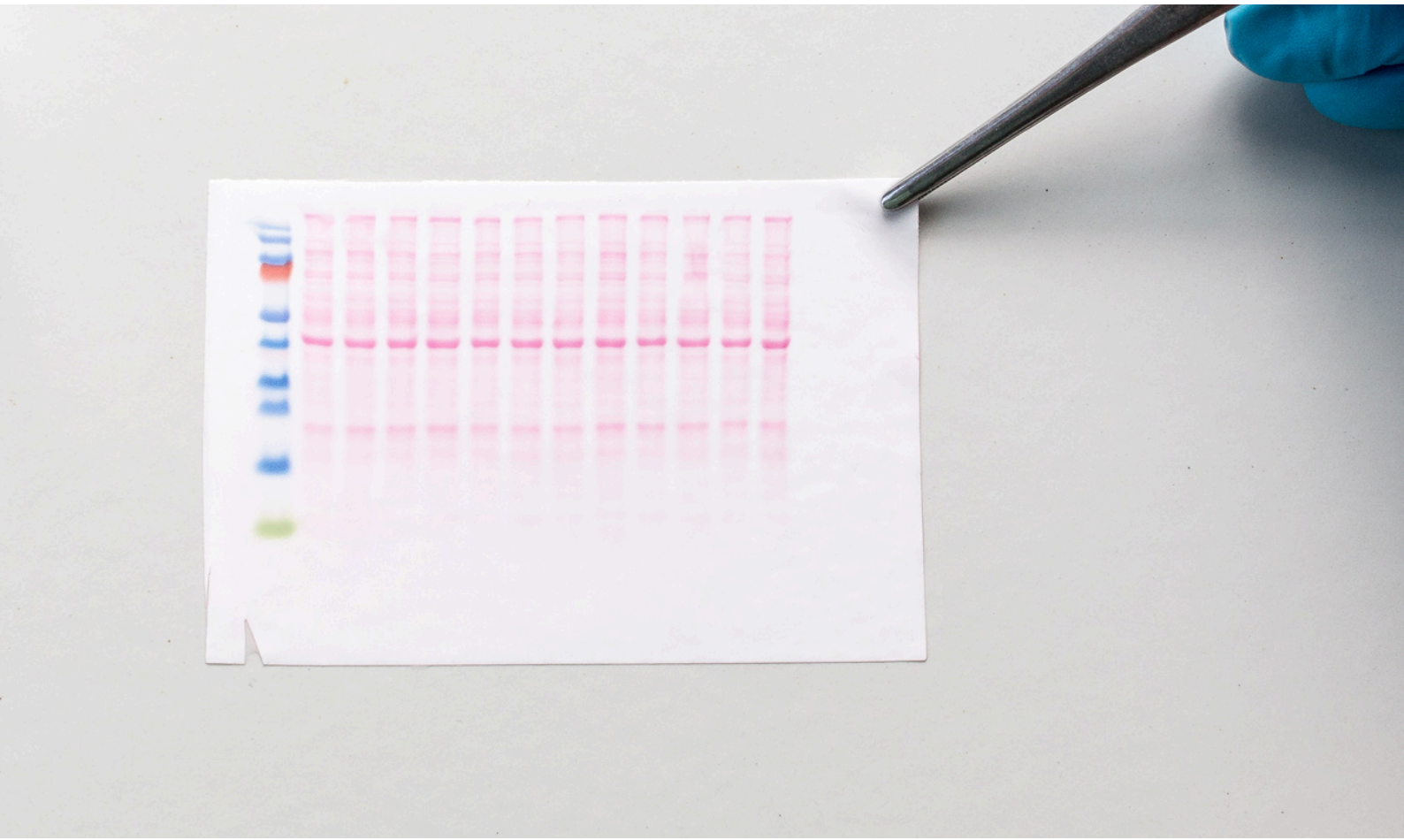
Western Blot Protocol



Cell Lysing Protocol

Sample Preparation, Electrophoresis, and Protein Transferring

Antibody Detection

Protocol Overview

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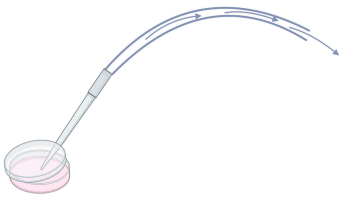
Cell Lysing Protocol

Material

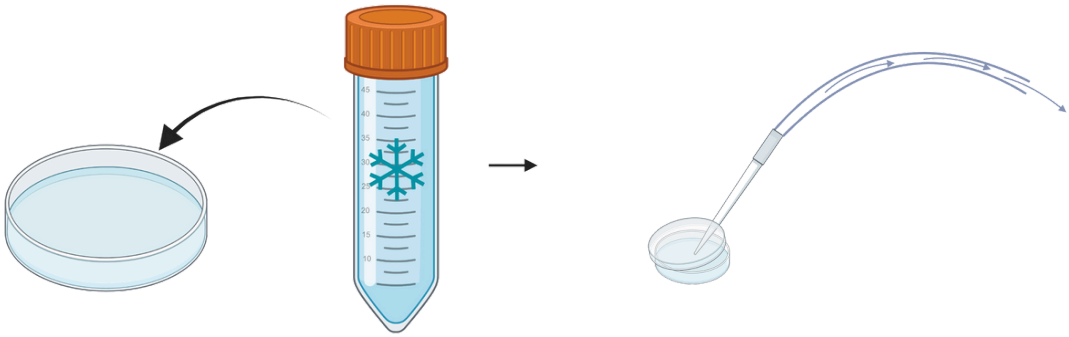
- Cell sample
- PBS (ice-cold)
- NP4O Cell Lysis Buffer (RIPA Lysis Buffer: 25mM Tris-HCl pH7.5, 150mM NaCl, 1% NP-40, 1mM EDTA pH8.0. Add fresh: 1 mM PMSF, 1 mM Na3VO4, and 1 X Protease Inhibitor Cocktail-P2714, Sigma)

Steps

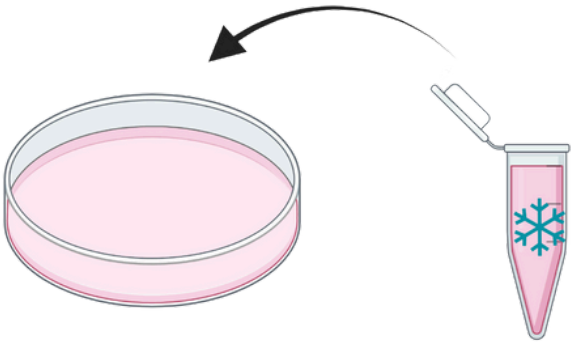
1 Remove the culture media by aspiration.



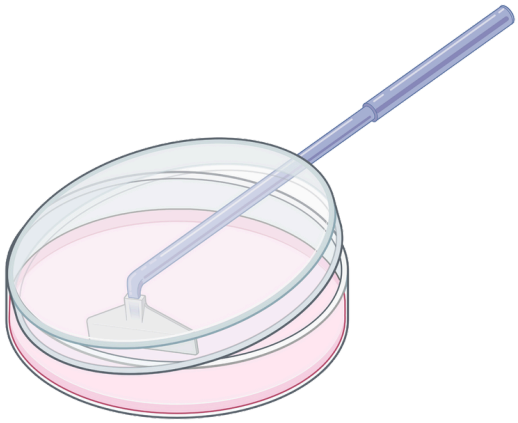
2 Wash cells in the dish with ice-cold PBS and aspirate off PBS (1X).



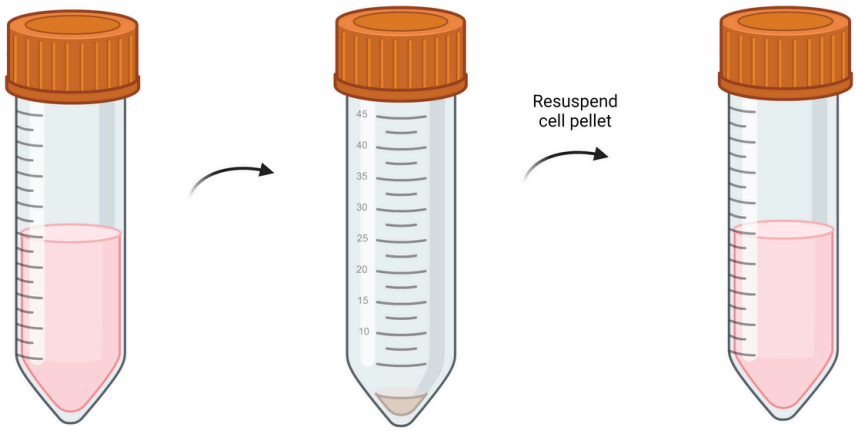
Add ice-cold NP40 Cell Lysis Buffer. We recommend adding 0.8 to 1 ml NP40 Cell Lysis buffer for 10-cm cell culture dish, when cells are over 70% confluent.



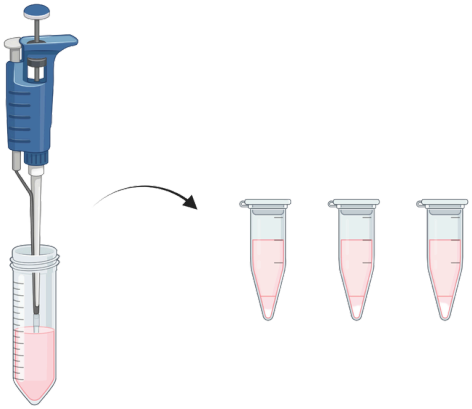
3 For adherent cells, scrap cells using cell scraper (Fisher brand Disposable Cell Lifter, Fisher Scientific, 08-773-1) under ice.



For suspension cells, pellet the cells, then resuspend in lysis buffer under ice.

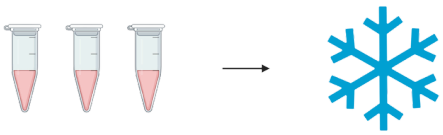


4 Transfer the cell lysis solution into eppendorf tubes after pipetting the cell pellets 15 times.



5 Determine the protein concentration by any commercially available reagent kit. We recommend using BCA Protein Assay kit (Thermo Scientific, 23227).

6 At this step, the sample can be divided into aliquots (for example, 100ug per tube) and stored at –80 °C for long-term.



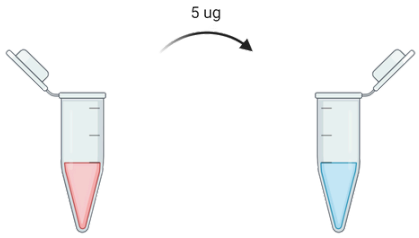
Sample Preparation, Electrophoresis, and Protein Transferring

Material

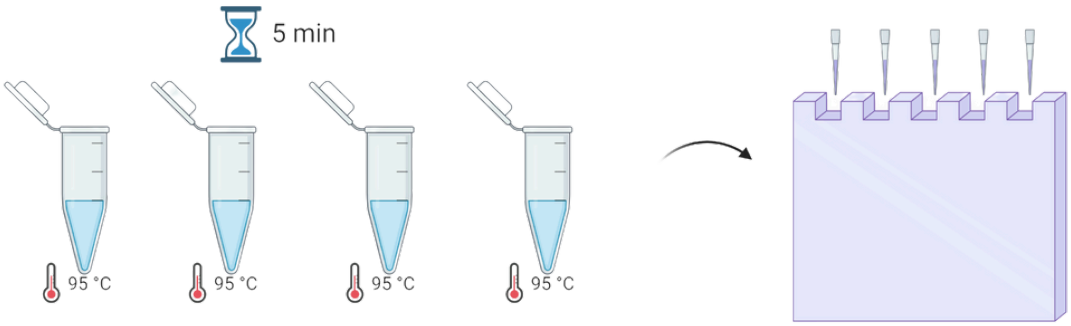
- Cell Lysate
- SDS Sample Buffer
- Pre-stained Protein Standard
- Molecular Weight Markers (when anti-tag antibodies are used)
- Pre-cast SDS polyacrylamide gel (NuPage 4 - 12 % Bis-Tris gel)
- Protein Transfer Buffer (Tris base 48.5 g, Glycine 240.2 g, Methanol 3.2 L, 10N NaOH 1.5 ml, add water to 16 L)
- Nitrocellulose Membrane
- Tri-Glycine Transfer Buffer
- PBS

Steps

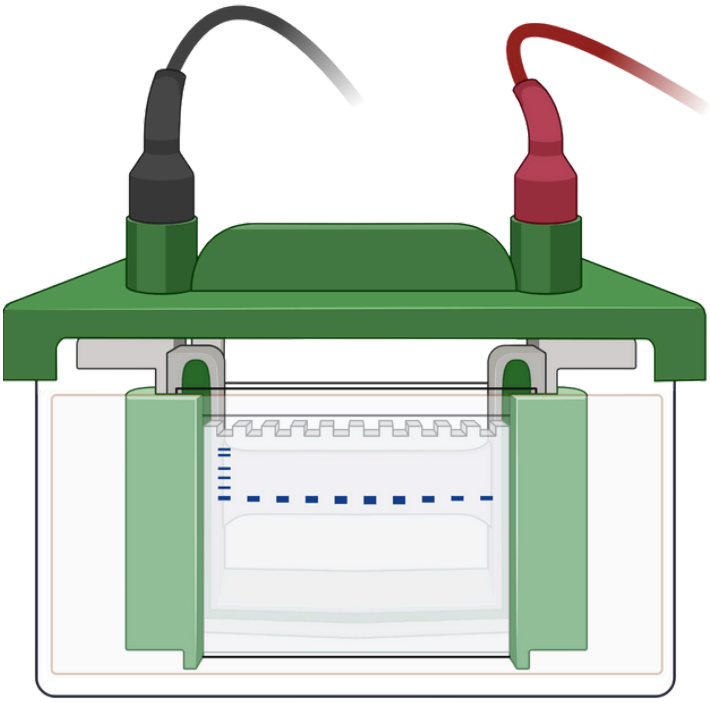
1. Add 5 µg of the above prepared cell lysate into SDS Sample Buffer in a volume of total 10 µL (for a mini gel, up to 15 ug of protein can be loaded per lane). Use the SDS Sample Buffer, which is included in the over-expression cell lysate package.



2. Boil the SDS samples at 95 °C for 5 min before loading. Prepare a pre-stained protein standard (See Blue Plus 2 Prestained Standard, Invitrogen, LC5925) as well. We suggest adding an OriGene MYC/DDK Tagged Western Blot Molecular Weight Markers (MWM1001) if anti-tag antibodies are used for Western blot experiments. 1 ug of MWM1001 is good for one loading.

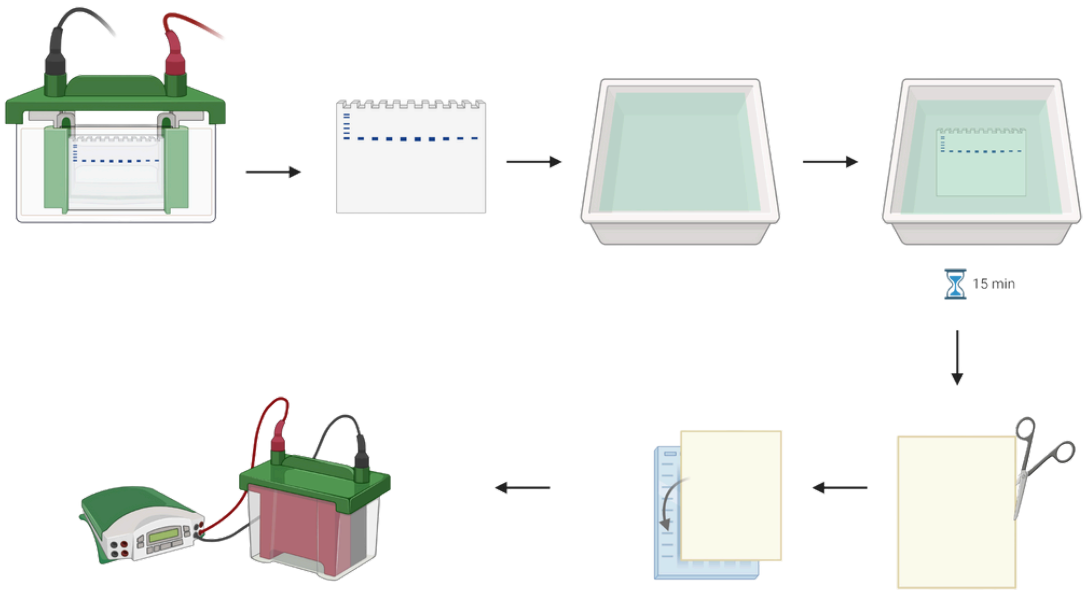


3 Run the samples on a pre-cast SDS polyacrylamide gel at 110 V (constant voltage) for 40 to 60 minutes until the dye reaches the bottom the gel. We recommend using NuPage 4-12% Bis-Tris gel (Invitrogen, NP0323BOX) for proteins with molecular weights smaller than 200 kD.

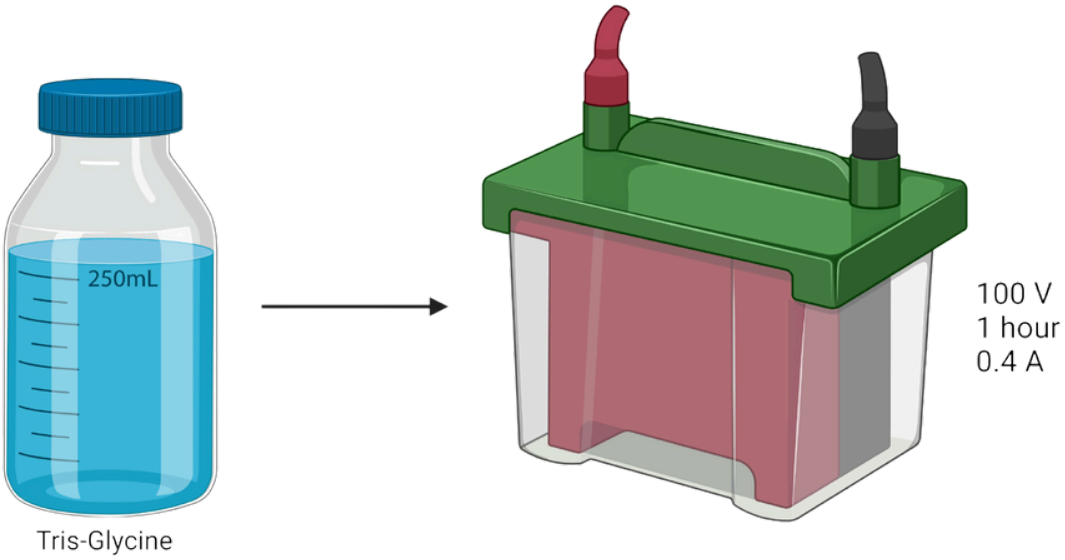


4

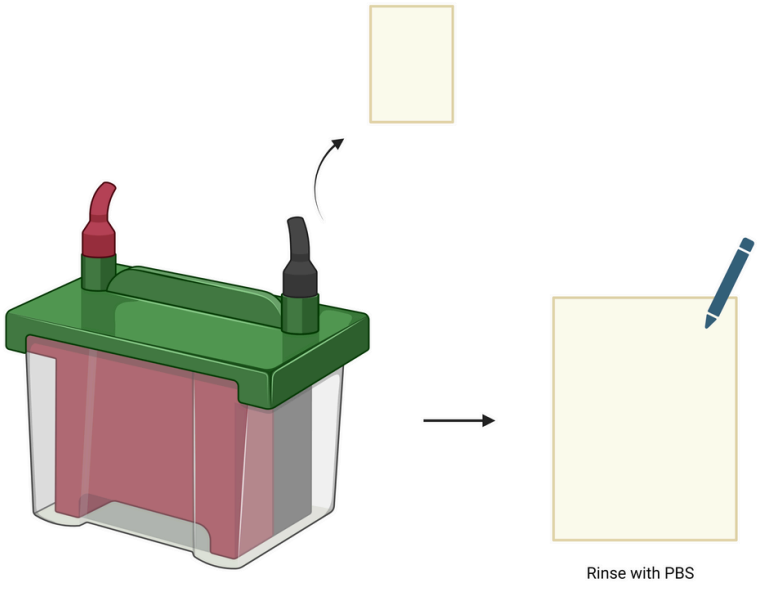
Remove the gel and soak in 1L of protein transfer buffer (made by mixing Tris base 48.5 g, Glycine 240.2 g, Methanol 3.2 L, 10N NaOH 1.5 ml, add water to 16 L) for 15 minutes. Cut the nitrocellulose membrane (ISC BioExpress, F-3139-3) to the similar size of the transfer area of the gel. Assemble the electroblotting cassette and place the electrodes in the blotting unit, according to the manufacturer's instructions.



5 Transfer in Tris-Glycine transfer buffer at 100 V for 1 hour at constant current (not to exceed 0.4 A).



6 Following transfer, remove the membrane from the blotting cassette and mark the orientation of the gel with a pencil. Rinse briefly with PBS.



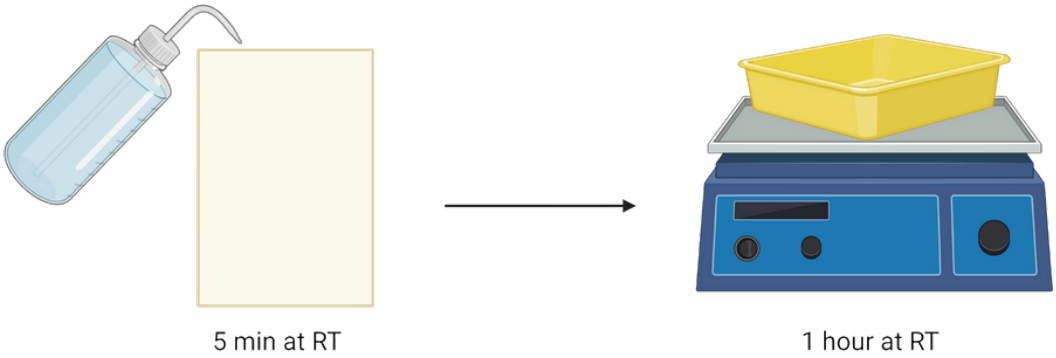
Antibody Detection

Material

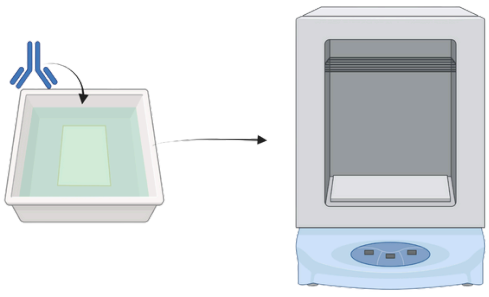
- TBST (10mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20)
- 5 % non-fat milk
- Primary Antibody
- HRP-conjugated secondary antibody (TA130003)
- Luminol Reagent (Detection Solution)
- Transparent plastic film
- Autoradiography film
- X-Ray film cassette

Steps

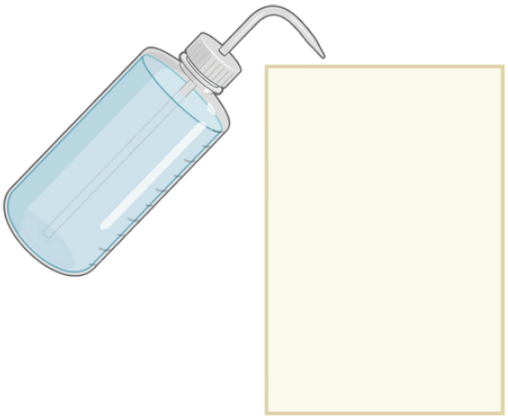
1 Wash the membrane with TBST (10mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20) once for 5 min at room temperature. Block non-specific binding on the membrane with freshly prepared 5% non-fat milk for 1 hour on a shaking platform at room temperature.



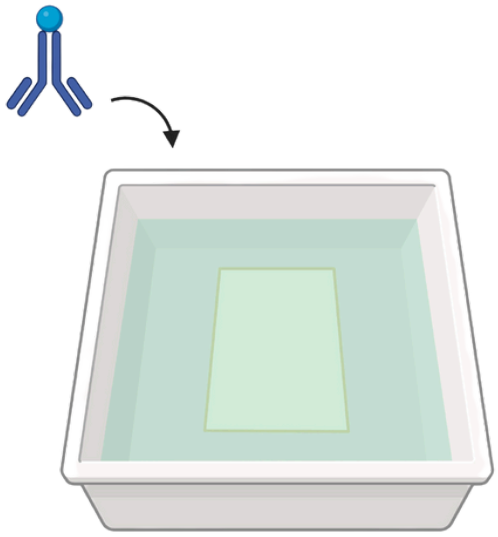
2 Incubate the membrane with a specific primary antibody diluted in TBST and 5% non-fat milk at the manufacturer's recommended dilution with gentle agitation at 4o °C overnight.



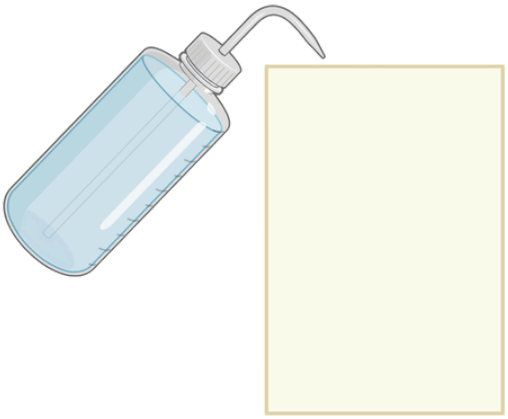
3 Wash three times for 5 min each with TBST.



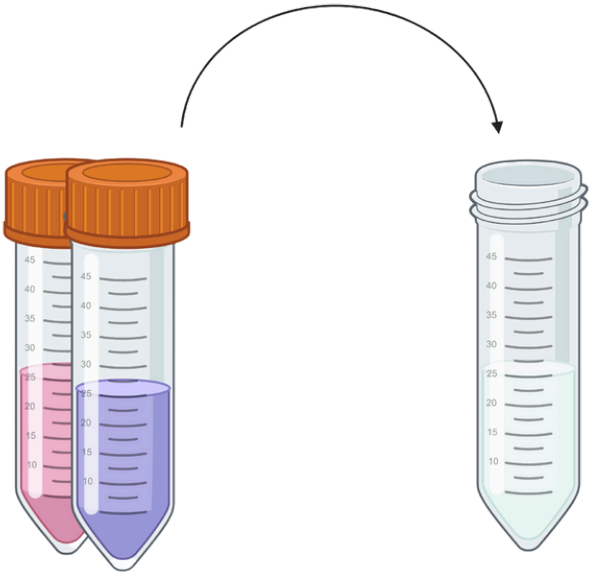
4 Incubate with an OriGene HRP-conjugated secondary antibody of your liking at 1:20,000 in TBST-5% non-fat milk for 1 hour at room temperature.



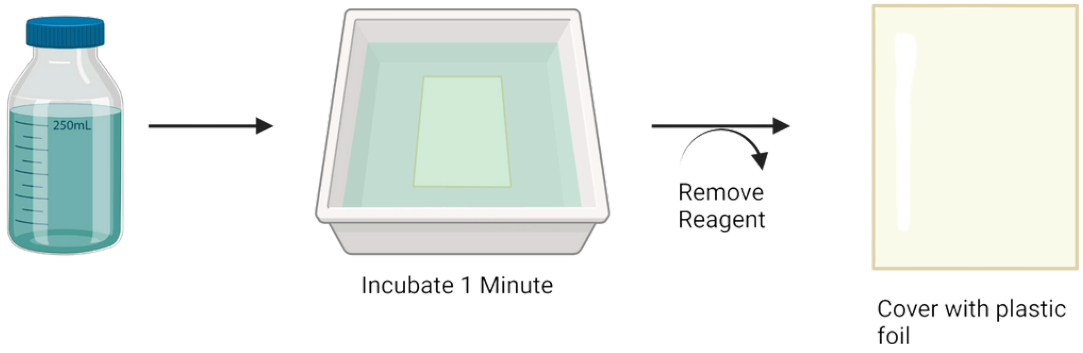
5 Wash three times again for 5 minutes each with TBST.



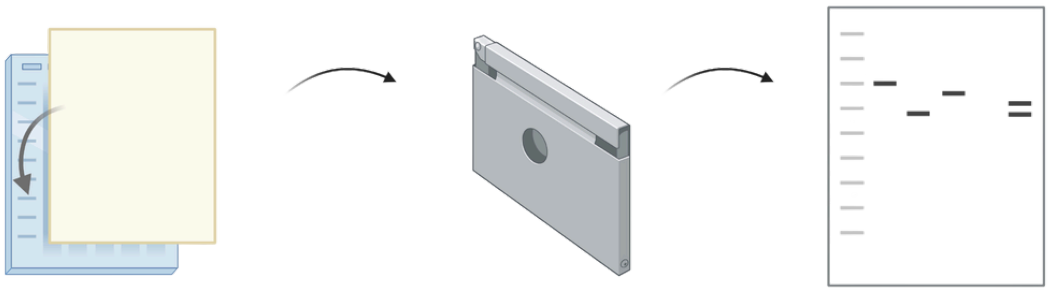
6 For detection, use Western Blot Luminol Reagent and prepare according to instructions.



7 Lay the membrane on a plastic surface with the protein side up. Add the mixed detection solution to the membrane. Incubate for 1 minute. Remove the excess solution and cover the membrane with transparent plastic.



8 Place the wrapped blot with protein side up in an X-ray film cassette. Place a sheet of X-ray autoradiography film on the top of the membrane. Close the cassette for 15 sec to 1 min. Remove the film for development. Add additional films if needed for longer or shorter exposures.



Western Blot Protocol Overview

Cell Lysing:

- Remove culture media and wash cells with ice-cold PBS.
- Add ice-cold NP40 Cell Lysis Buffer to the cells.
- For adherent cells, scrape cells; for suspension cells, pellet and resuspend in lysis buffer.
- Transfer the cell lysis solution to eppendorf tubes.
- Determine protein concentration using a BCA Protein Assay kit.
- Aliquot and store samples at −80°C for long-term storage.

Sample Preparation, Electrophoresis, and Protein Transferring:

- Mix cell lysate with SDS Sample Buffer and boil at 95°C for 5 minutes.
- Load samples onto a pre-cast SDS polyacrylamide gel and run at 110 V for 40-60 minutes.
- Soak the gel in protein transfer buffer and prepare the nitrocellulose membrane.
- Transfer proteins to the membrane at 100 V for 1 hour. Rinse the membrane with PBS.

Antibody Detection:

- Wash the membrane with TBST and block with 5% non-fat milk.
- Incubate with primary antibody overnight at 4°C.
- Wash the membrane and incubate with HRP-conjugated secondary antibody.
- Wash the membrane again.
- Detect using Western Blot Luminol Reagent and expose to X-ray film.

Notes

Think Western Blot, Think OriGene!

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