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[1]iGEM_NTHU_Taiwan

iGEM NTHU

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We use this protocol and it's working

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Abstract

We use Western blot to quantify and verify the protein’s expression level.

Materials

- Vortex mixer, microcentrifuge, rocking platform/shaker
- Dry heating block at **95 °C** (metal dry bath)
- Deionized water (MQ)
- Forceps, roller/glass rod, scissors/utility knife, straightedge/plate clip
- Shallow trays/dishes (for membrane incubation and washes)
- Insulated box/cooler (polystyrene), ice and ice packs
- PPE: lab coat, gloves, safety goggles (use fume hood for toxic/volatile reagents)
- Glass plates (large/small), spacers, clamps, comb(s), casting cassette
- Acrylamide solution
- Tris buffers (separate pH for resolving and stacking gels)
- SDS (sodium dodecyl sulfate)
- TEMED
- APS (ammonium persulfate)
- Gel tank and power supply
- **Running buffer** (e.g., Tris–Glycine–SDS)
- **Protein loading dye** (e.g., Laemmli sample buffer; final 1× in samples)
- Protein samples
- Protein molecular-weight marker/ladder
- Transfer tank and cassette (cathode/anode sides)
- Transfer buffer
- Sponges ×2
- Filter papers ×6
- Membrane ×1 (PVDF or nitrocellulose)
- Methanol (for PVDF activation)
- TBST (TBS with Tween-20)
- Nonfat dry milk (to prepare **5% w/v** blocking buffer)
- Primary antibody (diluted per datasheet in blocking/dilution buffer)
- Secondary antibody (typically HRP-conjugated)
- ECL chemiluminescent substrate
- Chemiluminescence imager/CCD system
- 96-well clear flat-bottom microplate
- Protein assay dye (e.g., Bradford-type or compatible)
- Protein standard (≥5 concentration points)
- Microplate reader (commonly **595 nm**)
- (Optional) Multichannel pipette

SDS-PAGE Gel Casting

1 Place the cassette **upside down**; insert spacers and mount the **small front** and **large back** glass plates. Fix tightly with clamps.

2 Add a little MQ water to check for leaks. If leak-free, discard the water and **blot completely dry** with filter paper.

3 In a conical tube, add **in order**: acrylamide → Tris (resolving pH) → SDS. Vortex to mix. **Do not add TEMED/APS yet.**

4 **Overlay ~800 µL MQ water** on the gel surface to exclude air.

5 Polymerize **≥30 min (recommended 60 min)** until a clear interface line appears. Decant the overlay and **blot the surface dry**.

6 In a Just before casting, add **TEMED and APS**, mix briefly, and pour on top of the resolving gel.second tube, add **in order**: acrylamide → Tris (stacking pH) → SDS; vortex.

7 Just before casting, add **TEMED and APS**, mix briefly, and pour on top of the resolving gel.

8 Immediately insert the **comb**, aligning the teeth with the top edge of the small plate; remove any trapped bubbles.

9 Allow to polymerize **20–30 min**. Remove the comb; the gel is ready for loading.

96-Well Plate Protein Quantification

10 Prepare a **5-point standard curve** from high to low (include 0 point), within the kit's linear range. If sample concentrations are unknown, **pre-dilute** to ensure readings fall within the linear range.

11 Dispense the designated volume of standard/sample into assigned wells.（Keep the pipette tip **below the liquid surface** and dispense along the wall to minimize **bubbles** and splashing.）

12 Add **Dye** to each well.

13 Read absorbance at the specified wavelength on a plate reader.

14 Build the **standard curve** from blank-subtracted absorbance of the standards.（Accept the curve only if **$R^2 \geq 0.95$**.）

SDS-PAGE Electrophoresis

15 Add loading dye to the **final desired concentration** , scaling with the planned load volume). Heat at **95 °C for 7 min**, then briefly spin down.

16 Mount the gel cassette on the apparatus. Level and secure; align the top edge with the reference mark.

17 Slowly add running buffer to the **inner chamber** first, ensuring **all well bubbles are expelled**. Add the remaining buffer to the **outer chamber**. Keep the **buffer level above the top edge of the small plate** throughout the run.

18 Load samples (and marker) **with the tip below the liquid surface**, dispensing **along the well wall** to avoid rupturing wells and forming bubbles.

19 Run at **constant current, 35 mA for ~30–40 min** (adjust for gel percentage/apparatus). Continuously monitor buffer level; do not let it drop below the small plate edge.

20 Stop when the tracking dye **approaches the gel bottom**. Decant buffer, gently pry open the cassette, and remove the gel. **Trim off the stacking gel** and cut a **corner notch near the first well** as an orientation mark.

Transfer

21 Immerse **sponges ×2, filter papers ×6, membrane ×1** fully in **transfer buffer** to pre-soak/equilibrate. If using **PVDF**, briefly wet in methanol first, then equilibrate in transfer buffer.

22 Place the transfer unit in an insulated box with ample ice/ice packs to maintain low temperature throughout the run.

23 Open the cassette and layer in order: **Sponge → Filter paper → Gel → Membrane → Filter paper → Sponge**. After placing the **gel** and **membrane**, gently roll to **expel all bubbles** and ensure full contact.

24 Close the cassette firmly (**press down and push in** to lock). Insert into the tank, confirming **black to black (cathode)** and **red to red (anode)** .

25 Add sufficient **transfer buffer** to cover the stack and electrodes; add ice packs as needed.
Run at **400 mA for 90 min** (adjust for gel thickness/protein size). **Do not let the membrane dry** at any point.

26 Disassemble the stack; lift the **membrane (keep it wet)** with forceps. Trim excess edges using a straightedge, and cut a **corner notch near lane 1** for orientation. Proceed immediately to blocking.

Membrane Blocking and TBST Washing

27 In a 50 mL tube, add **1 g** milk powder and bring to **20 mL** with TBST; mix until fully dissolved (**5% w/v**).

28 Pour blocking buffer into a shallow tray and **fully submerge the membrane** (mini-gels typically require ~**5 mL**, adjust to area).

29 Rock gently at room temperature for **1 h**, ensuring the membrane remains **fully wet** with no dry spots.

30 Wash **3×** with fresh TBST, **1 min each**, under gentle rocking. **Replace TBST for each wash.**

Adding antibodies

31 Incubate the membrane with the primary antibody (diluted per datasheet in blocking buffer) **overnight with gentle rocking** at 4 °C (or room temperature as recommended).

32 The next day, wash with **TBST 3×, 10 min each** under gentle rocking

33 Incubate with the appropriate secondary antibody (diluted per datasheet in blocking buffer) for **1 h with gentle rocking** at room temperature.

34 Wash with **TBST 3×, 10 min each** under gentle rocking.

35 Add **ECL substrate** to cover the membrane, incubate briefly per kit instructions, then **capture chemiluminescence** using an imager. *(Avoid drying the membrane; protect from strong light as needed.)*