Quantitative, Two-Color Western Blot Detection With Infrared Fluorescence

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Accurate and reproducible quantification of proteins on Western blots across a wide linear range is desirable, but difficult to achieve. Chemiluminescent detection is subject to enzyme/substrate kinetics that may affect performance, and use of antibodies labeled with visible fluorophores has not provided the necessary sensitivity for practical use. In this study, we investigated the use of antibodies directly labeled with near-infrared (IR) dyes for Western blot detection. Sensitive detection was achieved, presumably due to very low autofluorescence of membranes in the IR range compared to the visible light range, with 200-fold greater sensitivity than previous studies with visible fluorophores and greater sensitivity than chemiluminescence. We compared the linear range of IR detection to chemiluminescence in a dot blot assay, and found that IR fluorescence provided a 16-250 fold wider quantifiable linear range with increased reproducibility. Finally, we have demonstrated the use of two different IR fluorophores for simultaneous, multiplexed detection of two proteins at endogenous levels in whole cell lysates.

INTRODUCTION

Western blotting is a critical tool in protein analysis. Enhanced chemiluminescence has become the dominant technique for detection of proteins in Western format [1, 2, 3]. This technique uses an enzyme-conjugated antibody to cleave a chemiluminescent substrate and is usually detected with X-ray film or a CCD camera. Although it offers excellent sensitivity, this technique has limitations. Biological research is increasingly moving toward multiplexed analysis, but chemiluminescence is a one-color method that detects a single protein target and does not facilitate normalization or comparative analysis. Such studies generally require stripping and reprobing of blots [4] or comparison of separate blots. In addition, quantification with chemiluminescence could be compromised by the enzyme/substrate kinetics inherent to the process.

In recent years, studies have described the use of fluorescently-labeled antibody probes for Western detection, using fluorophores such as Cy®3, Cy®5, fluorescein, and the phycobilisome PBXL™-1 [5, 6, 7, 8]. Antibodies directly conjugated with visible fluorophores offer the potential for improved linearity and accuracy of quantification because no enzymatic amplification is involved. Some multiplexing has been performed with direct fluorescence [7]. However, these methods have not provided sufficient sensitivity to be practical for detection of endogenous protein levels, likely because the membranes have strong autofluorescence in the visible light region that can severely affect signal-to-noise.

Membranes and biomolecules have much lower autofluorescence in the longer-wavelength near-infrared (IR) region of the spectrum compared to visible wavelengths [9, 10]. The resulting low background should translate into greatly enhanced sensitivity if detection is performed in the IR range. Antibodies directly conjugated with IR fluorophores such as IRDye™ 800 (excita-

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tion 778 nm, emission 806 nm), IRDye™ 800CW (ex. 778, em. 806), Alexa Fluor® 680 (ex. 679, em. 702), and Cy®5.5 (ex. 675, em. 694) are available commercially. Because these dyes fall into two classes (approximately 700 nm and 800 nm emission, with 100 nm separation) they can be used simultaneously for multiplexed analysis of two targets.

In this report we describe the use of two IR fluorophores, IRDye 800 and Alexa Fluor 680, for detection of proteins in Western blot format. We compared the sensitivity and quantitative linear range of IR detection to conventional chemiluminescent detection. The use of two IR fluorophores for multiplex analysis of endogenous proteins in cell lysates was also demonstrated.

MATERIALS AND METHODS

Antibodies and Proteins

For transferrin (Tf) sensitivity experiments, rabbit anti-Tf was obtained from DAKO (Carpinteria, CA) and human apo-Tf from Calbiochem (San Diego, CA). The mouse monoclonal against the myc epitope tag used for the dot blot assay was obtained from Upstate Biotechnology (Lake Placid, NY). Two-color Westerns were performed with mouse anti-Syk and rabbit anti-ERK (Santa Cruz Biotechnology, Santa Cruz, CA). For detection, goat anti-mouse Alexa Fluor 680 (highly cross-adsorbed) was obtained from Molecular Probes (Eugene, OR), goat anti-mouse HRP was from Molecular Probes or Amersham Biosciences (Piscataway, NJ), and goat anti-rabbit IRDye 800 (highly cross-adsorbed) was from Rockland Immunochemicals (Gilbertsville, PA).

Cell extracts were prepared from cultured A431 cells (ATCC; Manassus, VA). Protein samples were electrophoresed on Tris-HCl Ready Gels® (Bio-Rad, Hercules, CA) and transferred to pure nitrocellulose (Osmonics, Minnetonka, MN) or PVDF membranes (Millipore, Bedford, MA). Odyssey® molecular weight marker (LI-COR Biosciences, Lincoln, NE) was used as a size standard.

Dot Blot Linearity Assay

Anti-myc mouse antibody was diluted in PBS + 0.05% Tween®-20 to 2.5 µg/µl. Two-fold serial dilutions were made, down to 0.3 pg/µl. Aliquots of 2 µl were spotted onto forty nitrocellulose membranes and allowed to dry. For each detection method, six membranes were processed. Membranes were blocked for 1 hr with Odyssey blocker (LI-COR Biosciences). Before all HRP antibody incubations, membranes were washed in PBS + 0.1% Tween-20 (PBST). To control for variability, master stocks of diluted antibodies and chemiluminescent substrates were made. After 1 hr incubation in secondary antibody, membranes were washed 4 x 5 min in PBST.

Chemiluminescent detection was performed using goat anti-mouse HRP, detected by a CCD camera or film exposure. Two chemiluminescent substrates (ECL™ and ECL Plus™ Western Blotting Detection Reagents; Amersham Biosciences) were used in parallel. Wider linear range was observed with ECL substrate, so only those data are shown and the antibody dilutions noted here were those used with this substrate. An EpiChemi™ II CCD camera (UVP; Upland, CA) was used for visualization. A second CCD camera system was tested in parallel but provided more limited linear range and sensitivity (not shown). A 1:15,000 dilution of HRP-labeled secondary antibody was used for chemiluminescent detection. Exposure times of 1, 5, 10, and 30 min were used to collect images, and quantification performed with the software provided. For film detection, a 1:15,000 dilution of HRP secondary antibody was used. Following substrate incubation, the group of membranes was exposed to film together. Quantification of films was performed with the CCD system using the white light source.

IR detection used a 1:2500 dilution of Alexa Fluor 680 goat anti-mouse and the Odyssey Infrared Imaging System (LI-COR Biosciences). All blots were imaged together. Scan resolution of the instrument ranges from 21-339 µm; in this study, blots were imaged at 169 µm. Quantification was performed with the analysis software provided.

Tf Detection and Two-Color Western Blotting

Blots were blocked in Odyssey blocker. Antibodies were diluted in blocker plus 0.1-0.2% Tween-20, except for HRP-labeled secondary, which was diluted in PBST because Odyssey blocker contains sodium azide. Blots were washed 4 x 5 min in PBST after primary and secondary antibody incubations. ECL substrate and film exposure were used for chemiluminescent detection of Tf. For

IR detection of two proteins, blots were incubated with both primary antibodies simultaneously, followed by both IR-labeled secondary antibodies simultaneously. Blots were imaged with the IR imager in both 700 and 800 nm channels in a single scan at 169 µm resolution.

For two-color detection, primary antibodies were from different types of host animal. Secondary antibodies were from the same type of host. Highly cross-adsorbed secondary antibodies were used to minimize cross-reactivity.

RESULTS

Sensitivity of IR Fluorescent Detection

To exploit the low background fluorescence of membranes in the IR range, we used a laser imaging instrument that images membranes, gels, and microplates in two IR channels, with simultaneous laser excitation at 680 nm and 780 nm. Emitted light was detected at approximately 720 nm and 820 nm, and 16-bit TIFF images were generated for each fluorescence channel. In this study, secondary antibodies labeled with IRDye 800 and Alexa Fluor 680 dyes were tested for Western detection. The spectral characteristics of these dyes allowed for good channel separation.

To assess Western sensitivity, we used purified human transferrin (Tf). Serial dilutions of Tf (10 ng to < 1 pg) were transferred to nitrocellulose after electrophoresis and detected with IR fluorescent Western methodology, with little modification to standard Western protocols. We found that 1.2 pg of Tf was reproducibly detected (Fig. 1A), while only 4.9-9.8 pg was detected with chemiluminescence. For comparison, in a study by Gingrich et al. [7] the limit of detection with visible fluorophores (Cy3, Cy5, or FITC) was reported as 250-500 pg for Western blots of Tf using the same source of anti-Tf primary antibody and a laser imager, while the limit for chemiluminescent detection was 125 pg.

Quantification of Dot Blots with IR Detection

Because of the enzyme/substrate kinetics intrinsic to chemiluminescent detection, we speculated that such data would be less quantitative than data generated with directly labeled fluorescent reagents. Direct fluorescence avoids kinetic and substrate availability caveats and involves no development or exposure times, so data should be more accurate and reproducible, with a broader quantifiable linear range. Therefore, we designed a controlled dot blot assay format to assess quantification and linearity of IR fluorescence and chemiluminescence.

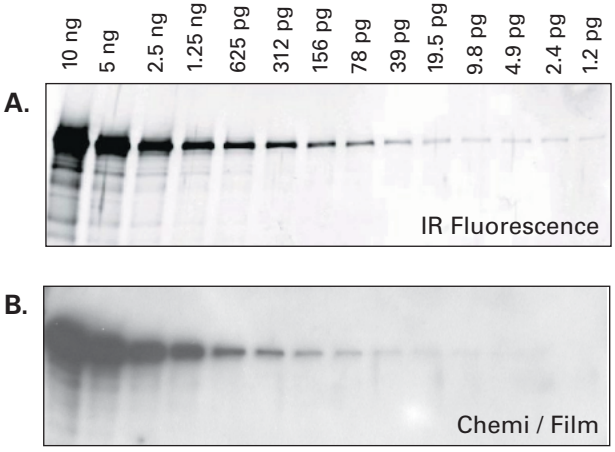

Figure 1. Western blot detection of transferrin. (A) Detection of Tf with IR fluorescence. Two-fold serial dilutions of purified Tf were loaded, starting with 10 ng. The blot was blocked and reacted with rabbit anti-human Tf, followed by Alexa Fluor 680 goat anti-rabbit. The blot was imaged in the 700 nm channel. All dilutions are visible, down to 1.2 pg. This band was quantified and determined to be >3 standard deviations above mean background. (B) Chemiluminescent detection. A duplicate blot of Tf dilutions was reacted with rabbit anti-Tf, then HRP-labeled goat anti-rabbit. Chemiluminescent substrate was applied and detected by film exposure for 3 min. Bands are not crisp, and "blow out" is seen for strong signals. In longer exposures the 4.9 pg band is visible, but background is high and bands become blurred.

In the dot blot assay, protein dilutions were spotted onto nitrocellulose to eliminate variability from electrophoresis and transfer. As target protein, we spotted serial dilutions of a mouse antibody against the myc epitope tag, which was detected with anti-mouse IgG secondary antibody. This experimental design simplified detection and further reduced variability by removing the primary antibody incubation step. For each detection method, six replicate blots were processed simultaneously so reproducibility could be assessed, and the mean and standard deviation were determined for each data point.

To assess IR detection, six blots were reacted with goat anti-mouse antibody labeled with Alexa Fluor 680. Fig. 2A shows a representative

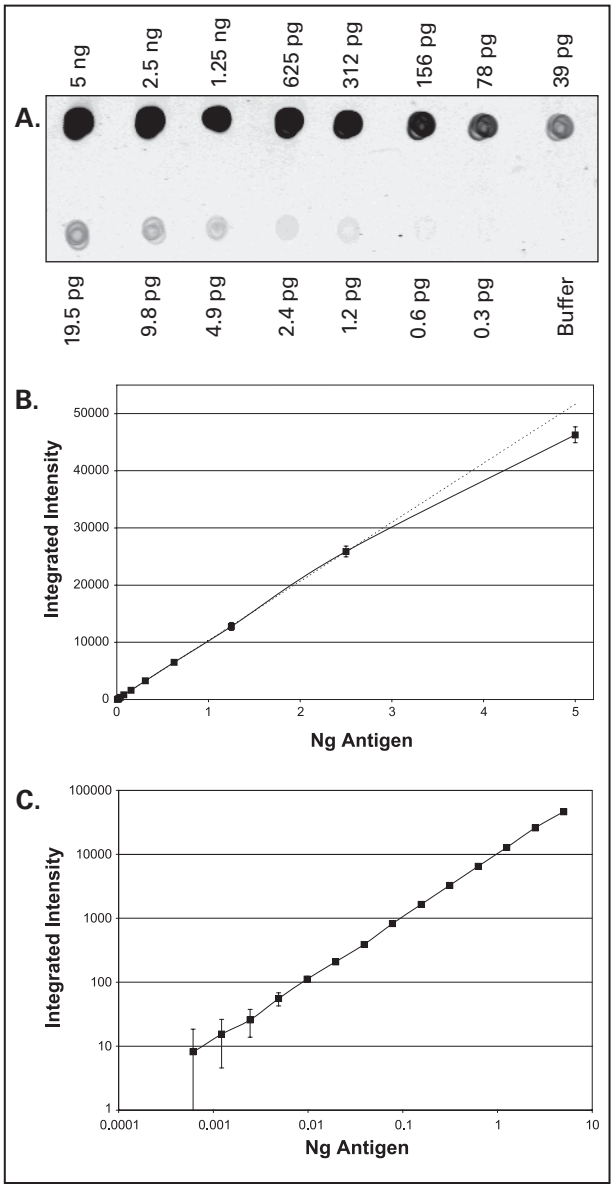

Figure 2. Linearity and reproducibility of IR detection in dot blot format. (A) Image of one of six replicate dot blots, showing detection of serial dilutions of antigen. Blots were detected with Alexa Fluor 680 goat anti-mouse. Amounts of anti-myc antibody spotted as antigen are indicated. (B) Quantification of integrated fluorescence intensity from IR detection. The mean and standard deviation for each data point are plotted on a linear scale. As described in the Results, the 5 ng protein spots showed lower signal then expected and fell slightly below the best-fit straight line (dashed line; linear regression over the range of 0.6 pg to 2.5 ng). Note that the intercept is zero. Error bars showing standard deviations are included for all data points. (C) The data from B was re-plotted on a log-log scale so compressed data points near the origin can be seen. Mean values form a straight line across the entire range. Error bars showing standard deviations are included, but are difficult to see for antigen amounts above ~5 pg because variation is small.

dot blot; Fig. 2B-C show quantification results. The limit of detection for IR laser imaging was approximately 0.6 pg. Linearity of the data sets was assessed using the square of the correlation coefficient (R^2). The R^2 value for the complete data set of six IR blots (5 ng to 0.6 pg of protein target) was 0.996, indicating excellent linear fit over this 8000-fold range of protein concentration. With all detection methods tested, the 5 ng spot was smeared, indicating that the protein binding capacity of the membrane was saturated and some protein target washed away. Quantification results for 5 ng data points with all detection methods were below predicted values. Recalculating R^2 using the 0.6 pg to 2.5 ng concentration range increased R^2 to 0.998, with a quantifiable linear range of greater than 4000 fold (3.6 orders of magnitude). IR fluorescent signal was directly proportional to the amount of antigen from the limit of detection to 2.5 ng. A two-fold increase in protein amount corresponded to a two-fold increase in signal. Standard deviations were small, indicating reproducible signal from blot to blot.

Quantification of Dot Blots with Chemiluminescent Detection

The dot blot assay was repeated using HRP-labeled anti-mouse secondary antibody and chemiluminescent substrates. Blots were detected with a CCD camera or film exposure, with multiple exposures performed. The limit of detection observed with the CCD system was approximately 9.8 pg, compared to 2.4 - 4.9 pg for film exposure.

Quantification data are shown in Fig. 3A-D. These data reveal a limited range through which the results could be considered linear, as well as ranges or exposure times for which the results were not linear. Exposure time had a significant effect on quantification, and saturation was observed on film exposures.

R^2 values were determined to assess the linearity of data from the CCD system (Fig. 3A-B). Linear range was greatest for the 30 min exposure, yielding $R^2 = 0.960$ for protein concentrations from 2.5 ng to 9.8 pg, which represents a 250-fold range. It should be noted that although the data set produced an R^2 value > 0.95, signal was not directly proportional to the amount of

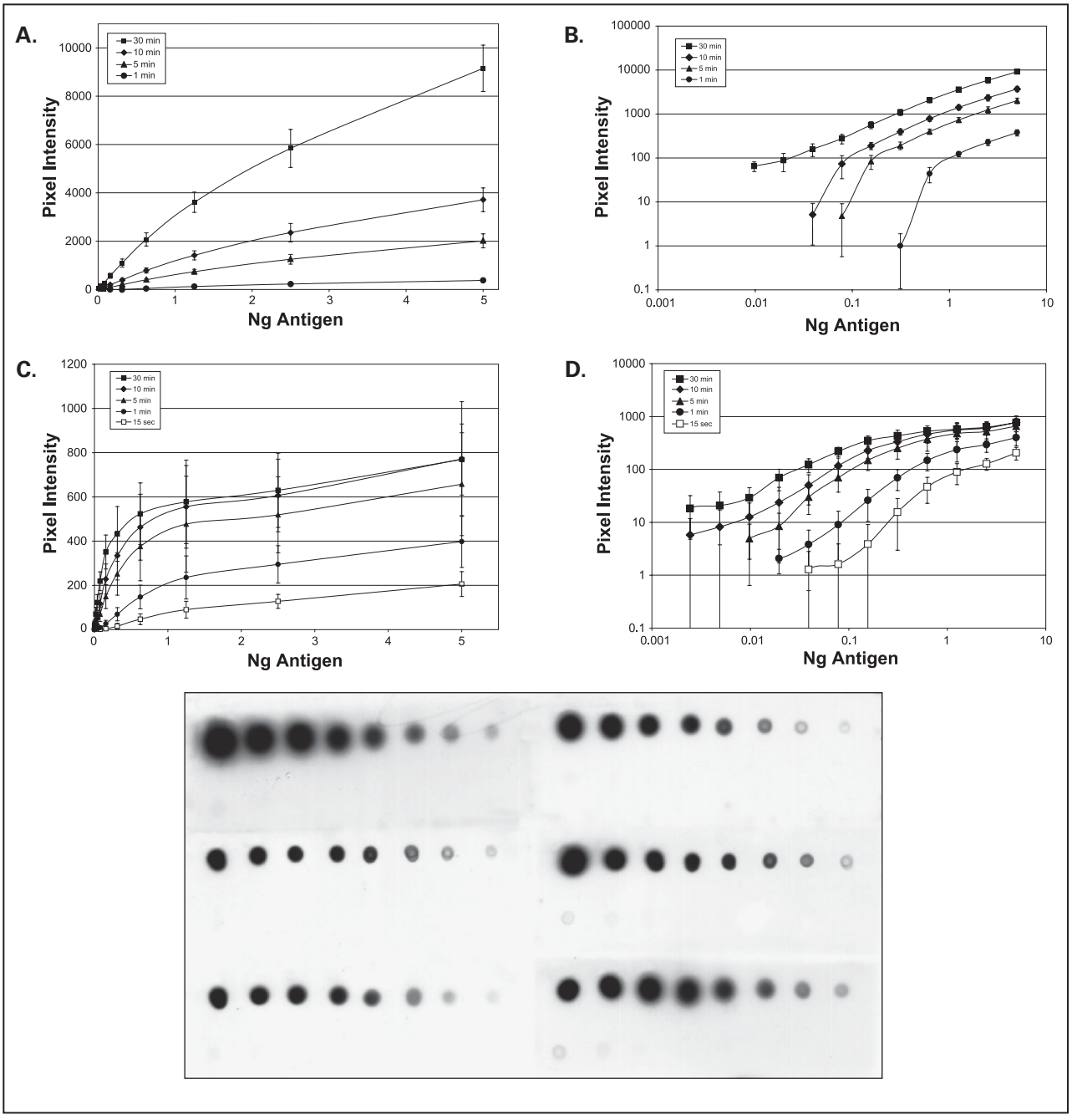

Figure 3. Linearity and reproducibility of dot blot detection using chemiluminescence. (A) CCD detection of chemiluminescence. The means and standard deviations of pixel intensity for six replicates are shown, plotted on a linear scale. Error bars indicate standard deviations. Various exposures (1, 5, 10, and 30 min) are shown. Standard deviations were larger than seen for IR detection. The shape of the curve varies for different exposure times. A 30 min exposure was required to obtain acceptable detection sensitivity. (B) The data from A was re-plotted on a log-log scale. The 30 min exposure provided the broadest linear range. Other exposures show a sharp drop in signal at lower antigen amounts, so linear range is narrower in these instances. (C) Quantification of chemiluminescence after film exposure, on a linear scale. These curves illustrate the limited linear range of film-based detection. Pronounced saturation is seen as amount of antigen increases. Error bars are often overlapping. (D) The data from C was re-plotted on a log-log scale. For each exposure, several consecutive points may approximate a straight line, but the range is limited. (E) Film detection of six chemiluminescent blots. Protein amounts are as in Fig. 2A. All blots were treated identically and detected simultaneously, but signal strength is variable. A 5 min exposure is shown.

antigen across this range (a two-fold increase in antigen amount did not always correspond to a two-fold increase in signal). Sensitivity was poor at shorter CCD exposure times, making linear range narrower. Film detection displayed more limited linearity than the CCD camera (Fig. 3C-D). It is difficult to fit a straight line to the film data for more than a few consecutive points, and the broadest linear range is about 16 fold, in keeping with published reports [11]. Reproducibility was poor, indicated by large error bars.

Simultaneous Detection of Two Targets on Western Blots using Two IR Fluorophores

One major advantage of fluorescent detection is the capacity for multiplex analysis. By selecting appropriate primary and secondary antibody reactivities and using spectrally distinct IR fluorophores, we analyzed mammalian cell lysates by two-color IR Western blotting. Simultaneous detection of Syk (a nonreceptor protein tyrosine kinase that resembles the Src family) and ERK (p44/42 MAP kinases, encoded by ERK1 and ERK2) in A431 cell lysates is shown in Fig. 4A. Mouse and rabbit primary antibodies were incubated with the blot together, and the same was done for dye-labeled goat anti-mouse and anti-rabbit secondary antibodies. After washing, both fluorophores were imaged simultaneously. In this image, Syk is shown in red and ERK in green. Detection is clean and specific, showing no cross-reactivity between the antibodies or against other cellular proteins. Both proteins were easily visualized at endogenous levels in less than 1 µl of total cell extract.

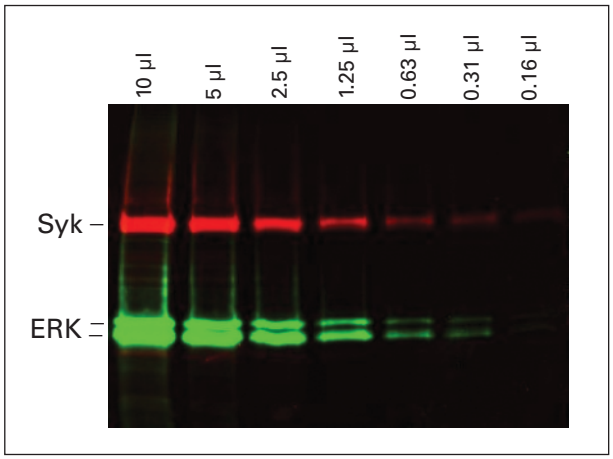

Figure 4. Multiplexed detection of proteins using two IR fluorophores. Detection of Syk and ERK in whole cell lysates. Two-fold dilutions of A431 cell extracts were loaded. The blot was detected with mouse anti-Syk and rabbit anti-ERK, followed by Alexa Fluor 680 goat anti-mouse (red) and IRDye 800 goat anti-rabbit (green). Both colors were imaged in a single scan.

DISCUSSION

The data presented here illustrate the use of IR fluorescence for Western detection. Although chemiluminescence is the predominant method and generally offers excellent sensitivity, it can be difficult to implement for applications that require accurate quantification. IR detection was shown here to provide high sensitivity and a wide quantitative linear range, with low blot-to-blot variability in signal.

In both the dot blot and Tf Western blot systems, we found that IR detection provided better sensitivity than chemiluminescence. IR detection was also 200-400 fold better in sensitivity of Tf detection (with a detection limit of 1.2 pg) than a published study that used secondary antibodies labeled with visible fluorophores [7]. This increase is almost certainly due to low background of membranes in the IR range. Furthermore, other published detection limits for Western detection with visible fluorophores (fluorescein, Cy5, and PBXL-1) with various imaging systems fall between 1 - 6 ng [5, 6, 8] indicating this is representative of blot imaging with visible fluorophores. While Western sensitivity is also dependent on the protein target and on the quality and affinity of the primary antibodies used, the Tf blotting described here duplicates work with the same antibody [7], enabling a nearly direct comparison of the technologies.

The data presented here indicate that chemiluminescent detection is only semi-quantitative (Fig 3A-E), with an unpredictable linear range of up to 250 fold. In contrast, IR detection was quantitative and sensitive with a wide, consistent linear range (Fig. 2 A-C). In the dot blot format, IR signal is linear over 3.6 orders of magnitude ($R^2 = 0.998$) with an intercept of zero, and signal is directly proportional to the amount of

antigen throughout this range. These characteristics permit accurate protein quantification and comparison.

During Western blotting, gel loading and transfer from gel to membrane can introduce error, and small amounts of protein may transfer inefficiently [12]. This limits the linearity that can be achieved after transfer. Despite these limitations, we have observed 500-fold linearity in detection of Tf with IR antibodies following electrophoresis and Western transfer, with $R^2 > 0.987$ (data not shown; protein concentration range from 5 ng to 10 pg). Below 10 pg, purified Tf transfers less efficiently and signal is not proportional.

Multiplexed detection offers many experimental benefits. With conventional techniques, detection of two proteins for comparison or normalization requires that either blots be stripped and re-probed or separate blots be compared. Either method introduces error: stripping can cause inconsistent protein loss that compromises quantification; inadequate stripping of antibodies gives rise to spurious or confusing bands; and inconsistencies in loading and transfer cause substantial blot-to-blot variation. The ability to quantitatively detect two targets unambiguously in the same sample lane makes analysis more accurate and increases experimental throughput (Fig. 4).

In summary, infrared fluorophores enable quantitative protein detection with a wide linear range. IR detection is accurate and reproducible, with sensitivity equal to or better than that of chemiluminescence. These characteristics, combined with the ability to analyze two proteins simultaneously, should make IR methodologies an important tool for proteomic analysis.

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REFERENCES

[1] A. Paladichuk. 1999. How the Western was won. *The Scientist* 13:18-21.

[2] W.F. Patton. 2000. A thousand points of light: the application of fluorescence detection technologies to two-dimensional gel electrophoresis and proteomics. *Electrophoresis* 21:1123-1144.

[3] D. Wilkinson. 2000. The Western lights. *The Scientist* 14:29-30.

[4] S.J. Kaufmann, C.M. Ewing, and J.H. Shaper. 1987. The erasable Western blot. *Anal. Chem.* 161:89-95.

[5] Amersham Biosciences. 2001. Application note #68: fluorescent Western blotting with the Typhoon Variable Mode Imager. Code 63-0043-05.

[6] J. Fradelizi, E. Friederich, M.C. Beckerle, and R.M. Golsteyn. 1999. Quantitative measurement of proteins by Western blotting with Cy5-coupled secondary antibodies. *BioTechniques* 26:484-494.

[7] J.C. Gingrich, D.R. Davis, and Q. Nguyen. 2000. Multiplex detection and quantitation of proteins on Western blots using fluorescent probes. *BioTechniques* 29:636-642.

[8] J.P. Morseman, M.W. Moss, S.J. Zoha, and F.C.T. Allnutt. 1999. PBXL-1: a new fluorochrome applied to detection of proteins on membranes. *BioTechniques* 26:559-563.

[9] G. Patonay and M.D. Antoine. 1991. Near-infrared fluorogenic labels: new approach to an old problem. *Anal. Chem.* 63:321A-327A.

[10] J. Sowell, L. Strekowski, and G. Patonay. 2002. DNA and protein applications of near-infrared dyes. *J. Biomed. Optics* 7:571-575.

[11] D. Huang and S.A. Amero. 1997. Measurement of antigen by enhanced chemiluminescent Western blot. *BioTechniques* 22:454-458.

[12] J.H. Bhavsar, J. Remmler, and P. Lobel. 1994. A method to increase efficiency and minimize anomalous electrophoretic transfer in protein blotting. *Anal. Biochem.* 221:234-242.

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