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Western Blotting Troubleshooter

Bio-Rad has been manufacturing equipment and reagents for western blotting for over 10 years. In that time, our Technical Service department has answered numerous question about western blotting. This publication will save your valuable time by answering commonly asked questions *before* you have a need to ask them.

Q: What is the difference between the Trans-Blot®, Trans-Blot SD semi-dry, and the Mini Trans-Blot® cells?

A: The Trans-Blot tank blotter with plate or wire electrodes and the super cooling coil provides the most versatility for blotting. Two types of transfer can be done in the tank, high intensity transfer and standard field transfer. A high intensity transfer is performed with the electrodes 4 cm apart, and a standard field transfer with the electrodes 8 cm apart. With cooling, a high intensity transfer can be accomplished in as little as 30 minutes. With a standard field, the Trans-Blot cell may be allowed to run overnight. This broad range of transfer times provides optimal quantitative transfer of proteins.

The Mini Trans-Blot cell can accomplish excellent transfer in as little as 1 hour. The electrode distance is fixed at 4 cm in the modular blot electrode assembly. The close electrode distance is capable of 1 hour transfers when the Bio-Ice cooling unit is used, or the system may be run overnight at low voltage.

The Trans-Blot SD semi-dry cell provides the highest field strength because the electrode distance is limited only by the gel and filter paper. It is for fast, high intensity transfers, as cooling is not available. It is most suited for medium range molecular weight proteins (10–100 Kd). Very small proteins may be blown through and very large proteins may not have enough time to transfer completely.

Q: What is the difference between nitrocellulose, supported nitrocellulose, and PVDF membrane?

A: There are three different types of membranes available for blotting proteins. Nitrocellulose is the most frequently used. It comes in two different pore sizes, 0.2 micron and 0.45 micron. Which size to use depends on the molecular weight of the protein of interest. For very small proteins, ≤15 kd, 0.2 micron is recommended. Methanol must be used in the transfer buffer when using nitrocellulose. Supported nitrocellulose is pure nitrocellulose cast on an inert synthetic support.

PVDF (polyvinylidene difluoride) is a hydrophobic support suitable for proteins. It has a higher capacity for proteins than nitrocellulose, and that helps prevent transfer of proteins through the membrane. The chemical resistant properties make PVDF the choice for sequencing. PVDF must be wetted in 100% methanol prior to use, but may be used with buffer which does not contain any methanol. PVDF membrane is recommended if SDS is used in the transfer buffer because of its high affinity and capacity for proteins.

Guide to Protein Blotting Membranes

Membrane	Binding Capacity (µg/cm^2)	Major Applications
Nitrocellulose	80–100	Immunoblotting
Supported Nitrocellulose	80–100	Immunoblotting,
PVDF	170–200	Immunoblotting, Protein Sequencing

Q: My buffer overheats during the transfer. What can I do to keep it from overheating?

A: As buffer overheats, it breaks down, causing even more overheating. This can become a serious problem in a short time. To avoid buffer breakdown, make sure that you have ade-

Blotting Cell Performance

Blotting Capabilities	Trans-Blot Cell with Wire Electrodes	Trans-Blot Cell with Plate Electrodes	Mini Trans-Blot Cell	Trans-Blot SD Cell
Electrode distance	8 cm—standard field 4 cm—high intensity	8 cm—standard field 4 cm—high intensity	4 cm	Determined by gel thickness*
Transfer time	2–5 hr—standard field 1–2 hr—high intensity	1–2 hr—standard field 30 min–1 hr—high intensity	1 hr	15–30 min—mini gels 30 min–1 hr—large gels
Cooling	Super cooling coil with refrigerated water recirculator required for high intensity transfers	Super cooling coil with refrigerated water recirculator required for high intensity; recommended for all applications that run over 100 mA	Bio-Ice cooling unit	Not necessary

quate cooling, that you only use buffer that is recommended for your transfer unit, and that you do not transfer under excessive power conditions.

1. Adequate cooling. For most tank transfers, the buffer should be cooled by recirculating chilled water or buffer through the buffer tank. Transferring in a cold room, or placing the transfer chamber in an ice bath, does not give sufficient cooling. This is because most transfer cell buffer chambers are made of plastic which doesn't allow efficient heat transfer. In addition, since the buffer nearest the gel is held relatively static by the gel-membrane sandwich, the buffer should be stirred during the transfer to maintain buffer circulation.

2. Buffer recommendations. The transfer buffer must be of a known ionic concentration to avoid overheating. If the ionic concentration is too high, transfers will either be at an artificially low voltage (if the transfer is under constant current conditions) or will overheat (if the transfer is at constant voltage). See Table 1 for recommended buffer formulations.

Table 1. Recommended Buffers for Western Blotting

Buffer Formulation	Reference	Application
1. 25 mM Tris 192 mM glycine, pH 8.3 20% methanol	1	SDS-PAGE gels
2. 48 mM Tris 39 mM glycine, pH 9.2 20% methanol	6	SDS-PAGE gels
3. 10 mM $NaHCO_3$ 3 mM Na_2CO_3, pH 9.9 20% methanol	7	Basic proteins from SDS-PAGE gels

3. Power Conditions. Heat generated in a transfer buffer is directly proportional to the power put through the buffer. The power in watts is equal to the voltage in volts multiplied by the current in amperes. Therefore

$$P=IE$$

where P represents power, I stands for current, and E stands for voltage. The voltage and current of a transfer are determined by the power conditions set on the power supply for the transfer and the resistance of the circuit (basically the buffer). Voltage, current, and resistance are related by the equation

$$V=IR$$

which can also be shown as

$$R=V/I$$

where I again stands for current, V represents voltage, and R is resistance. As the buffer breaks down, its resistance drops. If the voltage is set to be constant, when the resistance drops, the current will increase. Therefore, the power will increase and heating will result (see Figure 1A). However, if the current is set to be held constant and the resistance drops, so do the voltage and the power, causing the proteins to transfer more slowly (see Figure lB). Table 2 gives recommended power conditions.

Q: This immune detection procedure is too long. At which steps can I stop?

A: The best place to stop is after you have transferred the proteins onto a membrane. If you plan to continue with the proceedure the next day, you can store the blot in TBS (Tris buffered saline) at 4 °C. If you do not plan to probe the membrane within a day, dry the membrane and store it between two pieces of filter paper until you are ready to continue.

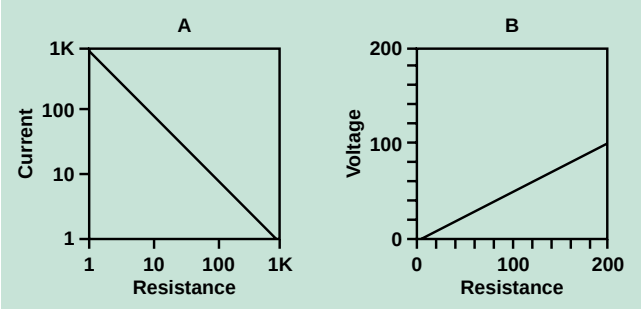

Fig. 1. Effects of resistance drop. (A) With constant voltage (1,000 V). (B) With constant current (500 mA).

Q: My blot is completely blank. Why don't I see any bands?

A: There are many factors affecting good band development.

1. Gel and membrane reversed. If the gel and membrane are reversed in the gel holder cassette or platform of the semi-dry cell, the proteins will be transferred out of the gel into the transfer buffer and be lost. For standard basic transfers, the gel should be on the cathode (black) side of the sandwich, and the membrane on the anode (red) side of the sandwich. For acidic transfers, the gel and membrane positions should be reversed.

2. Poor transfer efficiency. Transfer efficiency is affected by the size of the protein, the percentage of acrylamide in the gel, the strength of the electric field, the duration of transfer, and the pH of the buffer. Generally speaking, the larger the protein, the more slowly it will transfer. The best way to transfer large proteins is to blot with a high field strength.[10] Smaller proteins may be forced through the membrane if blotted for a long time with a high field strength. One way to avoid this problem is to blot onto 0.2 µm pore size nitrocellulose, or PVDF. If the isoelectric point of the protein is close to the pH of the buffer, the protein will not carry much charge, and therefore will not move in the electrophoretic field. If the protein of interest is very basic, either a carbonate buffer (pH 9.9),[7] a CAPS buffer (pH 11),[9] or an acidic buffer[6] is recommended.

Transfer efficiency can be determined by staining the gel after transfer, or by staining a second blot with a total protein stain, such as those in the Biotin-Blot Protein

Table 2. Recommended Power Conditions for Transfer in the Trans-Blot Tank Transfer Cells

Buffer Formulation	Low field Cooling optional Runs overnight	Standard field Cooling optional Runs 5 hr	High intensity field Cool to 0–4 °C Runs 30 min to 1 hr
25 mM Tris 192 mM glycine, pH 8.3 20% methanol	30 V, 0.1 A	60 V, 0.21 A	100 V, 0.36 A
48 mM Tris 39 mM glycine, pH 9.2 20% methanol	30 V, 0.1 A	60 V, 0.2 A	100 V, 0.43 A
10 mM $NaHCO_3$ 3 mM Na_2CO_3, pH 9.9 20% methanol	10 V, 0.1 A	20 V, 0.25 A	30 V, 0.44 A

Table 3. Recommended Power Conditions for Transfer in the Trans-Blot SD Semi-Dry Cell

Mini Gels	Large Gels
10–30 minutes 10–15 V, 5.5 mA/cm^2	30–60 minutes 15–25 V, 3 mA/cm^2

Any of the buffers from Table 2 may be used.

Detection Kit or the Enhanced Colloidal Gold Total Protein Detection Kit. To help you monitor transfer efficiency without staining the gel or a second blot, Bio-Rad provides prestained standards which are visible during the electrophoresis run and on the membrane after blotting.

3. Old or improperly stored reagents. Antibodies degrade with age, and will break down very rapidly with repeated freeze-thaw cycles. Substrates should be stored at -20 °C.

4. Impure or low titer antibodies. Primary antibody concentrations vary considerably. The dilution at which you need to use your first antibody must be determined empirically. Too much antibody is just as bad as too little antibody, as too much prevents binding of the antibody to the antigen. A general guideline is to dilute early bleed serum or tissue culture supernatant 1:100–l:l,000, and ascites fluid or serum from hyperimmunized animals 1:1,000–1:100,000. Bio-Rad's blotting grade second antibodies should be used at a 1:3,000 dilution.

5. Enzyme is inactivated. Sodium azide is a powerful inhibitor of horseradish peroxidase. Do not use azide in any of the solutions used in HRP conjugate western blotting. Sodium azide can be used with alkaline phosphatase conjugated antibodies without harmful effects. In addition, tap water or water deionized with polystyrene resins may inactivate the enzyme conjugate. Use only distilled, deionized water.

6. Tween-20 in washes. Tween-20 may interfere with some antibody-antibody interactions or may wash the protein of interest off the nitrocellulose. Although it should be used in washes at a final concentration of 0.05%–0.1%, even at this concentration it may cause binding problems. Tween-20 may be left out of all the washes except the first wash after blocking. However, this may result in increased background.

7. Detection system lacks sensitivity necessary to detect the amount of protein loaded. Make sure the amount of protein loaded will be within the range of sensitivity of the detection system.

Horseradish peroxidase	500 pg/band
Alkaline phosphatase	100 pg/band
Amplified alkaline phosphatase	5 pg/band
Colloidal gold	100 pg/band
Enhanced colloidal gold	10 pg/band
Immun-*Star*™ Chemiluminescence	10 pg/band

Q: I don't know how old my second antibody is. How stable are the conjugated antibodies? How should they be stored?

A: Bio-Rad's antibody conjugates are guaranteed for 1 year when stored at 4 °C, but will degrade rapidly if stored improperly. Alkaline phosphatase and horseradish peroxidase conjugated antibodies are shipped on dry ice. They can be stored at -20 °C until first used, then stored at 4 °C. Do not repeatedly freeze-thaw. The colloidal gold conjugates are shipped with ice packs, but not frozen. Upon arrival, store at 4 °C. Never freeze the colloidal gold conjugates. Do not store or carry out incubations with the colloidal gold conjugates in glass containers.

Q: I have background staining on my western blot. How can I avoid this?

A: Background staining can be due to a number of factors: insufficient blocking, excessive color development, insufficient washing, contaminated transfer buffer or apparatus, improper antibody dilution, or impure antibody.

1. Insufficient blocking. Any area on which either the primary or secondary antibody binds to the membrane will show substrate precipitation upon subsequent color development. To avoid non-specific binding, the membrane is incubated with blocking protein which binds to all free sites. The recommended blocker for nitrocellulose is 3% gelatin in TBS (Tris buffered saline). Incubate for 1 hour at

room temperature. Gelatin should not be incubated at 4 °C, as it will congeal. For low temperature blocking, use 3% BSA. The recommended blocker for PVDF membrane is 5% BLOTTO (nonfat dry milk) in TBS. BLOTTO is not recommended for use with Bio-Rad's biotinylated standards. Higher concentrations of BLOTTO can cause background problems.

2. Excessive color development. The length of time for color development varies depending upon the blot, the number of protein bands, and the desired sensitivity. If the membrane is left in the color development solution too long, excess precipitate formed by the enzyme can settle on the membrane and cause high background. The membrane should be allowed to remain in the color development solution until the protein bands are easily visible with little or no background.

3. Insufficient washing. The membrane should be washed at least twice after blocking and twice after each antibody step. Each wash should be 5 minutes long in a large volume of TBS-tween (0.05%–0.1% Tween-20). Alternative detergents can be used at very low concentrations. However, these stronger detergents can decrease binding of antibodies, and cause the antigen to be washed off the membrane. They are not generally recommended.

4. Contaminated transfer buffer or apparatus. Use detergent to clean all parts of the Trans-Blot transfer cell. The fiber pads used to hold the gel in the gel cassette are especially important to clean. Dirty fiber pads are a major cause of blot contamination. Also, discard transfer buffer after each transfer.

5. Improper antibody dilution. The second antibody should be used at a 1:3,000 dilution. The dilution of the primary antibody that will give maximum sensitivity with low background depends upon the source of the antibody and must be empirically determined. Generally, if the primary antibody is in serum from early bleeds or tissue culture supernatant, a 1:100–1:1,000 dilution is necessary. If the primary antibody is from hyperimmunized animals, or ascites fluid, a 1:1,000– 1:100,000 dilution is required. Insufficient dilution of either the primary or the secondary antibody can cause non-specific binding, and high background.

6. Impure second antibodies. Use only Bio-Rad's blotting grade affinity purified secondary antibodies. These antibodies are affinity purified, and cross adsorbed against other species of IgG to eliminate non-specific binding. Non-cross adsorbed antibodies from other sources are more likely to cross react with your primary antibody or other blotted proteins that are not of interest.

Instructions for the entire Immun-Blot® assay are provided with all Immun-Blot kits. Instructions for electrophoretic transfer are included with each Trans-Blot cell.

We hope these questions and answers have been informative and helpful. If you did not find the answer to your question here, contact your local Bio-Rad representative, or call our Technical Service Department at 1-800-4-BIORAD.

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