Enhanced Multiplex Western Blotting

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ABSTRACT

Rockland Immunochemicals and Bio-Rad Laboratories have jointly developed an enhanced multiplex fluorescent western blotting protocol for both the production of an immunoblot on PVDF membrane and determining the limit of fluorescent detection. 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 with additional analysis facilitated by using Quantity One® 1-D software developed by Bio-Rad Laboratories. We highlight two examples of the simultaneous detection of several human serum proteins, and the simultaneous detection of resting Akt1 and activated phosphorylated Akt1. Each blot was reacted with specific primary antibodies reactive with target proteins. The blots were probed simultaneously with two or three different DyLight™ conjugated secondary antibodies specific for primary antibodies each raised in a unique host animal (mouse, rabbit and goat). The resulting blots show enhanced detection of distinct target bands labeled by a unique identifying color.

INTRODUCTION

A typical western blot allows for detection of a single protein using a horseradish peroxidase or alkaline phosphatase conjugated antibody with a chemiluminescent substrate. In this application note, we present two separate multi-protein detection experiments that use a single blot and a fluorescent based detection system.

Human serum is a complex mixture of proteins including transthyretin, albumin, α-1 globulins (α-1 antitrypsin, α-1 glycoprotein, high density lipoprotein (HDL)), α-2 globulins (α-2 macroglobulin, antithrombin III, ceruloplasmin, haptoglobin), beta globulins (beta and prebeta lipoproteins LDL and VLDL), C3, C-reactive protein, hemoglobin, plasminogen, transferrin, gamma globulins and immunoglobulins [1]. For the first assay normal human serum was depleted of albumin (details on the albumin depletion assay are available). While several well suited antibodies exist in Rockland Immunochemicals' portfolio to study human serum proteins, we chose anti-transferrin and anti-α1-anti-trypsin for this study because the apparent molecular weights for these 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 and is the fourth most abundant plasma protein. Based on these values, we estimate the transferrin load in this assay to be approximately 0.5 – 1.0 µg. Note that under normal physiological conditions, serum transferrin is about 30% saturated with iron which affects its migration in SDS-PAGE. On average human serum also contains approximately 1.3 mg/mL α1-anti-trypsin which accounts for 1-2 % of the total amount of protein in serum. Similarly, by comparison, we estimate approximately 0.25 – 0.5 µg of α1-anti-trypsin in this assay. As a loading control we added exogenous glutathione-Stransferase (GST) to the sample as a third target protein. We were able to simultaneously detect each of the three components and visually identify each antigen by assigning a pseudo-color to each fluorochrome.

In a companion experiment we probed for the serine/threonine kinase Akt signaling molecule which is an important player in response to growth factor stimulation in eukaryotic cells. Akt regulates cellular survival and metabolism by binding and regulating several downstream effectors proteins such as NF-κB, the Bcl-2 family of proteins and murine double minute 2 (MDM2) [2] . Akt1 has also been implicated in tumor development. The clarification of its regulation by upstream kinases and phosphatases is an active area of study. Receptor tyrosine kinase stimulation, notably by epidermal growth factor (EGF), platelet derived growth factor (PDGF) and insulin-like growth factor (IGF) has been shown to lead to Akt activation [3,4]. Cells stimulated with these growth factors activate Akt through site specific phosphorylation of Thr-308 and Ser-473. In the second experiment presented here we examined Akt1 regulation by performing a Western blot probing for the phosphorylation status of Akt in differentially stimulated cells

METHODS

LOD Study: A 2-fold serial dilution of maltose binding protein (MBP) was made from 32 to 0.0012 ng to determine the limit of detection (LOD), which was subsequently loaded onto a 4 -20 % gradient Criterion™ PAGE gel for separation. Blots were blocked in 5% normal goat serum (NGS) in tris-buffered saline (TBS). Blots were treated with rabbit anti-MBP antibody overnight at 4 °C, followed by secondary DyLight™ 649 conjugated antibody at 1:10,000 (100ng/ml) for 1 h at RT. The membrane was rinsed in deionized water and was then soaked in methanol for 2 min and allowed to air dry in the dark prior to data collection.

Serum Multiplex Assay: In the human serum western blot assay, approximately 30 µg of albumin-depleted human serum was spiked with 80 ng of purified glutathione-S-transferase (GST). In the western blot assay for Akt1, 10 µg of NIH/3T3 cell lysate from untreated or PDGF stimulated cells was loaded per lane. All samples were loaded onto Criterion™ 4-20% gradient SDS-PAGE gels for separation. Precision Plus Protein™ WesternC™ fluorescent molecular weight standard was diluted 1:25 in sample buffer, and 5 µL of diluted marker was loaded per lane. Electrophoresis was run at 150 V for 1 h using the Laemmli buffer system. Proteins were transferred from the gel onto the PVDF membrane using the Criterion™ plate transfer cell at 100V for 30 min. Membranes were blocked in BLOTTO for 1 h.

Antibody conditions: anti-GST (mouse host), anti-transferrin (rabbit host), and anti-α1-antitrypsin (goat host), were diluted to 1 µg/mL in Blocking Buffer for Fluorescent Western Blotting. The blocked membrane was incubated with a cocktail of primary antibodies overnight at 4 °C. 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 1:20,000 in Blocking Buffer for Fluorescent Western Blotting and incubated with the membrane in opaque incubation trays at room temperature for 1 h. Prior to drying, the membranes were plunged into distilled water and soaked in methanol for 2 min. Fluorescent images were collected in the VersaDoc™ MP 4000 molecular imaging system according to the manufacturer's instructions. Data for each DyLight™ fluorophore were collected independently at the following excitation wavelengths; blue for the DyLight™ 488, green for the DyLight™ 549, or red for DyLight™ 649 (Table 1). Integration times of about 1-2 min were sufficient to produce bright, high-quality images. Each assay was run a minimum of 3 times.

LINEARITY & LOD IN DOT BLOT

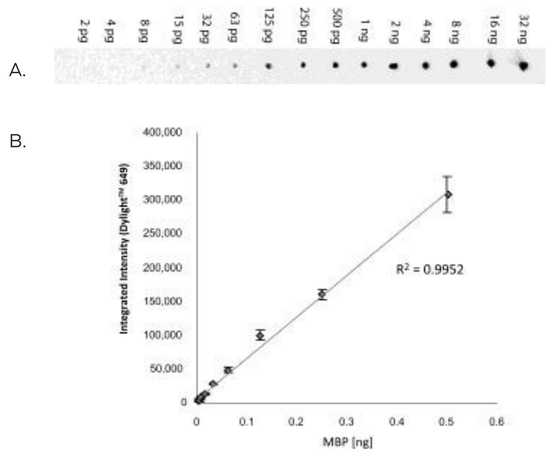


Figure 1 Linearity and reproducibility of fluorescent detection in dot blot format. A, Image of a MBP serial dilution 32 ng to 2 pg. Blots were detected with anti-MBP and probed with DyLight™649 anti-rabbit. The bar indicates linear range B. Integrated intensity of fluorescent counts plotted on a linear scale.

LINEARITY & LOD IN WESTERN BLOT

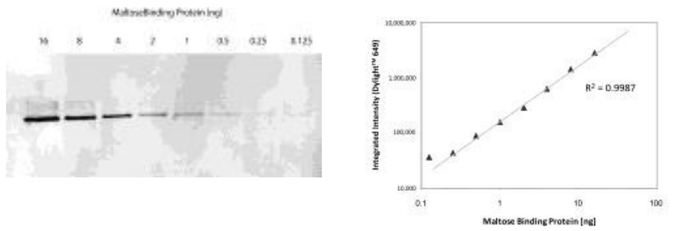


Figure 2. Limit of detection in western blot using DyLight™649. 16 ng of MBP were loaded onto SDS-PAGE as a 2-fold serial dilution. The blot was blocked with Blocking Buffer for Fluorescent Western Blotting followed by reaction with anti-MBP and secondary DyLight™ 649 anti-rabbit antibody. B. Linearity of detection observed from 16 ng to 250 pg.

DATA COLLECTION PARAMETERS

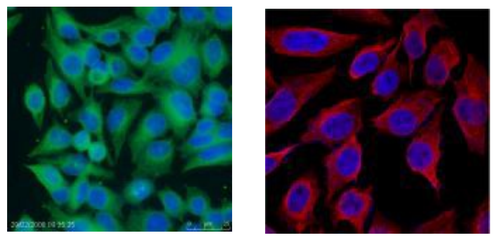
Table 1. Fluorescent detection settings for VersaDoc™ MP 4000

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

The protocol for enhanced multiplex Western blotting is summarized as follows:

- Perform SDS-PAGE and western blot transfer
- Block PVDF membrane and probe with Rockland's unique host primary antibodies
- Detect using DyLight™ conjugated secondary antibodies
- Excite and read fluorescent blot using VersaDoc™ MP 4000 molecular imaging system
- Analyze the data with Quantity One® software

LOW BACKGROUND STAINING OF DYLIGHT™ CONFIRMED BY IF



Dylight™ conjugated antibodies confer very low background when used in immunofluorescence. Hep2 cells stained with Rb-α-Tubulin diluted 1:2000 followed by DyLight™488 or DyLight™549 Gt-α-Rb IgG MX10 diluted 1:10,000. Counterstain was with bis-benzimide at 0.05 ug/ml. Data collected on Leica DMI6000B microscope. Left Dylight™488 is presented as blue staining; right Dylight™549 is presented as red staining.

MULTIPLEX WB OF HUMAN SERUM PROTEINS

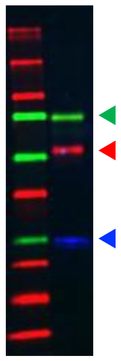


Figure 3. The enhanced multiplex fluorescent western blot shows staining of three distinct proteins present in human serum samples. Primary antibodies were incubated together in a single experiment. Note the extremely low background staining and high signal-to-noise for each band. The gel was loaded as follows: Lane 1 Precision Plus Protein™ WesternC™ molecular weight standards, Lane 2 - human serum spiked with GST. Each protein was detected with host specific DyLight™ conjugated antibodies. The arrows indicate transferrin (green), α1-anti-trypsin (red) and glutathione-S-transferase (blue), respectively.

SIMULTANEOUS DETECTION OF ACTIVATED AKT

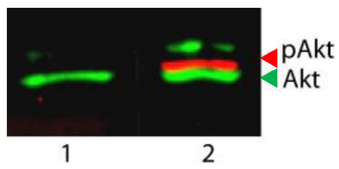


Figure 4. This fluorescent western blot shows simultaneous detection of unphosphorylated and phosphorylated Akt1 present in serum starved and PDGF stimulated NIH/3T3 whole cell lysates. Lane 1, unstimulated NIH/3T3 lysates contain inactive unphosphorylated Akt1, green band. Lane 2, PDGF stimulated NIH/3T3 lysate contains both inactive (green band) and activated phosphorylated Akt1 (red band). Both lanes were probed with rabbit anti-Akt (pan) and mouse anti-Akt pS473 specific antibodies followed by detection with DyLight™ 549 conjugated anti-rabbit IgG (green) and DyLight™ 649 conjugated anti-mouse IgG (red) secondary antibodies.

SUMMARY

The equipment, reagents and methods stated above enable multiplex detection of three targets simultaneously in a western blotting system with enhanced sensitivity and specificity as evidenced by very low background staining, high signal-to-noise ratios and differentiation between the active and inactive state of a key cell signaling pathway target. Distinct colors and robust signal result for the detection of serum samples using normal conditions for the detection of nanogram to picogram amounts of protein. Multiplex blotting can identify protein status or post-translational modification of proteins in a cellular context. The multi-color visualization presented here demonstrates how to produce clear and easily interpretable results, allowing a straightforward process for the identification of individual protein components in a complex sample. We additionally show low background immunofluorescence imaging using Dylight™ antibodies.

DYLIGHT FLUOROCHROME OPTICAL PROPERTIES

	Emission	Fluor	Ex/Em+	See Spectra	Spectrally Similar Dyes
	Blue	DyLight 405	400/420		Alexa™405, Cascade Blue
	Green	DyLight 488	493/518		Alexa™488, fluorescein, FITC
	Yellow	DyLight 549	562/578		Alexa™546, Alexa™555, Cy3,TRITC
	Red	DyLight 649	654/673		Alexa™647, Cy5
	Near IR	DyLight 680	692/712		Alexa™680, Cy5.5
	Infrared	DyLight 800	777/794		IRDye® 800

Left - Excitation (black) and emission spectra (color) of DyLight fluorescent dyes conjugated to affinity-purified secondary antibodies. This figure illustrates the relative shape and position of each fluorophore in the peak region of its excitation and emission following conjugation to antibodies.

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