



Feb 02, 2025

Western Blot protocol

DOI

<https://dx.doi.org/10.17504/protocols.io.bp2l622nkgqe/v1>

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Protocol Citation: Carolina Lopez 2025. Western Blot protocol. **protocols.io**

<https://dx.doi.org/10.17504/protocols.io.bp2l622nkgqe/v1>

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Protocol status: Working

We use this protocol and it's working

Created: May 29, 2024

Last Modified: February 02, 2025

Protocol Integer ID: 100848

Keywords: western blot protocol general western blot protocol, protocol

Abstract

General Western Blot Protocol

Protein Harvest

1 Prepare these items before starting this protocol:

I. Protein extraction:

- Cells or tissues
- NP-40 lysis buffer (200 μ L per sample): **See STEP I "NOTE" to select the appropriate protein extraction buffer**
 - NP-40 Surfact-Amps™ Detergent Solution (Thermo Fisher-85124)
 - 0.5M EDTA
 - 5M NaCl
 - 1MTris-HCl
 - Glycerol 10%
 - 20X phosphatase (4906837001 PhosSTOP™)
 - Halt™ Protease Inhibitor Cocktail (100X) (Thermo: 78429)
- Cell scrapers
- 1.5 mL Eppendorf tubes
- Microcentrifuge
- Ice

II. Protein concentration quantification:

Pierce™ BCA Protein Assay Kit (Thermo: 23225): **See STEP II "NOTE" to select the appropriate protein quantification method.**

- Immulon 4 HBX 96 well plate
- Paraffin
- Protein sample
- Microplate reader (Kau lab)

1.1 I. Protein extraction protocol:

1. Prepare the desired volume of NP-40 lysis buffer (200ul per sample in 6 well plates): Mix NP-40 lysis buffer, 2MM EDTA,150 mM NaCl, 50nM Tris-HCl, Glycerol 10%, 1x of 20X phosphatase, 1x of 100x Protease, NP40 for the remaining volume.

IMPORTANT: protease and phosphatase need to be added at the time of use, not for storage

**Note**

When it comes to protein extraction, the choice of buffer for cell lysis is crucial as it can significantly affect the yield and quality of the extracted protein. In the lab, one commonly used buffer for cell lysis is NP-40, which is a non-ionic detergent that can efficiently solubilize membrane-bound proteins. NP-40 is a good choice for proteins that are localized to cell membranes or other lipid-rich structures. However, for proteins that are in the cytosol, other buffers may be more appropriate. For example, RIPA buffer contains ionic detergents that can disrupt protein-protein interactions and extract a wider range of proteins. Meanwhile, Triton X-100 buffer can be used for gentle lysis of cells and preservation of protein-protein interactions. Ultimately, the choice of buffer for cell lysis depends on the specific protein of interest and the desired outcome of the experiment.

2. Harvest the cells or tissues: Rinse the cells or tissues with phosphate-buffered saline (PBS) to remove debris or contaminants. Add 200 μ L of NP-40 lysis buffer to the cells and using a cell scraper, gently scrape the cells to homogenize the solution.

3. Incubate sample on ice for 20min.

4. Centrifuge samples to remove debris: 13,000rpm for 20min at 4 degrees C.

5. Collect supernatant in a new Eppendorf tube (keep on ice)

IMPORTANT: samples can be saved on -20 until next step

1.2 **II. Protein concentration quantification protocol**

6. Measure protein concentration by BCA and record results using a Microplate reader (in the Kau's lab)

7. Store proteins at -20 until further use

Note

Protein quantification is an important step in many biochemical experiments, but the method used to measure protein concentration should be carefully chosen depending on the experiment being conducted. In the lab, BCA (bicinchoninic acid) assay is a commonly used method to measure protein concentration. BCA is a good choice as it is relatively sensitive, has low protein-to-protein variability, and is compatible with a wide range of protein samples. However, other methods may be more suitable for specific experiments. For example, the Bradford assay is a rapid and simple method that can be used to quantify proteins in complex mixtures but may be less accurate than the BCA assay for pure protein samples. The Lowry assay is another option that is particularly sensitive for proteins with high aromatic amino acid content. Additionally, for specific types of proteins, such as antibodies, measuring absorbance at 280 nm may be more appropriate. In summary, the choice of protein quantification method depends on the specific experiment and the nature of the protein being measured.

Western Blot

2h 15m 15s

2 **Prepare these items before starting this protocol:**



III. Western Blot:

- Protein sample
- 1x Running buffer (XT MES 20X- Biorad Cat#1610789)
- TBST (washing buffer)
- SDS-PAGE gel (10-15%, percentage depends on the size of your protein of interest)
- PVDF membrane
- Methanol
- Transfer buffer (Tris-glycine-methanol)
- Blocking buffer (5% non-fat dry milk in TBS-T)
- Primary antibody
- Secondary antibody conjugated with HRP (horseradish peroxidase)
- HRP reagent (Millipore: Cat# WBKLS0100)
- Electrophoresis tank
- Power supply
- Fast transfer machine
- Cut Blotting paper (6 per Western blot, 3.5 in W x 2.75 in H)
- Transfer membrane holder
- Shaker
- Rocker
- Western blot apparatus
- Chemiluminescence imaging system
- Ice
- PageRuler prestained protein ladder (P/N57318)
- Western blot boxes (small ones to stain and wash while rocking)
- 20X Reducing agent (Cat#1610792)
- 4X Loading buffer (Cat#161-0791)
- Stripping buffer (Thermo#46430)

2.1 III. Western Blot Protocol:

2h 15m 15s

1. Prepare the protein sample: Add 4x loading buffer and 20x of reducing agent to the protein sample. Boil for  00:05:00 at  95 °C . Spin down samples at 3,000 rpm for 1min. Cool on ice for at least 5min
2. Prepare the gel: Assemble the gel in the electrophoresis tank and add the **running buffer** (25mL XT MES running buffer (20X) and 475mL deionized H₂O). Pipette the wells to clean them and remove potential bubbles.
3. Load the protein sample (20-25 µL) and run the gel at constant voltage (100-150 V) until the dye front reaches the bottom of the gel (if using 100V, usually is 1 hr of waiting).

>> Prepare the transfer membrane: Cut the membrane to the size of the gel and in a plastic container, activate membrane in methanol for at least 5 minutes while on a shaker. Dump the methanol back into the bottle to be reused. Rinse the membrane in **transfer**

buffer (50mL Tris/Glycine buffer (10x), 100mL Methanol, and 350mL deionized H₂O. → 20% Methanol) while on a shaker and leave on transfer buffer until use.

4. After the gel electrophoresis is done, the gel should also be placed in transfer buffer for at least 6-5 min (to remove remaining running buffer)
5. Set up the fast transfer machine: Assemble the blotting sandwich (in the semi dry chamber) in the following order: Blot, Membrane, Gel, Blot. Roll to remove bubbles. Lock the chamber by placing the cover and twisting the lock to induce pressure. Set the rapid transfer machine: 1.5 mm Gel (2.5 A, 25 V, 13min).
6. Carefully remove the membrane from the transfer apparatus and immerse it in a container filled with ethanol for  00:00:15 . This step is intended to fix the transferred proteins onto the membrane and to remove any residual transfer buffer.
7. Cover the membrane with a sufficient volume of swift stain solution from the stock bottle to ensure complete coverage and immerse the membrane for 6-10 minutes.
8. Inspect the membrane regularly during this step to determine if the protein bands have been successfully stained. If the bands are not visible, the staining procedure should be continued until optimal staining is achieved.
9. Remove the excess swift stain solution from the membrane and immerse it in a D-stain solution for 1 minute, or if required to adequately remove any background staining. This step is designed to remove any non-specific staining and improve the contrast of the protein bands.

IMPORTANT: Steps 7-10 may be omitted if a pre-stained protein marker is used. The pre-stained marker allows for visual assessment of successful transfer by observing the protein bands directly on the membrane, thereby eliminating the need for additional staining and destaining steps.

10. Block the membrane: Remove the membrane from the transfer membrane holder and rinse it in **TBS-T** (1X TBS or PBS, 0.1% Tween20 (TBS/T) → 500 µL Tween20 in 500 mL PBS) (3 times). Block the membrane with blocking buffer for  01:00:00 at room temperature on a shaker.

IMPORTANT: Step 11 can also be extended overnight at a low temperature, such as in a cold room, to increase the effectiveness of the blocking process. If this is the case, it is important to ensure that the samples are kept in a shaker to prevent settling or precipitation.

11. Incubate with primary antibody: Dilute the primary antibody in blocking buffer (PBS/BSA (1g in 10 mL of TBST)) and incubate the membrane for 1-2 hours at room temperature on a shaker.

IMPORTANT: Step 12 can also be extended overnight at a low temperature, such as in a cold room, to increase the effectiveness of the antibody binding process. . If this is the case, it is important to ensure that the samples are kept in a shaker.

12. Wash the membrane: Rinse the membrane in TBS-T three times for  00:05:00

each time on a rocker.

13. Incubate with secondary antibody (1:10,000): Dilute the secondary antibody conjugated with HRP in 5% milk and incubate the membrane for  01:00:00 at room temperature on a shaker.

14. Wash the membrane: Rinse the membrane in TBS-T three times for  00:05:00 each time on a rocker.

15. For protein visualization,  1000 μL of peroxide and luminol HRP substrate (for 3-5min) should be added to the membrane. This can be achieved by mixing the reagents according to manufacturer's instructions (Kit in Mushu's fridge).

16. Place the membrane in the ChemiDoc imaging: Open the imaging software on the ChemiDoc system. Place the membrane in the imaging tray or holder, ensuring that the protein bands of interest are within the imaging area.

17. Adjust the imaging settings to optimize the signal-to-noise ratio and the dynamic range of the image. This may involve adjusting the exposure time, aperture, and other camera settings. Preview the image to ensure that the protein bands are clearly visible and in focus. Capture the image using the appropriate capture settings, such as the file format, resolution, and image size. Save the image to a designated location on the computer or network, along with any necessary metadata or labeling information.

IMPORTANT: The following steps aim to enable the testing of additional proteins using the same protein membrane, with a specific focus on quantifying housekeeping proteins such as GAPDH or alpha tubulin.

18. In case of needing to use the membrane for a new protein evaluation, strip the membrane: Using stock stripping buffer incubate the membrane in the stripping buffer for the recommended amount of time, which is usually 10-30 minutes at room temperature with gentle agitation. Wash the membrane thoroughly with buffer to remove any residual stripping buffer.

19. Block the membrane again with blocking buffer to reduce non-specific binding of new antibodies.

20. Incubate the membrane with new primary and secondary antibodies according to the experimental design (repeat from step 11).

Protocol references

Lab expert: Nicole Rivera

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Annotated by NSRE (2023-04-04)