Increase Western Blot Throughput with Multiplex Fluorescent Detection

Introduction

The most common method for analyzing protein expression levels is western blotting with detection of a single protein target, using horseradish peroxidase–conjugated or alkaline phosphatase–conjugated antibody probes combined with colorimetric or chemiluminescent detection (Bers and Garfin, 1985; Bronstein et al., 1992; Kricka, 1991; Thorpe et al., 1985). While these methods work well for studying a single target, they are unsuitable for analyzing multiple targets at the same time, particularly if the target proteins are of unknown or similar sizes. For analysis of multiple targets, the blot is typically stripped and reprobed for additional targets of interest (Kaufmann et al. 1987). Reprobing is time consuming, and often some of the target protein on the blot is lost as a result of the stripping procedure. If one protein is removed to a greater or lesser extent relative to another protein, the ability to quantitate the relative amounts of different proteins of interest is compromised.

Recently, there has been a trend towards the application of fluorescence-based detection of proteins on western blots. This approach allows for multiplex analyses and greater signal stability relative to chemiluminescent detection. Simultaneous probing reduces the time and labor required for western blot processing, and less sample is initially required, since only one blot has to be prepared (Gingrich et al. 2000). The combination of antibodies selected for minimal cross-reactivity with fluorescent detection methods enables quantitative analysis of two or more proteins. The fluorescent signal of a protein of interest can be normalized to a housekeeping protein internal standard to improve the accuracy of quantitation and to measure differences in protein expression levels (McDonald et al., 2006). In addition, with the emergence of highly photostable dyes, enhanced imaging instrumentation, and low-fluorescence membranes, the sensitivity of fluorescent immunodetection is comparable to that of chemiluminescent detection (Koticha et al., 2006). Generally, fluorescent stains provide a greater linear dynamic range for quantitation and are easier to visualize on charge-coupled device (CCD) camera–based and laser scanner–based imaging systems (Top et al., 2001).

The numerous advantages of fluorescent western blot detection include:

- Fast and quantitative detection of multiple proteins in a single experiment
- Sensitivity comparable to chemiluminescent detection
- Linear dynamic range up to 10 times greater than that of chemiluminescent detection
- Fewer experimental steps than chemiluminescent detection
- No substrate requirement, and therefore no risk of exhausting the substrate and causing a “dead zone” in the blot
- The ability to visualize and quantitate both phosphorylated and non-phosphorylated forms of individual proteins

This technical note is divided into three sections to help those who are new to fluorescent western blot detection quickly generate reliable and reproducible results. A complete protocol, including materials and their sources, is provided first. The Tips section will help ensure the success of initial experiments, and the Troubleshooting section will help you achieve continued success.

Section 1: Protocol

Materials

Gels and Membranes

Precast single-percentage or gradient gels such as Ready Gel® gels and Criterion™ gels or handcast polyacrylamide gels can be used, depending on the separation needs of the experiment.

A low fluorescence PVDF membrane such as Pall FluoroTrans or Millipore Immobilon FL should be used.

Description	Pall FluoroTrans Catalog #	Millipore Immobilon FL Catalog #
7 x 8.4 cm sheets	PVM020C-160	IPFL07810
10 x 15 cm sheets	PVM020C1015	
20 x 20 cm sheets	PVM020C2020	IPFL20200
26 cm x 3.3 or 3.5 m roll	PVM020C-099	IPFL00010

Figure 1 illustrates the low fluorescence background advantage provided by either the FluoroTrans or Immobilon PVDF membrane, which can reduce background fluorescence more than tenfold to greatly enhance the sensitivity of fluorescent detection. Nitrocellulose, Pall FluoroTrans W PVDF, and Millipore Immobilon-P PVDF membranes should not be used for this application, because of their high fluorescence backgrounds.

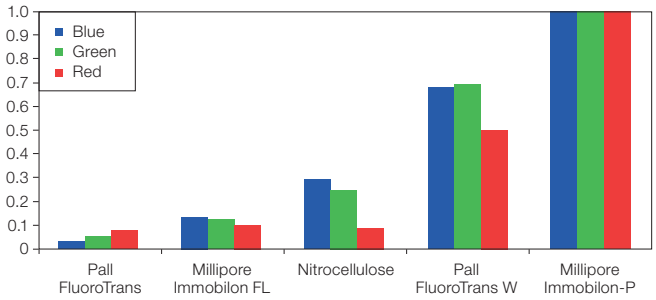

Fig. 1. Relative fluorescent background levels with various membrane types and excitation sources. Background fluorescence levels at appropriate emission wavelengths for each LED excitation source (blue, green, or red) were measured and normalized to those of Millipore Immobilon-P.

Antibodies and Molecular Weight Markers

Primary antibodies should be raised against the proteins of interest using different species for each protein (mouse, rabbit, or goat). Detection is provided by fluorescent secondary anti-IgG antibodies raised in species different from the primary antibody species, for example, fluorescently labeled goat anti-rabbit IgG.

Secondary antibodies labeled with fluorophores with different spectra can be chosen from different commercial sources. The secondary antibodies listed in Table 1 have been shown to be suitable for fluorescent western blot detection by the Molecular Imager® VersaDoc™ MP and Molecular Imager® PharosFX™ imaging systems from Bio-Rad.

Bio-Rad Precision Plus Protein™* Dual Color (catalog # 161-0374) and Precision Plus Protein™ WesternC™ standards (catalog # 161-0376) fluoresce with both green and red excitation (Figure 2). Precision Plus Protein Kaleidoscope™ standards (Bio-Rad) can also be used, as they contain two blue-excitable bands.

Table 1. Fluorescently labeled secondary antibodies and their spectral characteristics.

Fluorescently Labeled Antibody	Excitation/ Emission	Source	Catalog #
DyLight 488 anti-mouse	400/410	Rockland Immunochemicals	610-141-121
DyLight 549 anti-mouse	550/518	Rockland Immunochemicals	610-142-121
DyLight 649 anti-mouse	646/674	Rockland Immunochemicals	610-143-121
DyLight 488 anti-rabbit	500/520	Rockland Immunochemicals	611-141-122
DyLight 549 anti-rabbit	550/568	Rockland Immunochemicals	611-142-122
DyLight 649 anti-rabbit	646/674	Rockland Immunochemicals	611-143-122
Goat anti-mouse Alexa 633	632/647	Invitrogen	A21052
Goat anti-mouse Alexa 555	555/565	Invitrogen	A21424
Goat anti-mouse Alexa 488	500/520	Invitrogen	A11029
Goat anti-rabbit Alexa 633	632/647	Invitrogen	A21071
Goat anti-rabbit Alexa 555	555/565	Invitrogen	A21429
Goat anti-rabbit Alexa 488	500/520	Invitrogen	A11034
Sheep anti-mouse IgG Cy3	550/570	Sigma	C2181
Goat anti-rabbit IgG H+L Cy5	650/670	Abcam	ab6564

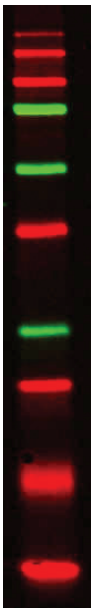

Fig. 2. Precision Plus Protein™ WesternC™ standards. Precision Plus Protein WesternC standards (5 µl) were separated on a Criterion gel, blotted using Pall FluoroTrans membrane, and imaged with the green and red LEDs of the VersaDoc MP 4000 system. The bands denote molecular masses of 250, 150, 100, 75, 50, 37, 25, 20, 15, and 10 kD.

Solutions and Buffers

Membrane wash buffer is composed of phosphate buffered saline plus 0.05–0.2% Tween (PBST). Membrane blocking buffer is composed of PBST plus 1% bovine serum albumin (BSA) or casein.

Transfer buffer is composed of Towbin Tris-glycine buffer (192 mM glycine, 25 mM Tris, pH 8.3) with 20% methanol (see the Protein Blotting Guide, Bio-Rad bulletin 2895 for buffer formulations for difficult (glycoproteins, high molecular weight, basic) proteins). PVDF membranes are prewetted using high quality, analytical grade methanol.

All incubations are performed in incubation dishes slightly larger than the membranes to minimize reagent volumes. These dishes should be dedicated to western blotting to minimize background problems. Staining trays are available for Bio-Rad Criterion gels (catalog # 345-9920).

A rocking platform is required to uniformly agitate the membranes in wash buffers and antibody solutions.

Western Blotting Transfer Apparatus

There are two main types of electrophoretic transfer procedures. In wet transfer, gels and membranes are submerged under transfer buffer in electrophoresis tanks; in semi-dry transfer, gels and membranes are sandwiched between buffer-wetted filter papers that are in direct contact with flat-plate electrodes. Submarine transfer systems are recommended for most routine protein work, for efficient and quantitative protein transfers, and for transfer of proteins of all sizes. Some large proteins may be poorly transferred with semi-dry blotting, which is most often used in laboratories processing large numbers of blots, because of the speed, ease of setup, and low buffer usage of semi-dry blotting. For more complete information on blotting transfer methods, please see the Protein Blotting Guide (Bio-Rad bulletin 2895).

Fluorescent Imaging System

Bio-Rad Molecular Imager VersaDoc MP 4000 and 5000 systems or a PharosFX system with external lasers can be used for fluorescent imaging (Figure 3). Both are open systems capable of a wide variety of applications in addition to fluorescent western blotting. Bio-Rad Quantity One® software provides predetermined filter and excitation source settings for blotting applications, making both systems easy to use. See Table 2 for a list of common filter and light source settings for blotting.

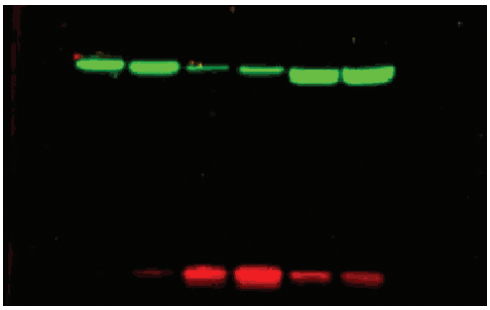

Fig. 3. Fluorescent western blot of human β-actin and phosphorylated cofilin imaged with the VersaDoc MP 4000 system. Mouse anti-β-actin (Sigma) and sheep anti-mouse IgG (whole molecule) labeled with Cy3 (Sigma) were used to image the actin band. Rabbit anti-p-cofilin 1 (Santa Cruz Biotechnology) and goat anti-rabbit IgG (H+L) labeled with Cy5 (Abcam) were used to image the phosphorylated cofilin band. Images were captured using a VersaDoc MP 4000 imaging system and Quantity One software. The blot was imaged using the Cy3 and Cy5 fluorophore settings in the Quantity One VersaDoc MP application selection. This experiment was performed to confirm the effective knockdown of ß-actin expression and an associated increase in the level of p-cofilin. Knockdown was performed using siLentMer™ Dicer-substrate siRNA duplexes (Bio-Rad). For details on the experiment, refer to Bio-Rad bulletin 5564.

Table 2. Predetermined filter and excitation source settings provided by Quantity One software.

VersaDoc MP Imager/Quantity One Software Settings	Excitation Source	Emission Filter	Applicable Dyes
Alexa 488/Cy2	Blue LED	530BP	DyLight 488, Alexa 488
Alexa 555/Cy3	Green LED	605BP	Cy3, DyLight 549, Alexa 532
Alexa 647/Cy5	Red LED	695BP	Cy5, DyLight 649, Alexa 635
PharosFX Imager/Quantity One Software Settings			
Alexa 488/Cy2	488 nm laser	530BP	DyLight 488, Alexa 488
Alexa 555/Cy3	532 nm laser	605BP	Cy3, DyLight 549, Alexa 532
Alexa 647/Cy5	635 nm laser	695BP	Cy5, DyLight 649, Alexa 635

Procedure

1. Transfer proteins from a polyacrylamide gel to a membrane with low background fluorescence (see pages 25–30 of the Protein Blotting Guide, Bio-Rad Bulletin 2895, for an overview of steps and protocols involved in performing protein transfer; detailed protocols can be found in the relevant blotting instrument instruction manuals).
2. Incubate the membrane in blocking buffer for 1 hr at room temperature. Use enough solution to completely cover the membrane (≥0.4 ml/cm^2). This is a convenient stopping point, as the membrane can be left in blocking solution overnight at 4°C.
3. Incubate the blot with primary antibody diluted 1:1,000 to 1:5,000 in blocking buffer for 1 hr. The appropriate dilution will depend on the primary antibody used and should be determined experimentally.
4. Incubate with wash buffer 5 x for 10 min each on a rocking platform.
5. Incubate blot with secondary antibody, which is usually diluted ~1:3,000 in blocking buffer, for 1 hr at room temperature in the dark. The appropriate dilution will depend on the secondary antibody used and should be determined experimentally.
6. Wash with PBST buffer 5 x for 5 min each on a rocking platform.
7. Rinse membrane in 100% methanol and air dry.
   Note: Do not use methanol with nitrocellulose membranes, as >40% methanol will dissolve them.
8. Capture fluorescence images using a VersaDoc MP system or the PharosFX system with the appropriate settings. All of the settings are application driven and preprogrammed in Quantity One software, so that the user needs only to select the appropriate application. Take an initial exposure, then examine the brightest band on the image with the View>Plot Density>Density at Cursor tool. The density at cursor should be slightly below saturation (65,535 counts) to maximize the dynamic range of the instrument. If this density is significantly lower than saturation, increase the exposure time when using a VersaDoc MP system, or use a higher gain setting (low sample intensity) if using the Pharos FX system; then reimage the blot. Bands that have a density at saturation will not provide quantitative data; the blot should be reimaged with a shorter exposure time or lower gain setting.
9. Use the Volume Analysis Quick Guide in Quantity One to quantitate each fluorescent band. Make sure volume objects are drawn to include background and to draw a background volume object to subtract background data.
10. Export results to an Excel spreadsheet for normalization, if needed.

Section 2: Tips for Better Results

Chose Antibodies With Distinctive Spectra

- Choose two antibodies with fluorescent labels having distinct nonoverlapping spectra. Use of a blue-excitable (Alexa 488) or a green-excitable (Alexa 555) fluorescent antibody with a red-excitable one (Alexa 633) will provide complete spectral separation and accurate quantitation of both antibodies on one blot. When using blue-, green-, and red-excitable labeled secondary antibodies on the same blot, crosstalk between the blue and green channels is about 3–5%
- Optimize primary and secondary antibody concentrations. This will produce better results and reduce cost in the long run (see pages 38–41 of the Protein Blotting Guide (Bio-Rad bulletin 2895) for additional information

Increase Sensitivity

- Use the narrowest well size possible on the gel to produce a more concentrated target protein band
- Store fluorescently labeled antibody stocks in the dark, and minimize exposure of membranes to strong light during secondary antibody incubations and washes. To minimize photobleaching and resultant loss of fluorescent signal, keep membranes in the dark if imaging will not be performed immediately after incubation
- Stripping and reprobing is not recommended for any type of blotting
- To improve the binding of the primary antibody, incubate overnight at 4°C
- Do not overblock the membranes; long blocking incubations (≥24 hr) can result in significant loss of available target protein from the membrane (DenHollander et al., 1989)

Reduce Fluorescent Background

- Bromophenol blue emits a strong fluorescent signal; either omit it from the loading buffer, run the bromophenol blue dye front off the polyacrylamide gel, or cut off the dye front before transfer
- Coomassie dyes will also produce a highly fluorescent background. Make sure that the incubation trays and other tools have not been used with Coomassie dye solutions
- Avoid other sources of fluorescent background such as gel fragments, Triton X-100, glove powder, or oil from skin. Always use clean forceps to manipulate membranes
- Remove the stacking gel, if applicable, because it is likely to adhere to the membrane after transfer and trap secondary antibodies that cause imaging artifacts
- Milk-based blocking solutions may contain IgG, which reacts with anti-goat antibodies. This can significantly increase background. Casein and BSA can be used instead
- Avoid extended incubations with the secondary antibody; they will increase background
- Do not use a ballpoint pen to write on membranes, as the ink can fluoresce. If the membrane must be marked, notch a corner or mark with a pencil
- Once the membrane has been wetted, use only smooth-tipped membrane forceps to move it. Excessive pressure on the membrane can lead to increased background levels
- Accurate pipetting is a must for quantitative results. Prewet the pipet tip 2–3 times before loading the protein sample onto the gel; make sure samples are at room temperature, and dispense them smoothly

Section 3: Troubleshooting

Problem	Cause	Solution
High background	Primary antibody concentration too high	Dilute the primary antibody within the appropriate dilution range, determined from optimization studies
	The primary antibody is nonspecifically binding	Validate specificity of primary antibody for target protein by other methods
	Insufficient washing	Increase number and volume of washes
	Inefficient blocking	Block the blot for at least 1 hr at room temperature, or overnight at 4°C in 1x blocking buffer
	Inadequate volume of antibody solution	Use enough solution to cover the entire membrane surface with liquid at all times
	Incorrect membrane used	Use only low fluorescent membranes (see the Materials section)
	Membrane was touched or bent	Only use smooth forceps to touch the membrane after hydration, and do not fold or bend the membrane
Weak signal or no signal	Insufficient quantities of antigen or primary antibody	Increase the amount of protein loaded onto the gel or the primary antibody concentration
	Inefficient protein transfer	Optimize transfer conditions (refer to the Protein Blotting Guide, Bio-Rad bulletin 2895)
	Incorrect emission filter used on imaging instrument	Refer to filter and application descriptions in Quantity One software
	Incorrect membrane used	Use only low fluorescent membranes (see the Materials section)
Uneven, blotchy, or speckled background	Unevenly hydrated PVDF membrane	Ensure that the membrane is completely covered with methanol for at least 15 sec before placing it into the buffer
	Inefficient protein transfer	Make sure there are no bubbles between the gel and the membrane and that there is even contact pressure over the entire gel/membrane interface
	Membrane stained with Ponceau S	Prestaining may cause high background even after this stain is washed off
Nonspecific bands	Antibodies cross-reacting	Change the antibody source, if possible
	Primary or secondary antibody concentration was too high	Dilute the primary antibody within an appropriate range, determined from optimization studies

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