Experimental Protocol for Multiplex Fluorescent Blotting Using the ChemiDoc™ MP Imaging System

Protocol

Bulletin 6570

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Bio-Rad provides instruments and reagents for multiplex fluorescent western blotting (MFWB), a powerful technique that is gaining increased attention in the life science community. Like any other assay, MFWB requires careful assay development to guarantee high-quality data. Here, we introduce a protocol independently generated by Dr. Stefanie Ritter, a postdoctoral fellow in Dr. Donald Rainie's lab at Emory University. We hope this protocol and the included tips will help other scientists have a successful experience with MFWB.

Background

Fluorescence-based multiplex western blot detection is particularly suited for simultaneous detection of phosphorylated and total protein populations, as it allows for accurate and easy signal normalization within the same sample on the same blot. Moreover, the ability to simultaneously detect two proteins of interest increases the information that can be collected from your sample. This is especially important if you have only enough sample for one or two western blots.

Fluorescence-based western blot detection is very sensitive but lacks the additional amplification achieved when using horseradish peroxidase (HRP) secondary antibodies and chemiluminescence-based detection. Importantly, the factors affecting signal-to-noise ratio after the detection of the protein(s) of interest must be optimized, including blocking conditions, antibody concentration, and the amount of experimental sample to be probed. Likewise, the fluorescence-based detection requires that care be taken to avoid exposing the membrane to dust and/or other environmental contaminants, which could introduce unwanted background.

The ChemiDoc MP Imaging System is a high-resolution gel documentation system that allows fast, easy quantification of gels and blots. It uses a new-generation lighttight enclosure (the universal hood III) that minimizes contaminants. It contains LED-based illumination to detect fluorescent secondary antibodies in the visible range. Here, we describe a protocol using the ChemiDoc MP Imaging System to detect levels of phosphorylated and total populations of the extracellular signal-regulated kinase (ERK) protein in brain samples. The detection of phospho-ERK (pERK) using either traditional chemiluminescence-based detection or fluorescence-based detection are compared as well.

Materials and Methods

- Harvest buffer
  - 150 mM NaCl
  - 20 mM Hepes
  - 1x Halt Protease and Phosphatase Inhibitor Cocktail and 1x EDTA (Thermo Fisher Scientific)
- 6x Laemmli buffer
- Rat brain tissue
- Tissue homogenizer
- 95°C heat block
- 4–20% Mini-PROTEAN® TGX™ Gels (Bio-Rad Laboratories)
- Tris-glycine SDS-PAGE running buffer
- Tris-glycine transfer buffer supplemented with 20% methanol
- PowerPac™ Power Supply and Mini Trans-Blot® Cell (Bio-Rad Laboratories)
- Precision Plus Protein™ Standards (Bio-Rad Laboratories)
- Nitrocellulose membranes (Bio-Rad Laboratories)
- LF-PVDF membranes (Bio-Rad Laboratories)
- Methanol
- 10x Tris-buffered saline (TBS)
  - 2 M Tris (25.4 g Trizma Cl + 4.72 g Trizma base)
  - 1.5 M NaCl (87.66 g)
  - Diluted in 1 L of dH_2O
- 1x TBS (10x diluted in dH_2O)
- 1x TBS/T (10x diluted in dH_2O w/ 0.1% Tween 20 added prior to use)
- Ponceau S
- 0.1 N NaOH
- Black or opaque blotting boxes (Rockland Immunochemicals/ LI-COR Biosciences)
  - Lid to prevent dust contamination and/or evaporation
- Bovine serum albumin (BSA)
- Primary antibodies (see protocol)
- Secondary antibodies (see protocol)
- Forceps
- SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific)
- ChemiDoc MP Imaging System (Bio-Rad Laboratories)

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Procedure

1. Homogenize and process brain tissue in harvest buffer.
2. Dilute samples in 6x Laemmli buffer, boil at 95°C for 5 min and load onto a 4–20% Mini-PROTEAN TGX Gel in the order listed in Figure 1.
3. Run protein samples using SDS-PAGE and transfer to LF-PVDF using standard transfer conditions.
4. Cut LF-PVDF membrane into two sections and reversibly stain with Ponceau S to examine transfer efficiency. Remove stain by washing the membrane with 0.1 N NaOH and rinse twice in 1x TBS.
5. Block membranes for 30 min in freshly prepared 3% BSA in TBS with agitation at room temperature (do not include Tween in this step).
6. Probe membranes with either mouse anti-pERK (1:500, Santa Cruz Biotechnology, Inc.) (Figures 1A and 1B) or rabbit anti-total ERK (tERK) (1:1,000, Cell Signaling Technology) (Figure 1C) diluted in 3% BSA in TBS/T. Process overnight with agitation at 4°C.
7. Return membranes to an orbital shaker for 10 min at room temperature.
8. Discard primary antibody solutions and wash membranes 3 times for 5 min in 1x TBS/T.
9. Incubate membranes with their respective secondary antibodies. The membranes shown in Figure 1 were probed with the following secondary antibodies: A, anti-mouse HRP (1:8,000, Vector Laboratories); B, anti-mouse 680 (Alexa Fluor 1:4,000); C, anti-rabbit 549 (DyLight 1:4,000). The antibodies were diluted in 3% BSA in TBS/T and the membranes were incubated at room temperature for one hour with gentle agitation.
10. Rinse membranes 3 times for 5 min in 1x TBS/T.
11. Incubate the membrane probed with HRP secondary antibody in SuperSignal West Pico Chemiluminescent Substrate for 5 min at room temperature with gentle agitation. Discard the substrate and image the blot on the ChemiDoc MP Imaging System using the Chemi Hi Sensitivity feature at a manual exposure of 1–60 sec, acquiring ten images throughout the duration of the imaging.

    Note: In Figure 1, the image captured at 6.8 sec was selected and saved, as it did not have any signal saturation.
12. Rinse the fluorescent membranes (Figures 1B and 1C) with 1x TBS and keep in TBS until ready to image on the ChemiDoc MP Imaging System. Image the fluorescent membrane using a protocol to detect the IR 680 (Autoexpose, Faint Bands, 30 sec) and DyLight 549 (manual exposure, 360 sec) secondary antibodies.

    Note: The manual exposure time was chosen to avoid signal saturation and to increase the information gathered in one image. The gain of the images was adjusted to minimize background and a gain was selected in which pERK bands at both 44 and 42 kD could be distinguished. A comparison of detection with fluorescence and chemiluminescence using the above protocol is shown in Figure 1.

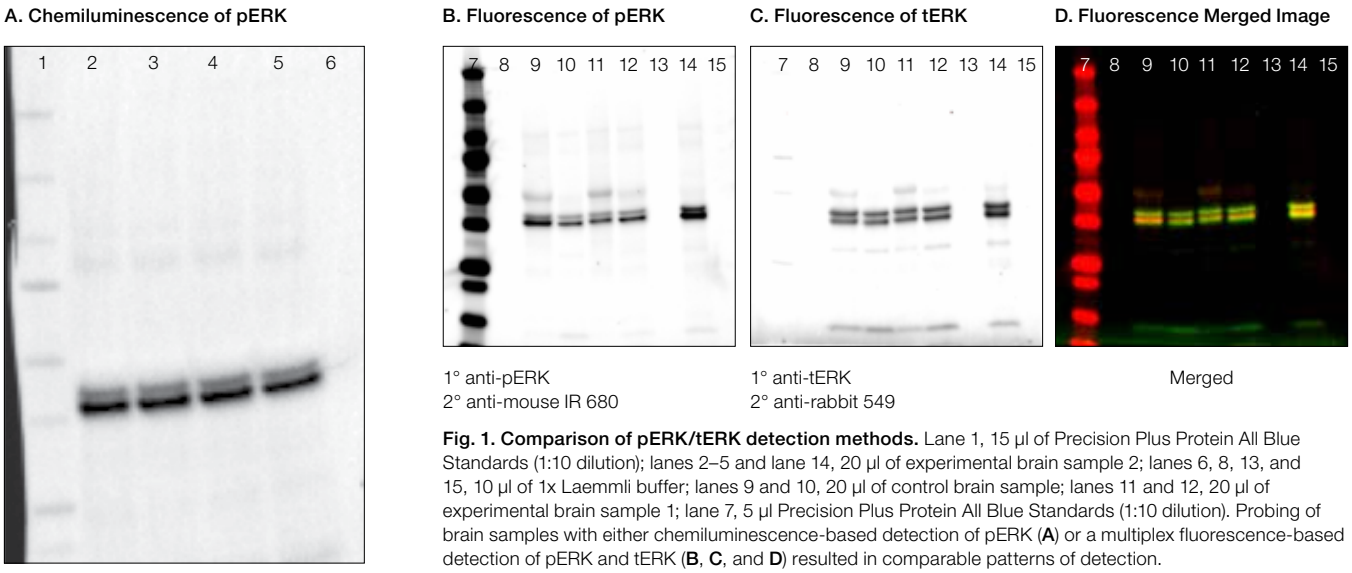

Fig. 1. Comparison of pERK/tERK detection methods. Lane 1, 15 µl of Precision Plus Protein All Blue Standards (1:10 dilution); lanes 2–5 and lane 14, 20 µl of experimental brain sample 2; lanes 6, 8, 13, and 15, 10 µl of 1x Laemmli buffer; lanes 9 and 10, 20 µl of control brain sample; lanes 11 and 12, 20 µl of experimental brain sample 1; lane 7, 5 µl Precision Plus Protein All Blue Standards (1:10 dilution). Probing of brain samples with either chemiluminescence-based detection of pERK (**A**) or a multiplex fluorescence-based detection of pERK and tERK (**B**, **C**, and **D**) resulted in comparable patterns of detection.

Important Information

Choice of Membrane

It is imperative to use a membrane with low autofluorescence for fluorescence-based antibody detection. Although nitrocellulose membranes are cheap and widely used, a number of companies have formulated low-fluorescence PVDF (LF-PVDF) membranes for use with fluorescence-based imaging. Nitrocellulose membranes autofluoresce in the visible and near infrared (near IR) spectrum, making background a more substantial issue and minimizing the detectable range useful for imaging (Figure 2A). In fact, nitrocellulose membranes may work only with the near IR or IR spectrum. In contrast, LF-PVDF membranes activated with methanol prior to imaging on the ChemiDoc MP Imaging System have little to no detectable background in the visible or near IR range (Figure 2B). Importantly, if a PVDF membrane is used, it will need to be activated with methanol. When using methanol, make sure the membrane does not dry out during the blotting and imaging process. Water may be sprinkled onto the membrane for the final imaging steps with the ChemiDoc MP Imaging System.

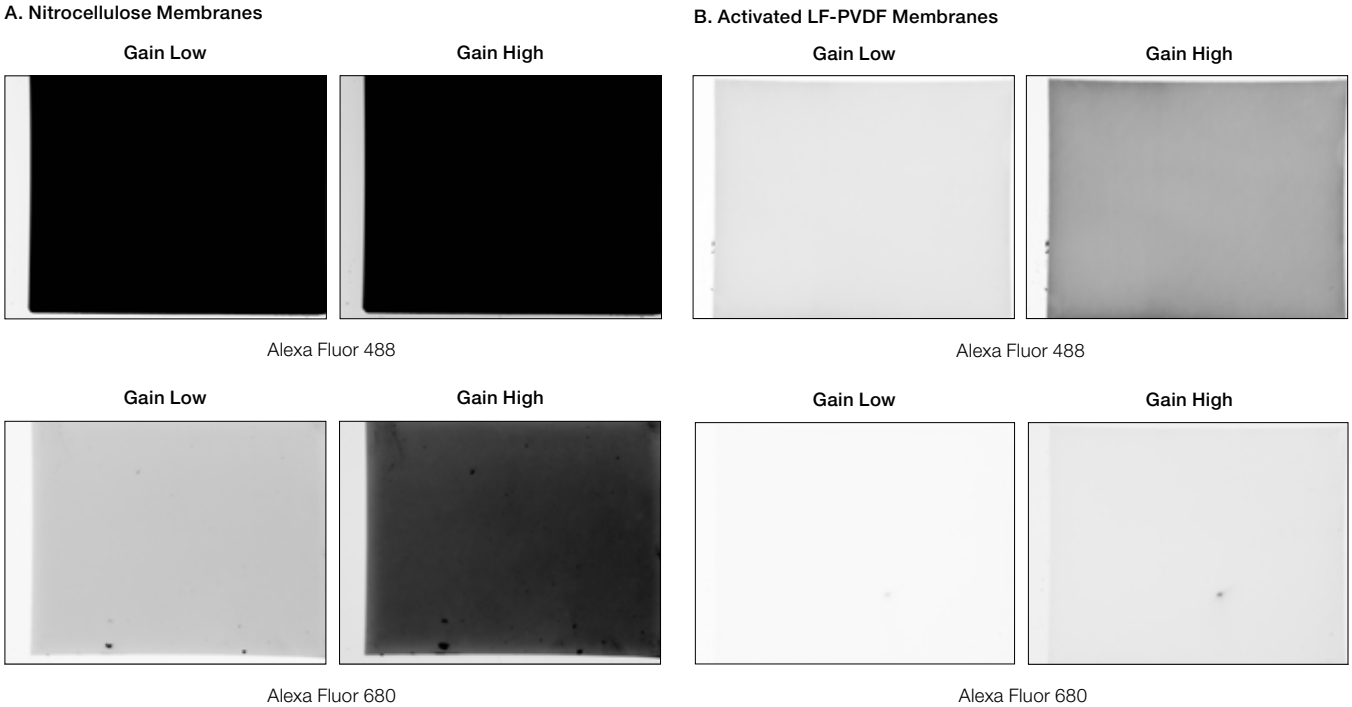

Fig. 2. Comparison of the autofluorescence of nitrocellulose and LF-PVDF membranes for fluorescence-based imaging. Images show autofluorescence of nitrocellulose membranes (**A**) and LF-PVDF membranes (**B**) in the visible (top) and infrared (bottom) spectra. **Note:** Low and high gain refers to the gain settings for the image as displayed in the Image Transform options of the ChemiDoc MP Imaging System. Images were captured at 30 sec, in accordance with timing required for routine imaging detection.

Importance of Blocking Buffers

Optimization of multiplex detection requires titrating the pERK and tERK antibody conditions in a variety of blocking buffers, including 5% milk diluted in a HEPES-buffered salt solution in H_2O, 5% casein blocker in PBS or TBS, LI-COR Odyssey buffer, Rockland fluorescence buffer, or 3–5% BSA diluted in PBS or TBS. Generally, TBS is useful when working with phosphoproteins and is recommended by Cell Signaling Technologies (purveyor of most phosphoprotein antibodies used in this study). The goal is to increase the sensitivity of the antibody for the protein of interest while achieving specificity of the signal.

As shown in Figure 3, the same sample probed with the same amount of primary antibody can vary widely in its pattern of detection depending on blocking and incubation conditions. This highlights that an antibody must be optimized on a case-by-case basis to achieve optimal results for imaging.

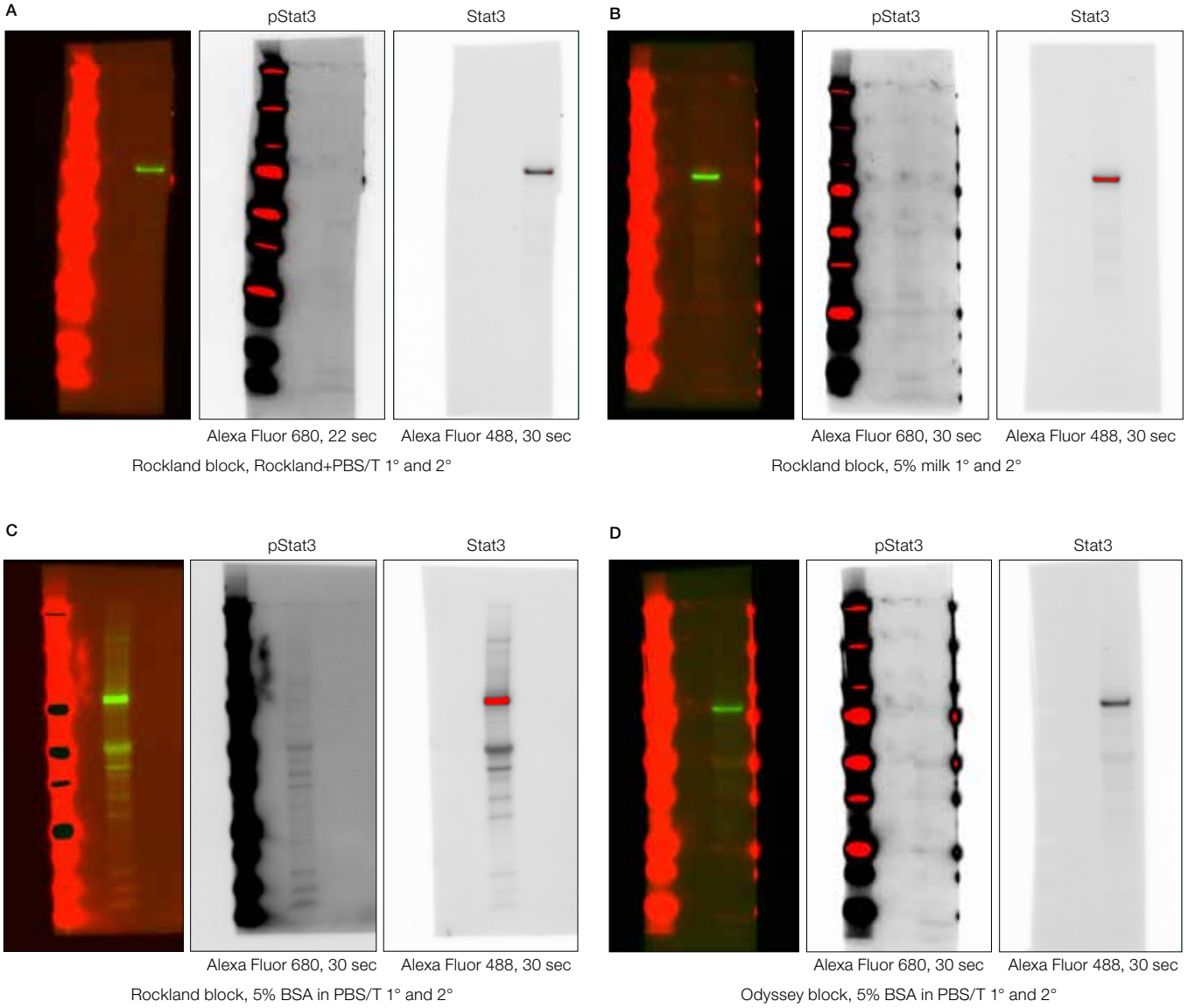

Fig. 3. Comparison of the effect of blocking conditions on multiplex fluorescent detection of pStat3 and Stat3. Alternating samples of the Precision Plus Protein Unstained Standards and 20 µg of brain lysate were subjected to SDS-PAGE, transferred to LF-PVDF, and cut into four separate membranes for blocking buffer optimization. The membranes were blocked in various buffers such as Rockland blocking buffer (**A**–**C**) or LI-COR Odyssey blocking buffer (**D**). After a 30 min incubation in blocking buffer, the membranes were incubated with rabbit anti-pStat3 and mouse anti-Stat3 antibodies (Cell Signaling Technology) diluted in various buffers, 1:1 dilution of Rockland and PBST (**A**), 5% nonfat milk in 20 mM HEPES and 150 mM NaCl w/0.1% Tween (**B**), or 5% BSA in PBS/T (**C** and **D**), and incubated overnight at 4°C with agitation. Approximately 18 hr later, the primary antibody solutions were discarded, the membranes were washed 3x for 5 min in either PBS/T (**A**, **C**, and **D**) or milk (**B**), and the primary antibodies were detected with Alexa Fluor 680 (pStat3, 1:4,000 dilution) or Alexa Fluor 488 (Stat3, 1:4,000 dilution) in the same diluent as the primary antibody. **Note:** pStat3 images were transformed to emphasize the faint banding pattern across conditions, while Stat3 images were not transformed and thus represent the entire dynamic range for that image capture. Additionally, Stat3 may be directly compared across the four conditions, as the exposure time for the Stat3 channel (Alexa Fluor 488) is identical for all four. Overall, transitioning from traditional film and chemiluminescence-based western blot detection to fluorescence-based detection will most likely require adjusting the protocol and titrating antibodies for the new method of detection. It is imperative to optimize the signal-to-noise ratio of the antibody, especially for fluorescence-based detection, in order to measure a clear signal for the protein of interest.

General Tips

1. Wear gloves at all times while handling membranes. Use freshly cleaned boxes for incubations (clean with ethanol and dH_2O prior to use). Keep blot boxes closed while incubating to avoid dust exposure. Cap all liquid reagents when not in use and filter solutions, if necessary, to remove particulates (might be helpful for milk-based blocking solutions).

2. Optimize blocking and primary antibody conditions. See Figure 3 for specifics. Membranes may be blocked for 30–60 minutes prior to adding the primary antibody. Generally, incubating primary antibodies overnight at 4°C will facilitate detection of the protein of interest. If necessary, store the antibody at 4°C and use within a few days. However, bacteria/mold might begin to grow in the antibody container, which could lead to an artifact signal on the blots. Ideally, use fresh antibodies for imaging.

3. Secondary antibody concentrations may also be optimized. For example, Stat3 can be detected when using Alexa Fluor 488 at 1:4,000 and 1:10,000 dilutions. The final concentration, again, will depend on the signal-to-noise ratio.

4. While exposing the blots, choose exposure times that are just prior to signal saturation. For example, although the ERK bands shown in Figure 1 were exposed for 30 seconds using the Autoexpose feature for Faint Bands, a longer exposure time of 360 seconds could be used to capture more information in the image. However, there may be changes to the densitometry measurements between the two exposure times or changes to the Image Transformation features. This could be empirically determined.

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