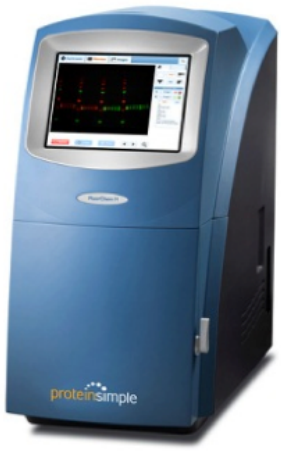
Identification of Co-migrating Proteins on the FluorChem M System

Introduction



Western blotting is an essential tool in protein research. Traditionally, proteins are labeled with secondary antibodies tagged with chemiluminescent substrates and are detected using photographic film. Chemiluminescent Western blotting has been a dominant method of detection for identification of proteins in cell or tissue samples for decades. While it offers significant advantages over other methods it also has certain limitations, one being the ability to analyze multiple proteins on the same blot.

Fluorescent imaging technology gives researchers the ability to move beyond the traditional Western blot assay by offering the flexibility to multiplex and detect co-migrating proteins. Native and phosphorylated isoforms can be probed simultaneously and quantified without the additional time and signal loss in sequential stripping and re-probing.

The technique involves the use of fluorescently-conjugated secondary antibodies to label the proteins of interest. Antibodies directly labeled with fluorophores such as Cy3, Cy5, and FITC have different fluorescent excitation and emission properties that allow for simultaneous detection using a digital imager. Unlike chemiluminescent detection, which involves an enzymatic reaction that decays with time, visible fluorescent signals are much more stable over time and can therefore be detected with high accuracy and linearity.

The FluorChem M system, equipped with advanced fluorescent imaging technology, allows researchers to analyze and detect multiple proteins simultaneously on the same blot (**Figure 1**).

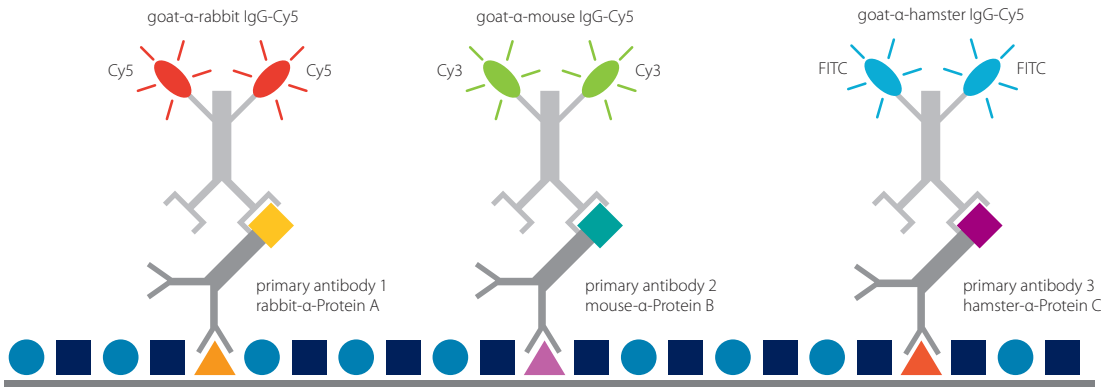


FIGURE 1. Multicolor fluorescent Western blot detection. Three primary antibodies are shown, binding to three independent antigens (triangles) on a membrane surface. Each primary antibody is tagged with a secondary antibody that has been directly conjugated to a fluorescent dye such as Cy5 (red), Cy3 (green), or FITC (blue) as shown.

In this application note, we detail the use of two different fluorophores, MultiFluor™ Green and MultiFluor Red, to analyze concentrations of ERK 1 and ERK 2 in HeLa lysates using the FluorChem M system. The ERK protein molecule and its phosphorylated pERK form were used as the co-migrating proteins of interest. We will also demonstrate the use of direct multicolor fluorescent detection to extend linearity and dynamic range – resulting in superior band clarity and proportional quantitative accuracy.

Materials

- **MultiFluor Western Blotting Kit:** 2-color Red (Goat Anti-Rabbit) and Green (Goat Anti-Mouse), P/N WB201. Includes fluorescently-conjugated secondary antibodies, PVDF membranes, blocking buffer and washing solution.
- **FluorChem M imaging system:** P/N 92-15312-00

Methods

Western Blot Preparation

HeLa cells were cultured under low and high ERK phosphorylation conditions. The lysates in RIPA buffer were denatured with heat and DTT, and then loaded with Laemmli buffer into a 10% SDS-PAGE gel along with a molecular weight standard. Lanes were loaded in this order from left to right: 5 µg high phosphorylation, 2 µg high phosphorylation, 2 µg mixture of high/low phosphorylation, 2 µg low phosphorylation, and 5 µg low phosphorylation. After separation, the proteins were transferred to a low-fluorescence PVDF membrane (included in the MultiFluor Western Blotting Kit) using a standard wet transfer protocol. Subsequent antibody incubations and washes were carried out according to the MultiFluor Western Blotting Kit protocol and detailed as follows.

Blocking

Added 20 mL of MultiFluor Blocking Solution to the Incubation bag. Blocked the membrane in MultiFluor Blocking Solution for 1 hour at room temperature (RT).

Primary Antibody Incubation & Primary Wash

Diluted the primary antibodies, anti-ERK1/2 and anti-pERK1/2 in 8 mL of MultiFluor Blocking Solution. Removed Blocking Solution from Incubation bag and added the diluted primary antibody solution into the Incubation bag to incubate for 60 minutes at RT with gentle rocking. After 60 minutes, the primary antibody solution was removed from the Incubation bag and replaced with 25 mL of 1X MultiFluor Antibody Wash Solution. The Incubation bag was placed on the rocker for 5 minutes. The Antibody Wash Solution was removed and the wash was repeated two more times.

Secondary Antibody Incubation & Secondary Wash

Removed the Antibody Wash Solution from the Incubation bag and replaced with 10 µL of MultiFluor Red and 10 µL of MultiFluor Green diluted in 10 mL of 1X MultiFluor Antibody Wash Solution. The blot was covered with a light shield and incubated for 60 minutes at RT with gentle rocking. After 60 minutes, the secondary antibody solution was removed from the Incubation bag and replaced with 25 mL of 1X MultiFluor Antibody Wash Solution. The Incubation bag was placed on the rocker for 5 minutes. The Antibody Wash Solution was removed and the wash was repeated four more times.

Final Wash

Removed the Antibody Wash Solution from the Incubation bag and added 25 mL of 1X MultiFluor Final Wash Solution. The bag was incubated on the rocker for 5 minutes, covered from light. The wash step was repeated once more. After the wash, the blot was removed from the Incubation bag and placed on a blotting paper. The resulting Western blot was dried for 5 minutes before imaging with the FluorChem M system.

To assess the linearity and dynamic range of fluorescent detection, a separate Western blot was prepared using serial dilutions of human Transferrin (TF). TF was diluted two-fold from 1.0 µg to 0.5 pg and was tagged with the MultiFluor Green secondary antibody. Serial dilutions of TF (1.0 µg to 0.5 pg) were transferred to a low fluorescent PVDF membrane and processed under standard Western blotting protocol.

Image Acquisition

The multiplex Western blot was imaged with the FluorChem M system using the standard MultiFluor Red Green protocol which acquires data on these two channels sequentially at standard resolution. ERK1/2, probed with MultiFluor Green, was detected with the Green epi-excitation channel using the Red filter. pERK 1/2, probed with MultiFluor Red, was detected with the Red epi-excitation channel using the Far Red filter. The exposure time was automatically calculated on the FluorChem M system for optimal image acquisition without saturation. Data for each channel were automatically overlaid on-screen.

Transferrin was detected on the FluorChem M system using the same MultiFluor Green filter as described previously. Exposure time was automatically calculated using the auto-exposure setting. A log plot was generated from the serial dilutions of TF to evaluate dynamic range and linearity.

Results

To demonstrate the capability of the FluorChem M system to resolve each secondary antibody labeled with either a green or red fluorophore, the bands captured for the native and phosphorylated ERK proteins are shown in **Figure 2**.

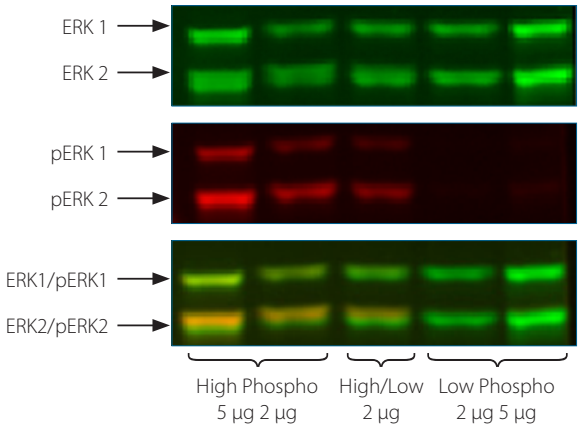


FIGURE 2. HeLa lysates with anti-ERK1/2 and anti-pERK1/2 primary antibodies followed by MultiFluor Green and MultiFluor Red secondary antibodies. The independent images for each fluorescent channel were obtained sequentially and a composite image was generated by overlaying the individual images. (Top) ERK1/2 labeled with MultiFluor Green; green color shown. (Middle) pERK1/2 labeled with MultiFluor Red; red color shown. (Bottom) Overlaid image of ERK1/2 and pERK1/2.

ERK 1/2 bands are present in all lanes, with a weaker signal as expected with a lower amount of protein loaded. pERK 1/2 bands are present only in the lanes from samples from either high phosphorylation or the mixture of high and low phosphorylation.

Linearity and dynamic range were assessed using serial dilutions (1.0 µg to 0.5 pg) of purified human Transferrin (TF). We found that the linear range was above 3 orders of magnitude with a 0.5 pg limit of detection. The dynamic range of detection was approximately 5 logs (**Figure 3**).

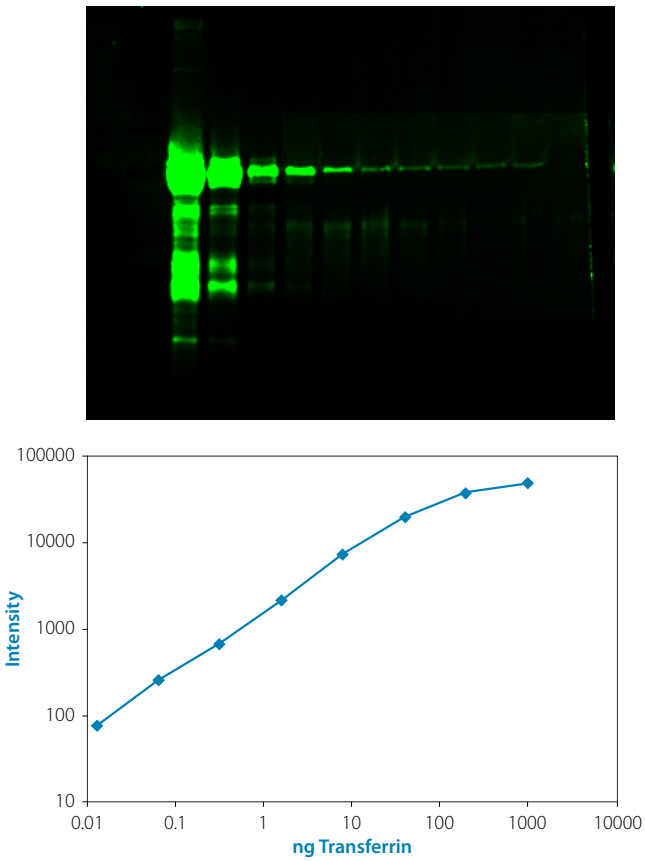


FIGURE 3. (Top) Serial dilutions of TF from 1.0 µg to 0.5 pg were detected by Western blot using MultiFluor Green secondary antibody. (Bottom) Log plot of serial dilutions of TF on Western blot detected by MultiFluor Green secondary antibody.

Conclusion

Western blotting has proven to be an indispensable tool in protein research. While chemiluminescent detection offers excellent sensitivity, it lacks the ability to multiplex without the need to strip and re-probe. Advancements in Western blotting techniques have allowed researchers to accurately measure co-migrating proteins with the use of fluorophores to analyze multiple proteins simultaneously without the additional assay time and signal loss. The FluorChem M imaging system offers the ability to detect and analyze multiple targets that overlap on the same blot. Co-migrating proteins, such as native and phosphorylated isoforms, can be probed with fluorescently conjugated secondary antibodies to detect subtle mobility shifts. The data described shows multiplex detection of the ERK and pERK proteins labeled with the MultiFluor secondary antibodies, demonstrating the ability of the FluorChem M system to resolve co-migrating proteins on separate channels. The ability to detect multiple proteins simultaneously allows researchers to save time and precious samples. The need to strip and re-probe is eliminated, resulting in higher data integrity and shorter assay times. Fluorescent imaging with the FluorChem M system provides excellent linearity and dynamic range. This was demonstrated using serial dilutions of human TF tagged with MultiFluor Green. The quantitative linear range was above 3 orders of magnitude with an LOD of 0.5 pg. The dynamic range of human TF was approximately 5 logs.

In summary, the FluorChem M imaging system is a complete solution to multiplex the traditional Western blotting technique. This is particularly useful for analysis of co-migrating proteins where there are both high and low concentrations of proteins present in one sample.

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Toll-free: (888) 607-9692
Tel: (408) 510-5500
Fax: (408) 510-5599
orders@proteinsimple.com
proteinsimple.com

95-4000-00 / Rev A.