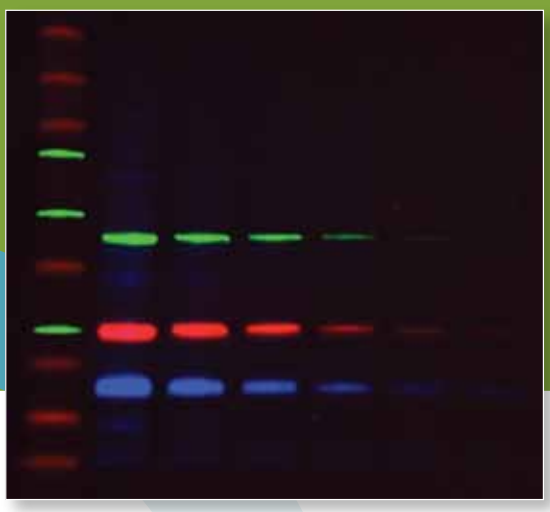
Multiplex Fluorescent Blot Detection: A Troubleshooting Guide

BIO-RAD

Bio-Rad's Handy Introduction to Fluorescent Blotting

This guide is intended as a brief introduction to fluorescent western blotting for researchers currently using chemiluminescent or colorimetric detection. Most western blotting protocols can be readily adapted for fluorescent detection but may require optimization.



Fluorescent detection offers several advantages compared to other methods:

Multiplexing

Use of multiple fluorophores for simultaneous detection of several target proteins makes stripping and reprobing unnecessary.

Dynamic range

A tenfold increase in dynamic range over chemiluminescent detection offers better linearity within detection limits.

Quantitative

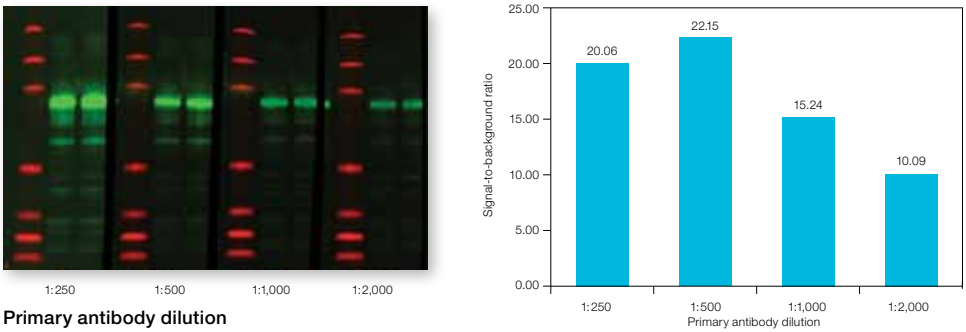
Fluorescent detection is more quantitative than enzyme-based detection methods.

Stability

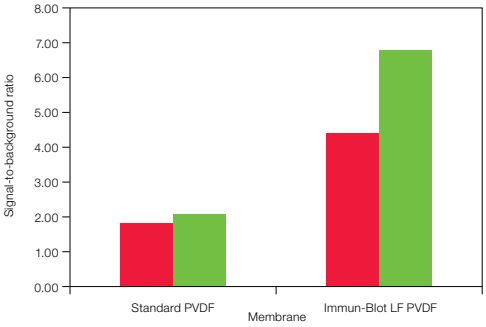
Most fluorescent molecules provide excellent stability, allowing blots to be archived and re-imaged at a later date.

Tips for Fluorescent Blotting

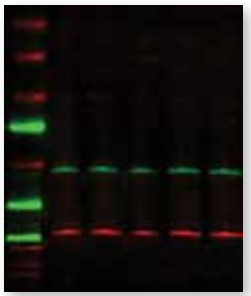
- Antibody concentrations should be optimized by incubating the membrane in several dilutions of each antibody. Select the dilution that yields the highest signal-to-background ratio
- When adapting a chemiluminescent protocol for fluorescent detection, primary antibody concentrations may need to be increased; two- to fivefold increases are common. Secondary antibody concentrations may also have to be optimized; a good starting point is a 1:5,000 dilution. Check the manufacturer's recommendations when using specific antibodies
- In order to maximize the signal-to-background ratio, use a membrane with low autofluorescence, such as the Immun-Blot® low fluorescence (LF) PVDF membrane
- Many blocking buffers can be successfully used for fluorescent detection. We recommend 0.5–5% casein, up to 5% nonfat dry milk, or up to 3% BSA dissolved in TTBS
- Particulates in buffers can settle on membranes and create fluorescent artifacts. Use only high-quality reagents and filter sterilize all buffers
- Use blunt forceps to handle the membrane from the edges. Avoid scratching or creasing the membrane, which can produce artifacts during fluorescent detection
- Use a pencil to mark membranes because many inks fluoresce
- Bromophenol blue will fluoresce. Ensure that the dye front has migrated away from the sample, cut off the portion of gel containing the dye front, or omit bromophenol blue from the sample buffer
- It is not necessary to perform immunodetection in the dark; normal room lighting will not significantly photobleach fluorescently labeled antibodies. However, stocks of fluorescently labeled antibodies should be stored in the dark
- Use powder-free nitrile gloves when handling the membrane to minimize artifacts and fingerprints on the blot



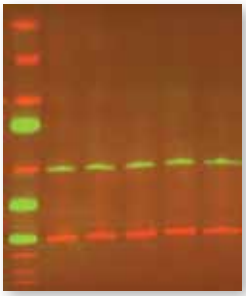
Optimizing antibody concentration is critical for best results. A gel was loaded with a protein sample containing transferrin (~80 kD) and blotted onto an Immun-Blot LF PVDF membrane. This blot was cut into strips and each strip was probed with a different concentration of primary antibody. The intensity of the transferrin band was compared to the background intensity of the membrane. For this antibody, a 1:500 dilution provides the optimal signal-to-background ratio. A high concentration of antibody increases background, yielding a lower overall signal-to-background ratio while low concentrations of antibody yield lower overall signal intensity. Higher antibody concentrations also increase nonspecific binding.



Standard PVDF membranes (bottom right) give higher auto fluorescence. The Immun-Blot LF PVDF membrane (top right) has lower autofluorescence and higher signal-to-background ratios in each channel, allowing detection of lower quantities of target protein. Both blots were handled and imaged using the same parameters on the ChemiDoc™ MP system using the DyLight 549 (green) and 650 (red) channels.



Immun-Blot LF PVDF membrane



Standard PVDF membrane

Tips for Multiplexing

- Use primary antibodies from different host species (for example, mouse and rabbit). Antibodies produced from two closely related species (such as rat and mouse) often give cross-reactivity, even when the antibodies are cross-adsorbed
- Use secondary antibodies that are highly cross-adsorbed against other species to avoid cross-reactivity
- Avoid cross-channel fluorescence by using fluorophore conjugates with optically distinct spectra
- Always optimize the detection of each target individually before simultaneously detecting multiple targets. Since some primary antibodies may be nonspecific and yield multiple bands on a blot, single target detection will help determine the banding pattern of each antibody prior to a multiplex experiment
- Most membranes show higher background with shorter wavelength excitation light. Detect your strongest target in the blue channel, your middle target in green, and reserve the red channel for your weakest target

Multiplex image | Red channel | Green channel

Non–cross adsorbed antibodies may bind nonspecifically and result in channel cross-talk. In this example, a protein sample containing glutathione S-transferase (green signal in left panel) and soybean trypsin inhibitor (red signal in left panel) were run on a gel, blotted, and immunodetected with a rabbit antibody against glutathione S-transferase (25 kD) and a mouse antibody against soybean trypsin inhibitor (21 kD). The non–cross adsorbed anti-rabbit secondary antibody (green) is weakly detecting the mouse primary antibody (green arrow at 21 kD).

Multiplexing with Stain-Free Gels

Stain-free gels are formulated with a trihalo compound that becomes covalently bound to tryptophan residues in proteins when the gel is activated with UV light. The labeled tryptophan adducts then fluoresce when excited by UV light. This allows for the direct visualization of total protein samples in gels and on blots.

- The stain-free fluorescent adduct is excitable with short-wavelength excitation light and will be visible when imaging blue fluorophores. For multiplex blot applications, use the red and green channels for detecting proteins of interest and reserve the blue channel for total protein detection
- Since Qdot nanocrystals and stain-free imaging both utilize UV excitation, Qdots cannot be used with proteins activated in stain-free gels. If stain-free total protein imaging is desired, use fluorophores that are not excitable with UV light

Fluorophore Selection

Recommended Fluorophores*	Red	Green	Blue
Number of Targets	Fluor A	Fluor B	Fluor C
1	Qdot 705 or DyLight 650	—	—
2	Qdot 705 or DyLight 650	Qdot 605 or DyLight 549	—
3**	Qdot 705 or DyLight 650	Qdot 605 or DyLight 549	Qdot 525 or DyLight 488
1+stain-free	DyLight 650	—	—
2+stain-free	DyLight 650	DyLight 550	—

* These fluorophores have been validated for use with Bio-Rad's ChemiDoc MP system; other fluorophores with similar excitation and emission spectra may also be used.

** Note: Qdot secondary antibodies may not be available for all three of your host species. In this case, DyLight 650 can be replaced with Qdot 705.

Compatible Fluorophores*

	Fluorophore	Excitation Filter/Bandpass, nm	Emission Filter/Bandpass, nm
UV	Stain-free	UV-B transilluminator	590/110
Blue	Cy2	470/30	530/28 (532/28)
Blue	Alexa 488		
Blue	DyLight 488		
Blue	Fluorescein		
Blue	Qdot 525		
Green	Qdot 605	470/30	605/50 (607/50)
Green	Qdot 625		
Green	Cy3	530/28	
Green	Alexa 546		
Green	DyLight 549 or 550		
Green	Rhodamine		
Red	Cy5	625/30	695/55 (697/55)
Red	Alexa 647		
Red	DyLight 649 or 650		
IR	Cy5.5		
IR	Alexa 680		
IR	DyLight 680		
IR	IRDye 680		
IR	Qdot 705	470/30	

* Note: This is a partial list. Other fluorophores with similar excitation and emission profiles will also be compatible.

Immunodetection Protocol Example

Materials:

- Immun-Blot LF PVDF membrane with transferred proteins
- Methanol or ethanol (necessary for wetting/rewetting dry PVDF membranes)
- Blocking buffer (Tris-buffered saline with 0.05% Tween 20 and an additional 1% casein)
- Wash buffer (Tris-buffered saline with 0.05% Tween 20)
- Primary antibody, diluted in blocking buffer
  - For multiplexing applications, add all primary antibodies to the same blocking solution
- Fluorescently labeled secondary antibody, diluted in wash buffer
  - For multiplexing applications, add all secondary antibodies to the same solution

Procedure:

Wash buffer volumes should be at least 20 ml for mini blots and 100 ml for Criterion™ blots. Block and antibody solution volumes should be enough to cover the membrane. Generally, this is at least 10 ml for mini gel blots and 25 ml for Criterion blots.

1. After protein transfer, equilibrate membranes in wash buffer for 3 min. Dried PVDF membranes should be briefly rewet in methanol prior to equilibration in wash buffer.
2. Incubate the blot protein side up in blocking buffer for 1 hr with continuous agitation.
3. Incubate the blot in diluted primary antibody solution for 1 hr with continuous agitation.
4. Wash the blot in wash buffer 5 times for 5 min each with continuous agitation.
5. Incubate the blot in diluted secondary antibody solution for 1 hr with continuous agitation.
6. Wash the blot in wash buffer 6 times for 5 min each with continuous agitation.
7. If recommended for your imager or fluorescent conjugate, the blot may be imaged while still wet. Alternatively, dip the blot in methanol and allow to air dry for imaging or long-term storage.

Storage of Fluorescently Detected Membranes

After immunodetection, membranes may be stored for analysis at a later date. If reprobing is not desired, blots can be dipped for a few seconds in methanol before hanging them to dry. This displaces the water with methanol, shortening drying time and also reducing background. Blots can then be placed in a plastic sheet protector to prevent contamination. Store the blots in the dark or wrapped in foil to prevent photobleaching of fluorophores. Depending on the fluorophore, properly stored blots can be imaged months later without significant loss of signal.

Buffers and Solutions

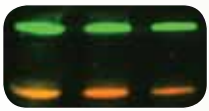
Wash buffer, Tris-buffered saline with Tween 20 (TTBS)	
(20 mM Tris, 500 mM NaCl, 0.05% Tween 20, pH 7.4)	
Tris	2.4 g
NaCl	29.2 g
10% Tween 20	5 ml

Adjust volume to 1 L with water.

Blocking buffer	
(20 mM Tris, 500 mM NaCl, 0.05% Tween 20, 1% casein, pH 7.4)	
TTBS Buffer	100 ml
Casein	1 g

Troubleshooting

Cross-channel fluorescence or extra bands

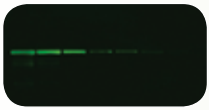


- Fluorophores not optically distinct. Ensure that secondary antibodies are conjugated to fluorophores with nonoverlapping emission spectra and that appropriate emission filters are used during image acquisitions
- Primary antibodies from same or related species. Check that each primary antibody in the multiplex set is from a different species (for example, mouse and rabbit). Avoid using two closely related species such as rat and mouse, as even cross-adsorbed antibodies from similar species may still cross-react
- Primary antibody may be nonspecific. Reduce concentration or use purified antibody
- Secondary antibody may be nonspecific. Use cross-adsorbed secondary antibodies. Run control omitting primary antibody
- If one signal of the multiplex set is much stronger than the others, the signal may be bleeding through to the other channels. Try reducing the concentration of the stronger primary antibody or swap the fluorophores on the secondary antibodies

Low overall signal

- Antibody concentrations too low. When adapting a chemiluminescent protocol for fluorescent detection, antibody concentrations may have to be increased. Typically, a two- to fourfold increase is a good starting point
- Concentration of labeled antibody too high. Extremely high concentrations of labeled antibody may also yield low signal due to quenching via Förster resonance energy transfer (FRET)
- Incomplete transfer of proteins to blot. High molecular weight proteins may require a longer transfer time. Verify transfer efficiency by using stain-free gels and monitor gel and membrane post transfer
- Protein load on gel too low. Some proteins elute less readily from the gel and require higher loads of protein to produce satisfactory blots

Uneven signal levels



- Air bubbles between gel and membrane. Air bubbles will produce blank spots on the membrane. Roll the gel and membrane to expel any trapped air during assembly of the transfer sandwich. Transfer artifacts can be monitored by using stain-free gels
- Agitation during antibody incubation may be insufficient. Ensure incubations occur with enough volume to completely cover the membrane and incubate and wash with continuous, vigorous agitation
- Use a membrane with low autofluorescence

Troubleshooting (contd.)

High overall background

- Membranes tend to have higher inherent fluorescence with shorter wavelength excitation light. Detect the weakest target using the red channel
- Blocking may be insufficient. Increase the concentration of the blocking agent or duration of the blocking step, or try an alternate blocking agent
- Washing may be insufficient. Increase the number or duration of wash steps
- Antibody concentrations may be too high. The concentration of primary or secondary antibodies may have to be optimized
- Adding 0.01% SDS to the secondary antibody solution during incubation may help reduce nonspecific binding to the blot

Uneven background levels

- PVDF membrane may have been improperly wetted or may have dried during handling. Ensure that the membrane is fully wetted in methanol prior to equilibration in aqueous solution and ensure that the membrane stays wet
- Wash steps may have occurred in insufficient volume. Ensure that incubations occur with enough volume to completely cover the membrane and wash with vigorous agitation

Fluorescent artifacts seen on blot

- Scratches or creases on the membrane can produce artifacts. Always use blunt forceps to handle the membrane and grasp the membrane at the edges
- Fingerprints on membrane. Do not touch membranes with bare hands. Some gloves leave fluorescent residues on membranes. Use powder-free nitrile gloves and forceps to handle the membrane
- Particulate contaminants in solutions may appear as fluorescent speckles on the blot. Filter buffers and reagents to remove contamination
- Do not use a pen to mark the membrane as some inks fluoresce. Use a pencil instead
- Bromophenol blue can produce a fluorescent signal when transferred to the membrane. Ensure that the dye front has migrated away from the protein of interest or use a loading dye without bromophenol blue

For additional suggestions on optimizing blotting conditions, refer to the Bio-Rad Protein Blotting Guide (Bulletin 2895).

Order Information

Catalog #	Description
Imaging System and Accessories	
170-8280	**ChemiDoc MP System**, gel imaging system, PC or Mac, includes darkroom, UV transilluminator, epi-white illumination, camera, power supply, cables, Image Lab™ software
170-8283	**ChemiDoc MP Red LED Module Kit**, pkg of 2 epi-red LED modules and 1 red emission filter, for use with applications requiring red fluorophore detection
170-8284	**ChemiDoc MP Green LED Module Kit**, pkg of 2 epi-green LED modules and 1 green emission filter, for use with applications requiring green fluorophore detection
170-8285	**ChemiDoc MP Blue LED Module Kit**, pkg of 2 epi-blue LED modules and 1 blue emission filter, for use with applications requiring blue fluorophore detection
Membranes	
162-0260	**Immun-Blot Low Fluorescence PVDF/Filter Paper Sets**, pkg of 10, 7 x 8.5 cm, precut low fluorescence PVDF/filter paper for immunoblotting
162-0261	**Immun-Blot Low Fluorescence PVDF/Filter Paper Sets**, pkg of 20, 7 x 8.5 cm, precut low fluorescence PVDF/filter paper for immunoblotting
162-0262	**Immun-Blot Low Fluorescence PVDF/Filter Paper Sets**, pkg of 10, 8.5 x 13.5 cm, precut low fluorescence PVDF/filter paper for immunoblotting
162-0263	**Immun-Blot Low Fluorescence PVDF/Filter Paper Sets**, pkg of 20, 8.5 x 13.5 cm, precut low fluorescence PVDF/filter paper for immunoblotting
162-0264	**Immun-Blot Low Fluorescence PVDF membrane**, pkg of 1 roll, 28 cm x 3.8 m, low fluorescence PVDF membrane for immunoblotting

Description	 10-Well 30 µl	 10-Well 50 µl	 15-Well 15 µl	 IPG/prep 450 µl	 12-Well 20 µl	 8+1-Well 30 µl
Mini-PROTEAN® TGX Stain-Free™ Precast Gels						
7.5%	456-8023	456-8024	456-8026	456-8021	456-8025	456-8029
10%	456-8033	456-8034	456-8036	456-8031	456-8035	456-8039
12%	456-8043	456-8044	456-8046	456-8041	456-8045	456-8049
Any kD™	456-8123	456-8124	456-8126	456-8121	456-8125	456-8129

All formats are available as both ten packs (catalog numbers listed) and two packs. To order as a two pack, add an "S" to the end of the catalog number for the corresponding ten pack.

Description	 12+2*-Well 45 µl	 18-Well 30 µl	 26-Well 15 µl	 Prep+2*-Well 800 µl	 IPG+1*-Well 11 cm IPG Strip
Criterion™ TGX Stain-Free™ Precast Gels**					
7.5%	567-8023	567-8024	567-8025	—	—
10%	567-8033	567-8034	567-8035	—	—
12%	567-8043	567-8044	567-8045	—	—
18%	567-8073	567-8074	567-8075	567-8072	567-8071
4–15%	567-8083	567-8084	567-8085	567-8082	567-8081
4–20%	567-8093	567-8094	567-8095	567-8092	567-8091
8–16%	567-8103	567-8104	567-8105	567-8102	567-8101
10–20%	567-8113	567-8114	567-8115	567-8112	567-8111
Any kD	567-8123	567-8124	567-8125	567-8122	567-8121

* Reference well accommodates 15 µl of markers/standards.

** Criterion TGX Stain-Free gels are sold as a single gel.

Reagents

161-0781	**10% Tween 20**, 1 L, detergent
161-0783	**1x Phosphate Buffered Saline with 1% Casein**, 1 L, blocking reagent
166-2403	**10x Phosphate Buffered Saline**, 100 ml, 10x PBS
161-0782	**1x Tris Buffered Saline with 1% Casein**, 1 L, blocking reagent
170-6435	**10x Tris Buffered Saline**, 1 L, 10x TBS

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