Western Blotting Protocol (Immunoblotting Protocol)

Western Blotting refers to the electrophoretic transfer of proteins from sodium dodecyl sulfate polyacrylamide gels to sheets of PVDF or nitrocellullose membrane, followed by immunodetection of proteins using antibodies with fluorescent or chemiluminescent detection. Click the steps of the Western Blotting protocol below to view the relevant details of each step:

Western Blotting Workflow Steps:

1. **Sample Preparation: Cell Lysis and Protein Extraction**
2. **SDS-PAGE Gel Electrophoresis**
3. **Membrane Transfer (Tank Transfer)**
4. **Membrane Transfer (Semi-dry Transfer)**
5. **Immunodetection**
6. **30-minute Immunodetection Protocol using the SNAP i.d.® System**
7. **Troubleshooting Guide**

Recipes for Western Blotting Buffers and Solutions

Membrane Transfer (Tank Transfer) Protocol

Materials

- Blotting paper, extra thick
- Transfer membrane (for example, **Immobilon® PVDF Membranes**)
- **SB10 omniPAGE mini electroblotting system** or other tank transfer system
- **Power supply, 250 volt AC input 110 V US 3-pin plug** (Product No. **PS2501**)
- **Power supply 250V AC input 220 V EU 2-pin plug** (Product No. **PS2502**)

Setup

1. Prepare sufficient transfer buffer to fill the transfer tank, plus an additional 200 mL to equilibrate the gel and membrane, and wet the filter paper.
2. Remove the gel from its cassette; trim away any stacking gel and wells.
3. Immerse the gel in transfer buffer for 10 to 30 minutes.
4. Soak filter papers in transfer buffer for at least 30 seconds.
5. Prepare the membrane:
    a. If using PVDF membrane, let the membrane in methanol for 15 seconds. Membrane should uniformly change from opaque to semi-transparent.
    b. Carefully place the membrane in Milli-Q® water and soak for 2 minutes.
    c. Carefully place the membrane in transfer buffer and let equilibrate for at least 5 minutes.

Transfer Stack Assembly

1. Open the cassette holder.

   Important : To ensure an even transfer, remove air bubbles between layers by carefully rolling a pipette or a stirring rod over the surface of each layer in the stack. Do not apply excessive pressure to prevent damaging the membrane and gel.
2. Place a foam (fiber) pad on one side of the cassette.
3. Place one sheet of filter paper on top of the pad.
4. Place the gel on top of the filter paper.
5. Place the membrane on top of the gel.
6. Place a second sheet of filter paper on top of the stack.
7. Place second foam pad on top of the filter paper.
8. Close the cassette holder.

Method for Protein Transfer (Tank Transfer)

1. Place the cassette holder in the transfer tank so that the gel side of the cassette holder is facing the cathode (–) and the membrane side is facing the anode (+).
2. Add adequate buffer into the tank to cover the cassette holder.

Related Links

- Sample Preparation for Western Blotting: Cell Lysis and Protein Extraction
- 30-minute Immunodetection Protocol Using the SNAP i.d.® 2.0 System
- Western Blotting Home Page
- Western Blotting Literature & Resources
- Antibody Explorer: Search primary and secondary antibodies

Categories

- AGE
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3. Insert the black cathode lead (–) into the cathode jack and the red anode lead (+) into the anode jack on the transfer unit.
4. Connect the anode lead and cathode lead to their corresponding power outputs.
5. If available, set up the cooling unit on the tank transfer unit according to the manufacturer's instructions.
6. Turn on the system for 1 to 2 hours at 6 to 8 V/cm inter-electrode distance. Follow the tank manufacturer's guidelines, for optimization details.
7. After the transfer is complete, remove the cassette holder from the tank.
8. Using forceps, carefully disassemble the transfer stack.

Tips for Successful Western Blotting Transfer

Tip: In both types of transfer systems (tank and semi-dry), extra caution should be taken to prevent introduction of air bubbles anywhere between the filter paper, gel or membrane.
Tip: Transfer proteins at constant current. If transferring at constant voltage, monitor current to make sure it doesn't exceed 0.4 amp. Start from 100 V and reduce voltage if current is too high.
Tip: Both sides of Immobilon® transfer membranes work equally well. The appearance of either side (shiny or dull) has no effect on the transfer and detection efficiency of the membrane.
Tip: For samples containing small peptides, equilibration of the gel in transfer buffer should be limited to less than 10 minutes.
Tip: Gels can be transferred individually or multiple gels can be transferred in a single stack.

Semi-dry Membrane Transfer Protocol

Materials

- Blotting paper, extra thick
- Transfer membrane (for example, **Immobilon® PVDF Membranes**)
- 14.2000 volt power supply
- **Semi-dry blotter** (Product No. **B2529**)

Setup

1. Prepare 200 mL of each anode buffer and 400 mL of cathode buffer.
2. Remove the gel from its cassette; trim away any stacking gel.
3. Immerse the gel in 200 mL of cathode buffer for 15 minutes.
4. Soak two pieces of filter paper in anode buffer I for at least 30 seconds.
5. Soak one piece of filter paper in anode buffer II for at least 30 seconds.
6. Soak three pieces of filter paper in cathode buffer for at least 30 seconds.
7. Prepare the membrane:
    a. Wet the membrane in methanol for 15 seconds. The membrane should uniformly change from opaque to semitransparent.
    b. Carefully place the membrane in Milli-Q® water and soak for 2 minutes.
    c. Carefully place the membrane in anode buffer II and let equilibrate for at least 5 minutes.

For single transfers:

1. Place the anode electrode plate on a level bench top.
2. Place two pieces of filter paper soaked in anode buffer I in the center of the plate.
3. Place the filter paper soaked in anode buffer II on top of the first two sheets.
4. Place the membrane on top of the filter papers.
5. Place the gel on top of the membrane.
6. Place the three pieces of filter paper soaked in cathode buffer on top of the membrane.
7. Place the cathode electrode plate on top of the stack.

For multiple transfers:

1. Place the anode electrode plate on a level bench top.
2. Place two pieces of filter paper soaked in anode buffer I in the center of the plate.
3. Place the filter paper soaked in anode buffer II on top of the first two sheets.
4. Place the membrane on top of the filter papers.
5. Place the gel on top of the membrane.

   For the last gel, go to step 10.
6. Place a piece of filter paper soaked in cathode buffer on top of the gel.
7. Place a piece of dialysis membrane on top of the filter paper.
8. Place a piece of filter paper soaked in anode buffer II on top of the dialysis membrane.
9. Repeat steps 4 through 8 until all gels (up to the maximum for the unit) have been incorporated into the stack.
10. Place three pieces of filter paper soaked in cathode buffer on top of the last gel.
11. Place the cathode electrode plate on top of the stack.

IMPORTANT: Do not bump the cathode plate cover during the run since it could disturb the alignment of the transfer stack and cause inaccurate results.

Method for Protein Transfer (Semi-dry)

1. Insert the black cathode lead (–) into the cathode plate jack.
2. Insert the red anode lead (+) into the anode plate jack.
3. Connect the anode lead and cathode lead to their corresponding power supply outputs.
4. Turn on the power supply.
5. Set the current and let it run for the time indicated in the following chart:

Current Density	Time Limit
0.8 mA/cm^2*	1-2 hours
1.2 mA/cm^2	1 hour
2.5 mA/cm^2	30-45 min
4.0 mA/cm^2	10-30 min

*The surface area (cm^2) is calculated from the dimensions of the footprint of the stack on the anode plate. This value is independent of the number of gels in the stack.

Traditional Immunodetection

Equipment Needed

- **Primary & Secondary Antibodies**
- **Orbital shaker*** (Product No. **Z367605**)
- Troughs 2-3 mm depth, slightly larger than the size of your blot

*Shaker and Troughs only required for traditional immunodetection; for rapid, vacuum-driven immunodetection using the SNAP i.d.® system, refer to the **SNAP i.d.® immunodetection protocol**.

Antibody Incubations

1. Place the blot in the blocking solution and incubate with agitation for 1 hour.
2. Place the blot in the primary antibody solution, for example **Monoclonal Anti-Beta-Actin Antibody** and incubate with agitation for 1 hour. The solution should move freely across the surface of the membrane.
3. Place the blot in PBS and wash for 10 minutes. Repeat twice with fresh buffer.
4. Place the blot in the **secondary antibody solution** and incubate with agitation for 1 hour at RT or 37 °C.
5. Place the blot in PBS and wash for 10 minutes. Repeat twice with fresh buffer.
6. Proceed with chromogenic, chemiluminescent, or fluorescent detection. Also view related resources for protein detection:

   Chromogenic and Chemiluminescent Detection of Proteins

   Immunodetection Using BCIP/NBT Substrate

Chemiluminescent Detection

Follow manufacturer's instructions.

1. Prepare the substrate according to manufacturer's instructions.
2. Place the blot in a container and add substrate to completely cover the membrane. Incubate for 1 minute.
3. Drain excess substrate.
4. Place the blot on a clean piece of glass and wrap in plastic wrap.

   Note: A cut-to-size sheet protector or a freezer bag can also be used.
5. Gently smooth out any air bubbles.
6. In a dark room, place the wrapped membrane in a film cassette.
7. Place a sheet of autoradiography film on top and close the cassette.
8. Expose film. Multiple exposures of 15 seconds to 30 minutes should be done to determine the optimum exposure time; 1 to 5 minutes is common.

Fluorescent Detection

Required Equipment

- Proteins blotted onto **Immobilon®-FL transfer membrane** and probed with antibodies.
- Mylar® wrap.
- Fluorescent imaging equipment.

The following is a general protocol for fluorescent immunodetection. For optimal results, refer to manufacturer's protocol provided with the reagents.

Note : If using chemifluorescent reagents, follow reagent manufacturer's directions.

1. Place the blot in diluted fluorescent dye-labeled secondary antibody solution and incubate for 1 hour with gentle agitation.
2. Wash the blot with wash buffer 3–5 times for 5 minutes each.
3. Place the blot onto a piece of clean filter paper to dry.
4. If using a wrap, use Mylar®. Do not use Saran™ wrap because it permits light to shine through and quench fluorescence.
5. Image the blot using an appropriate fluorescence scanner.

Western Blotting Troubleshooting Guide

Problem	Possible Cause	Solution
No signal or weak signal or non-specific bands	Substrate or conjugate weak or no longer active due to age or improper storage	Test conjugate and substrate for activity. For example, add enzyme conjugate to substrate solution. It should change color.
	If chemiluminescent detection is being used, the film development solution may have expired	Use fresh film development solutions.
	Incorrect substrate for application	Make sure that the substrate selected is appropriate for the enzyme conjugate.
	Substrate prepared incorrectly	Follow instructions that accompany the substrate. Make sure that fresh H_2O_2 is added if necessary.
	Incorrect dilution of primary or secondary antibody	Check the literature or data sheet for recommended dilutions for the antibodies being used. Try a range of dilutions.More is not always better, especially with sensitive detection systems such as chemiluminescence. Most of our antibodies are tested with colorimetric substrates that are not as sensitive. For this reason, the antibody may need to be diluted 5-10 times more if chemiluminescent detection is being used.
	Incorrect primary antibody for the application	Make sure the antibody has been shown to work in immunoblotting. Not all antibodies work in all applications. Primary antibody may not be capable of reacting with the protein of interest from the species being studied. Check the literature/data sheet and protein sequence information.
	Protein of interest is not present or is present in low amounts	If the positive control worked, check the amount of protein loaded. If necessary, try enriching the amount of protein of interest in the sample loaded by immunoprecipitation or by purification. Consult the literature for the best source for the protein of interest. See "Poor protein transfer" below.
	Inappropriate secondary antibody for the application	The secondary antibody may not be capable of binding to the primary antibody. Test this by spotting primary antibody on a small piece of membrane. When the spot dries, block, then probe with diluted secondary, wash and develop with substrate. A spot should appear if the secondary bound to the primary.
	Incubation times inadequate	Incubate at least one hour with primary antibody.
	Enzyme inhibitor present	Sodium azide will inhibit peroxidase reactions.
	Over washing	Shorten wash times, omit detergents from washing buffers.
	Poor protein transfer	Check transfer of the proteins to the membrane by staining the membrane with **Ponceau S** (Product No. **P7170**) prior to blocking.Make sure membrane wets uniformly before transfer. Test transfer times. Small proteins (< 10,000) may have transferred through the membrane (try including a second membrane behind the first). Larger proteins may require longer transfer times. Check transfer buffer - high methanol concentrations may prevent transfer of the protein from the gel. 0.005-0.01% SDS in the transfer buffer may increase the transfer of protein from the gel, but it can also interfere with protein binding to the membrane. If the pI of the protein is >9.0 try using CAPS, pH 9 as the transfer buffer.
High background or Non-specific bands	Insufficient blocking	Try different blocking strategies: Longer blocking times, higher % of blocker, a different blocker, inclusion of blocker protein in antibody dilutions or try a fresh batch of the same blocker.
	Primary antibody may not be specific enough	Try a monoclonal or an affinity purified polyclonal antibody.
	High concentration of primary antibody	Check literature or data sheet for recommended dilution of primary. Try a range of dilutions in order to optimize your system.
	High concentration of secondary antibody	Test this by running an extra sample lane and omitting the primary antibody incubation from the procedure. If this "secondary only"control results in nonspecific staining, try further dilutions of the secondary antibody or try another secondary.
	Secondary antibody is cross-reacting with other proteins in the sample	Dilute the secondary antibody in buffer containing 1-5% normal serum from the same species as the sample
	Multiple bands	Make sure protease inhibitors are present from the first step of sample preparation.

	may be proteolytic fragments of the protein of interest	Store and handle sample preparations to reduce the chance of proteolysis. Try fresh samples when possible.
	Antibody incubation times longer than necessary	Shorten the incubation times.
	Overincubation with colorimetric substrate solution	Decrease the staining time. Expose the membrane to substrate until a positive signal is seen. Stop the reaction by washing the membrane before the back ground develops.
	Inadequate washing	Increase the number or stringency of the washes.
	Contaminating enzymes present in sample	Test sample with substrate alone to check for contaminating enzyme activity in protein sample.

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