Transfer for 1 hour at 15 volts (or 250mA) at 4°C on a stir plate. Bigger proteins might take longer to transfer. For the Mini-Transblot, run at 100V for 1 hour with the cold pack and pre-chilled buffer.
- Immerse the membrane in Amido-Black stain for 5 minutes.
- Destain 4 times for 5 minutes each with destaining buffer.
- Wash membrane with TBST and then immerse membrane in blocking buffer (5% BSA or 3-5% nonfat milk/TBST) to block membrane for 2 hours at room temperature or 4°C overnight.

6. Antibody detection:

- Incubate membrane with primary antibody at recommended dilution in blocking buffer overnight at 4°C or at room temperature for 1 hour.
- Wash membrane with TBST at room temperature 4 times, 5 minutes per each.
- Incubate membrane with secondary antibody at recommended dilution in blocking buffer for 1 hour at room temperature.
- Wash membrane with TBST at room temperature 4 times, 5 minutes per each.
- Detect using ECL system of choice.

7. Stripping blot for re-staining:

- Rinse blot with TBST.
- Place blot into Kapak bag cut to slightly larger than blot.
- Add 5-10 ml of Stripping Buffer.
- Remove as much air as possible and seal bag.
- Immerse blot bag into 80°C water bath and incubate for 20 minutes.
- Rinse blot with TBST.
- Block blot membrane again with 5% BSA or 3-5% nonfat milk/TBST at room temperature for 2 hours or at 4 °C overnight.