Enhanced Multiplex Fluorescent Western Blotting

David P. Chimento[1], Angela M. Zaorski[1], Kevin McDonald[2], Charles Martin[2], and Carl A. Ascoli[1], [1]Rockland Immunochemicals, Inc., PO Box 326, Gilbertsville, PA 19525 USA; [2]Bio-Rad Laboratories, Inc., Hercules, CA 94547 USA

Introduction

Fluorescence-based detection is becoming the method of choice to detect proteins on western blots. Because fluorescent probes with different specific spectral ranges are available, this approach can provide multiplex analyses and greater signal stability than chemiluminescent detection. Additional advantages are that simultaneous probing reduces the time and labor required for western blot processing and, because only one blot has to be prepared, less starting sample is required (Gingrich et al. 2000). The combination of antibodies selected for minimal cross-reactivity and fluorescent detection methods enables quantitative analysis of two or more proteins on a single blot.

Rockland Immunochemicals and Bio-Rad Laboratories jointly developed an enhanced multiplex fluorescent western blotting protocol for producing immunoblots on PVDF membranes. The membrane is probed with specific primary antibodies and DyLight fluorochrome–conjugated secondary antibodies developed by Rockland Immunochemicals. Optimized detection is achieved using the Molecular Imager® VersaDoc™ MP 4000 imaging system and Quantity One® 1-D analysis software developed by Bio-Rad Laboratories.

In this application note, we describe two separate multiprotein detection experiments that each used a single blot and a fluorescence-based detection system. The first experiment involved the simultaneous detection of several human serum proteins, and the second experiment involved the simultaneous detection of resting and activated Akt1.

Methods

PAGE of the maltose binding protein (MBP) was performed on a 4–20% gradient Criterion™ gel. The gel was transferred onto PVDF membrane using a Criterion blotter (Bio-Rad). Membranes were blocked in 5% normal goat serum (NGS) in Tris-buffered saline (TBS) and probed with rabbit anti-MBP antibody overnight at 4°C, followed by DyLight 649 conjugated secondary antibody (Rockland Immunochemicals) at a concentration of 1:10,000 (100 ng/ml) for 1 hr at room temperature. The membranes were rinsed in deionized water, soaked in methanol for 2 min, and allowed to air-dry in the dark prior to data collection.

In the western blot assay for Akt1, 10 µg of NIH/3T3 cell lysate from untreated or PDGF (platelet-derived growth factor) stimulated cells was loaded per lane.

For serum and NIH/3T3 cell lysates, PAGE was performed at 150 V for 1 hr on 4–20% gradient Criterion gels using the Laemmli buffer system. Precision Plus Protein™ WesternC™ molecular weight standards were diluted 1:25 in sample buffer, and 5 µL of diluted marker was loaded per lane. After electrophoresis, the gels were rinsed in Tris/glycine/methanol transfer buffer for 5 min. Prior to transfer, the PVDF membranes were prewetted in 100% methanol followed by incubation in transfer buffer for 10 min. Proteins were transferred from the gel onto a PVDF membrane using the Criterion plate transfer cell at 100 V for 30 min. Membranes were blocked in nonfat dry milk (BLOTTO, Immunoanalytical Grade, Rockland Immunochemicals) for 1 hr. Primary and secondary antibodies were from Rockland Immunochemicals. Primary antibodies anti–glutathione-S-transferase (GST) (mouse host), anti-transferrin (rabbit host), and anti–α1-anti-trypsin (goat host) were diluted to 1 µg/mL in blocking buffer for fluorescent western blotting. The blocked membranes were incubated with a cocktail of primary antibodies overnight at 4°C. After incubation, the membrane was washed in Tris-Tween buffered saline (TTBS). Secondary antibodies DyLight 488 conjugated anti-MOUSE IgG, DyLight 549 conjugated anti-RABBIT IgG, and DyLight 649 conjugated anti-GOAT IgG (all raised in donkey) were diluted at 1:20,000 in blocking buffer for fluorescent western blotting and incubated with the membrane in opaque incubation trays at room temperature for 1 hr. The membrane was washed 4 times in TTBS (5 min/wash) and rinsed 2 times in TBS. Prior to drying, the membranes were plunged into distilled water and soaked in methanol for 2 min. The membranes were then dried on filter paper for 2–3 min. Fluorescent images were collected using the VersaDoc MP 4000 imaging system (Bio-Rad). Data for each DyLight fluorophore were collected independently at the excitation wavelength of blue for the DyLight 488, of green for the DyLight 549, and of red for DyLight 649 (Table 1).

Integration times of 1–2 min were sufficient to produce high quality images. Each assay was run a minimum of three times.

Results

Table 1. Fluorescent detection settings for the VersaDoc MP 4000 imaging system.

Fluorophore	Excitation (nm)	Emission Filter (bandpass)
DyLight 488 (blue)	470	530
DyLight 549 (green)	530	605
DyLight 649 (red)	625	695

Linearity and Limit of Detection in Dot Blot Format

To determine the limit of detection (LOD), a twofold serial dilution of MBP was made from 32–0.002 ng. We first performed a dot blot by directly spotting protein onto the membrane, which eliminates variability due to transfer of the protein from a gel. Samples (2 µl each) from a twofold serial dilution of MBP were spotted onto a nitrocellulose membrane, and the blot was processed as described in the Methods section. Fluorescence data were collected using the VersaDoc MP 4000 imaging system with instrument settings at 1 x 1 bin, 1x gain with an exposure time of 180 sec, using the red LED and the BP695 emission filter. Figure 1A shows that the LOD for MBP directly spotted on the membrane was 2 pg. Figure 1B demonstrates a linear range for quantitation of 500–2 pg; the square of the correlation coefficient R^2 was 0.995, indicating a good quality linear fit.

LOD on Western Blot

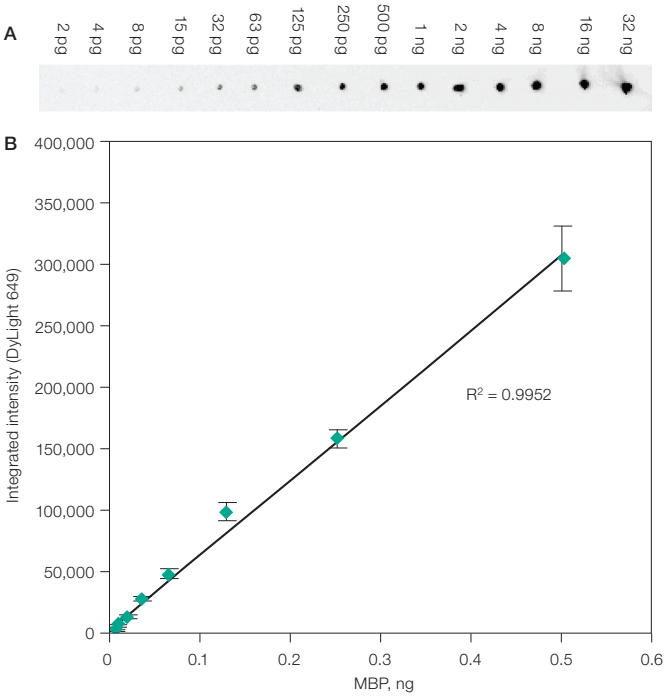

Fig. 1. Linearity and reproducibility of fluorescent detection in dot blot format. A, blot containing spots of 32 ng to 2 pg of MBP protein was probed with anti-MBP antibodies and revealed with DyLight 649 anti-rabbit antibodies. **B**, integrated intensity of fluorescence vs. quantity of protein plotted on a linear scale.

To determine the LOD and linear detection range in a western blot experiment, we probed blots containing the serial dilution of MPB as described. Quantification of the fluorescence data with Quantity One software showed a linear range of 16–0.25 ng (Figure 2).

Multiplex Detection of Serum Proteins

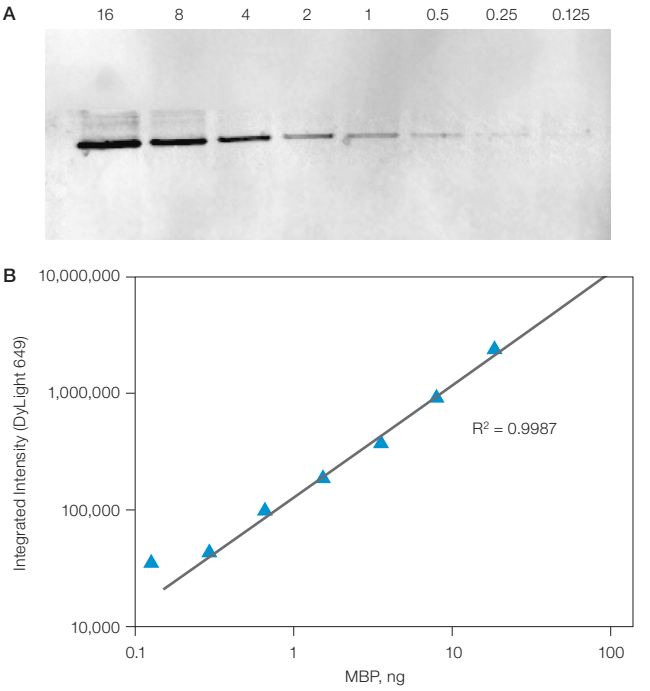

Fig. 2. LOD on western blot using DyLight 649. A, serial dilutions of MBP were loaded onto an SDS-polyacrylamide gel. The blot was probed with anti-MBP antibodies and secondary DyLight 649 anti-rabbit antibodies. **B**, linearity of fluorescence signal detection is observed from 16 ng to 250 pg.

Multiplex detection of proteins on a western blot was tested using human serum depleted of albumin. Anti-transferrin and anti–α1-anti-trypsin antibodies were used for this study because the apparent molecular weights for their targets are well separated by conventional SDS-PAGE techniques.

Human serum contains approximately 2.5 mg/ml transferrin, which accounts for about 4% of the protein content in serum. Based on these values, we estimated the transferrin load in this assay to be 5.0–10 µg. Under normal physiological conditions, serum transferrin is about 30% saturated with iron, which affects transferrin migration in SDS-PAGE. Human serum contains roughly 1.3 mg/mL of α1-anti-trypsin, comprising 1–2% of the total amount of protein in serum. Similarly, we estimated that 2.5–5.0 µg of α1-anti-trypsin was loaded on the gel for this assay. We added 80 ng of purified GST as a loading control to the albumin-depleted human serum sample prior to loading.

The membrane was probed simultaneously with antibodies specific for transferrin, for α1-anti-trypsin, and for GST. As shown in Figure 3, we were able to simultaneously detect each of the three components and visually identify each

antigen by assigning a pseudo color to each fluorochrome. For transferrin, a dominant band was observed at about 80 kD. Although α1-anti-trypsin is reported to be 52 kD in size, its apparent migration was closer to 56 kD in this system. GST, which is typically a 28 kD protein, was detected as a doublet at 26 and 28 kD, suggesting a slight breakdown of the protein. This image demonstrates both the extremely low background staining and the high sensitivity and quality of the image. These enhanced attributes are a result of the quality of the reagents, primary antibody specificity, DyLight secondary antibody signal intensity, and the overall performance of the VersaDoc MP 4000 imaging system.

Multiplex Detection of Resting and Activated Forms of Akt

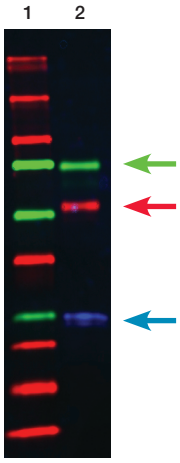

Fig. 3. Multiplex fluorescent western blot of human serum samples. Lane 1, Precision Plus Protein WesternC molecular weight standards. **Lane 2**, 30 µg albumin-depleted human serum spiked with GST. Each protein was detected with host specific DyLight conjugated antibodies; arrows indicate transferrin (green), α1-anti-trypsin (red), and GST (blue).

In the second experiment, we probed for the serine/threonine kinase Akt1 signaling molecule, which is important in the response of eukaryotic cells to growth factor stimulation. Akt1 regulates cellular survival and metabolism by binding and regulating several downstream effectors, proteins such as NF-κB, the Bcl-2 family, and murine double minute 2 (MDM2) (Song et al. 2005). Akt1 has also been implicated in tumor development. The clarification of its regulation by upstream kinases and phosphatases is an active area of study. Stimulation of receptor tyrosine kinase by, notably, epidermal growth factor (EGF), PDGF, and insulin-like growth factor (IGF), has been shown to lead to Akt1 activation (Franke et al. 1997, Franke et al. 1995). Cells stimulated with these growth factors activate Akt through site-specific phosphorylation of Thr-308 and Ser-473. We examined Akt1 regulation by performing a western blot probing for the phosphorylation status of Akt1 in differentially stimulated cells.

Figure 4 illustrates multiplex western blot detection of both the activated (phosphorylated) and inactive (unphosphorylated) Akt1. By using a pan-reactive rabbit polyclonal anti-Akt1 and phosphospecific mouse monoclonal anti-Akt1-pThr473, we demonstrated that both forms of the protein were detected simultaneously. In lane 1, unstimulated NIH/3T3 cells were probed with both a pan-reactive polyclonal Akt and pS473 specific monoclonal antibody. In this sample, Akt1 is present in an unphosphorylated or inactive state, which was detected by the pan-reactive antibody (green band). In lane 2, PDGF-stimulated NIH/3T3 cells probed with both Akt1 antibodies show that Akt1 is present in both the inactive unphosphorylated state and the active phosphorylated state. This is shown by the detection of unphosphorylated Akt1 by the pan-reactive antibody (green band) and by the detection of phosphorylated Akt1 by the phosphospecific antibody (red band). Phosphorylated Akt1 migrates slightly more slowly than the unphosphorylated Akt1 due to charge differences between the two molecules. The epitopes for pan-reactive and pS473 specific anti-Akt1 antibodies are distinct. This image confirms the sensitivity and specificity of all reagents in detecting specific activated states of Akt1.

Conclusions

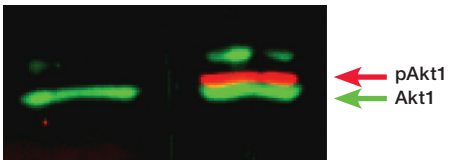

Fig. 4. Simulaneous detection of unphosphorylated and phosphoryated Akt1 in NIH/3T3 cell lysates. The blot was probed with rabbit anti-Akt1 (pan-reactive) and mouse anti-Akt1 pS473 specific antibodies followed by detection with DyLight 549 conjugated anti-rabbit IgG (green) and DyLight 649 conjugated anti-mouse IgG (red) secondary antibodies. **Lane 1**, unstimulated NIH/3T3 lysates. **Lane 2**, PDGF-stimulated NIH/3T3 lysate. pAkt1, phosphorylated Akt1.

The equipment and reagents used in this work allowed multiplex detection of three targets simultaneously in a western blotting system with enhanced sensitivity and specificity as shown by very low background staining, high signal-to-noise ratios, and differentiation between the active and inactive states of a key cell-signaling-pathway protein. Distinct colors and robust signal were seen in the detection of serum samples using normal conditions for the detection of nanogram to picogram amounts of protein. Multiplex blotting can identify the protein status or posttranslational modification of proteins in a cellular context. The multicolor visualization presented here demonstrates that clear, easy to interpret results can be produced in a straightforward process for the identification of individual protein components in a complex sample.

References

Franke TF et al. (1997). Pi3k: Downstream aktion blocks apoptosis. Cell 88, 435-437.

Franke TF et al. (1995). The protein kinase encoded by the akt proto-oncogene is a target of the pdgf-activated phosphatidylinositol 3-kinase. Cell 81, 727-736.

Gingrich JC et al. (2000). Multiplex detection and quantitation of proteins on western blots using fluorescent probes. Biotechniques 29, 636-642.

Song G et al. (2005). The activation of akt/pkb signaling pathway and cell survival. J Cell Mol Med 9, 59-71.

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