Imaging Chemiluminescent Western Blots With the FluorChem® Q Provides Superior Quantitative Capacity Relative to Film

Abstract

Chemiluminescence is the most frequently used detection method in Western blotting, and the chemiluminescent signal is typically detected using film. Historically, digital imaging has been considered less sensitive and therefore not suitable for detecting faint signals such as chemiluminescence. This note demonstrates that the FluorChem HD2 and FluorChem Q imaging systems can approach the sensitivity of film, while far exceeding film with respect to linear dynamic range and the ability to obtain quantitative information from the image.

Introduction

Western blotting relies on the use of labeled antibodies to detect the presence of a target protein on a membrane. Chemiluminescence is by far the most commonly used detection method in Western blotting, much more prevalent than alternatives relying on radioactivity, or newer fluorescence-based assays (1). Chemiluminescence is a low-light signal and is traditionally detected using film, which is thought to be a sensitive and relatively quick detection method. Digital imaging is an attractive alternative since it does not require a darkroom, a developer machine, or the toxic chemicals involved in developing film. Historically, digital imaging has been considered less sensitive than film, and therefore not a suitable replacement for film when detecting chemiluminescence. However, improvements in digital imaging, including the development of higher resolution cameras combined with the ability to "bin" adjacent pixels to increase sensitivity, and the ability to cool cameras to very low temperatures to reduce background noise, make digital imaging under low-light conditions an effective solution. This application note demonstrates that the digital imaging solution present in the FluorChem HD2 and FluorChem Q instruments can compete with film for imaging times, and is far superior to film with respect to the linearity of the relationship between the chemiluminescent signal and protein quantity.

Methods

Slot blots

Six replicate slot blots were prepared containing a two-fold serial dilution, from 5 ng to 0.15 pg, of an HRP conjugated goat-anti-rabbit IgG (Vector Labs). The antibody contained approximately 3 moles of HRP per mole of IgG. The serial dilutions were prepared in PBS containing 10% methanol and 5µg/mL BSA as a carrier protein.

PVDF membranes (Westran® Clear Signal, Whatman, Inc., cat. No 10485289) were pre-soaked in methanol, then water, then PBS containing 10% methanol. 200 µL of each sample was loaded per slot, and filtered using a vacuum operated slot blot apparatus (Life Technologies). Membranes were removed from the apparatus, washed twice with PBS containing 0.05% Tween-20 (PBST), and blocked with a 2% solution of ECL Advance blocker (GE RPN418V) in PBST for 30 minutes at room temperature. Membranes were washed twice in PBS.

The layout of each slot blot was as follows:

5 ng	20 pg
2.5 ng	10 pg
1.25 ng	4.9 pg
625 pg	2.4 pg
313 pg	1.2 pg
156 pg	0.6 pg
78 pg	0.3 pg
39 pg	0.15 pg

Imaging

Of the six slot blots created, three slot blots were imaged with film, and three were imaged digitally on the FluorChem Q. (The FluorChem HD2 meets the performance of the FluorChem Q for chemiluminescent imaging.) For all blots, HRP signal was developed using Chemiglow®(Cell Biosciences) according to the manufacturer's instructions. Equal volumes of reagents were mixed, and the mixture applied to the blots for five minutes. The identical time elapsed between completion of the incubation with Chemiglow, and imaging with either film or the digital imaging system.

For imaging on film, BioMax LightFilm (1788207) was used, and the film was developed in a Kodak 2000A Processor. After the completion of the Chemiglow incubation, three slot blots were imaged simultaneously with the following exposures: 10 sec, 30 sec, 1 min, 5 min, and 20 min. A final 10 sec and 30 sec image were taken to check for signal decay over the course of the experiment. The signal was found to change very little over the course of image collection. The total time in the darkroom was 39 minutes. To generate a digital file of the exposed film for analysis, the developed film was imaged on the FluorChem Q using the white light table and the Ethidium bromide filter to increase contrast.

Flat field calibration was also performed on the acquired image to ensure quantitative accuracy.

Digital imaging of the second set of slot blots was conducted on the FluorChem Q. The software AlphaView™Q was programmed to collect the following images: 10 sec, 30 sec, 1 min, and 5 min, each with 1x1, 3x3, and 8x8 binning. A 20 min exposure was taken with 1x1 and 3x3 binning. Final 10 sec and 30 sec images were taken about 20 minutes later to check signal stability. Total imaging time was about 60 minutes.

Analysis of the images was conducted with ImageJ, free software

available from NIH (2). AlphaView was not used to analyze the images in order to remove any bias toward the chemiluminescent images captured with AlphaView, instead of with film.

Images were opened in ImageJ, and bands were designated by drawing a box around them. The same sized box was used for each band. For the FluorChem Q images, background was measured from a single box adjacent to the slot blot, and subtracted from the value for each band. For the film images, background varied across the blot, so a separate background box was measured adjacent to each band of the slot blot.

Results

Binning increases sensitivity with an acceptable loss of resolution

Binning involves virtually "pooling" the signal from adjacent pixels. Increased binning will increase sensitivity with a concurrent reduction in resolution. The user can determine the optimal balance of sensitivity and resolution for their specific application. X-ray film presents a similar tradeoff between resolution and sensitivity; although it has a higher sensitivity than other forms of film, it also has a lower resolution. Binning is easily adjusted in the FluorChem Q software from a drop down menu when acquiring the image (Figure 1).

Normal/Ultra = 1X1 binning

Medium/High = 2X2 binning

High/Medium = 3X3 binning

Fast/Low = 4X4 binning

Super Speed = 8X8 binning

Figure 1. Binning settings in AlphaView Q software are designated as Speed/Resolution settings. The corresponding bin values are listed next to each Speed/Resolution settings.

Figure 2 shows the effect of binning on image resolution. In this figure, the same slot blot is shown, imaged on the FluorChem Q for 60 seconds with 1x1, 3x3, or 8x8 binning. It can be seen that the signal becomes stronger as sensitivity increases with increased binning. The resolution also begins to decrease with increased binning, visible as slightly pixilated bands in the 8x8 binned image. The image collected with 3x3 binning demonstrates a significant increase in sensitivity with no visible pixilation, providing an image suitable for publication.

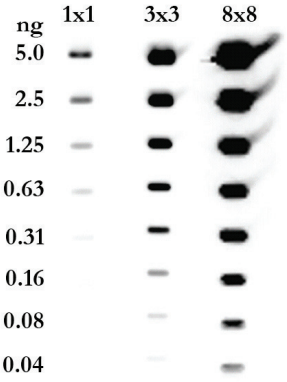

Figure 2. Demonstration of the effect of binning on image resolution. The same slot blot was imaged on the FluorChem Q for 60 seconds with either 1 x 1, 3 x 3, or 8 x 8 binning. The increase in sensitivity achieved with each increase in binning is visible. By 8 x 8 bin mode, some loss of resolution, a necessary trade-off when pooling pixels to increase sensitivity, becomes apparent as slightly increased pixilation of the bands.

Figure 3 demonstrates how increasing the degree of binning can greatly reduce the imaging time. Figure 3 shows the same blot imaged either for 5 minutes with 1x1 binning (left) or 10 seconds with 8x8 binning (right). The intensity of the bands is greater in the 10 sec exposure, with a concomitant 30-fold reduction in imaging time.

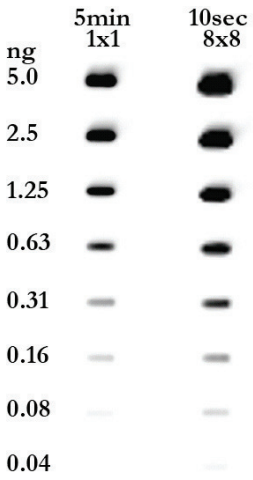

Figure 3. Effect of binning on imaging time. The same slot blot was imaged on the FluorChem Q for 5 minutes with 1 x 1 binning (left), or for 10 seconds with 8 x 8 binning (right). The 10 second image has stronger signals intensity than the 5 minute image, demonstrating the increased sensitivity that is achieved with increased binning.

The FluorChem Q uses binning technology to meet the speed of film

The band containing the lowest protein amount on the slot blot, 0.15 pg, was detected with a 20 minute exposure to either film or the FluorChem Q imager (Figure 4). Shown in Figure 4 are the lower range of the dilution series for each of the three replicate slot blots imaged with the FluorChem Q (left) or by exposure to film (right). At this exposure time, the higher end of the dilution range was saturated for all images and so is not shown. The FluorChem Q image was captured with 3x3 binning.

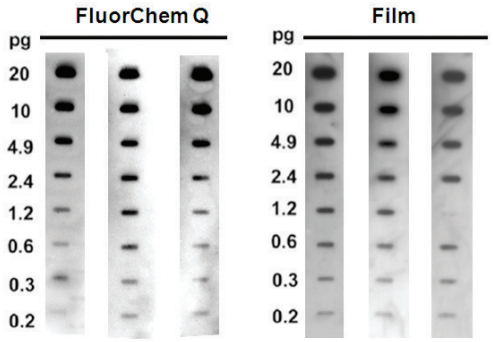

Figure 4. Six replicate slot blots containing 2-fold serial dilutions of an HRP-labeled antibody were prepared, and three were imaged on the FluorChem Q, and three were imaged by exposure to film. Shown are 20 minute exposures, with 3 x 3 binning on the FluorChem Q (left) and to film (right).

Digital imaging provides a greater linear range and more accurate quantification than film.

In general, for the same exposure time, the intensity of the bands was greater with film than with the digital camera, even with 8x8 binning.

However, film also demonstrated a higher background level, and significantly narrower linear range than the digital images. Film is known to be a non-linear measuring device, which is largely responsible for Western blotting being known as a semi-quantitative technique. The high and variable background observed in the film images makes accurate quantitation of band intensities difficult. Figure 5 shows plots of band density vs. protein quantity for a slot blot imaged with the FluorChem Q (Figure 5a), or with film (Figure 5f) for 10 seconds. The plot for the FluorChem Q image (Figure 5b) demonstrates that the band intensities are linear over nearly the entire protein range, from 0.15 pg to 5 ng. (Figures 5b and expanded scale in 5c). In contrast, even after background correction, the plot of the film image (Figure 5d) has a very narrow linear dynamic range, and the band intensities begin to saturate very quickly. The band density was linear with respect to protein quantity only in the range of 39 pg to approximately 160 pg (visible in the expanded scale of the plot shown in Figure 5e). The film image exhibits higher signal intensities than the digital image, such that the bands containing the highest protein amounts are saturated. However, even in the range of the lowest protein amounts, the relationship of band density to protein quantity is not linear for the film image.

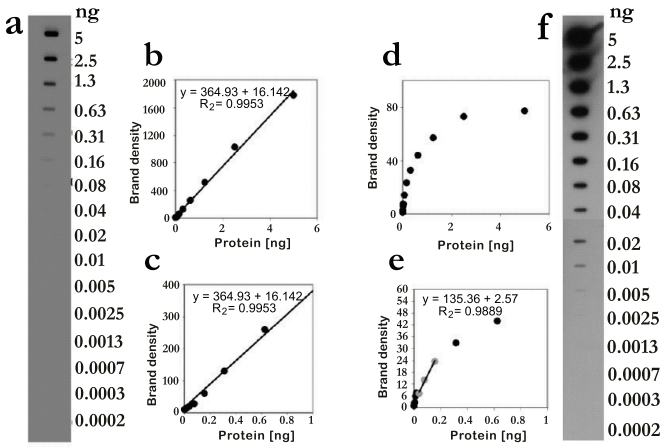

Figure 5. Linearity of the images with respect to protein concentration. The density of each band of a slot blot imaged with the FluorChem Q using 1X1 bin (a) or film (f) for 10 seconds was measured, and plotted against protein quantity in that band. The plot of digitally imaged blot is linear over the entire range from 0.15 pg to 5 ng, with an r^2 value of the 0.9953. An expanded view of (b) is shown in (c). The plot of the film image (d) has a very small linear range, extending only from approximately 39 pg to 156 pg, or 4-fold (visible in the expanded graph in (e)).

Conclusions

Digital imaging with the CCD camera of the FluorChem HD2 and FluorChem Q provides an accurate and sensitive means to image chemiluminescent blots. Though film may be more sensitive for a given exposure time, it also saturates more quickly and has a significantly narrower linear range than digital images. With a significantly wider linear response, digital images are preferable for applications requiring accurate quantification of the quantity of protein in a band on a Western blot. Additionally, adjustment of the binning setting can significantly reduce imaging time, with only negligible losses in image resolution, making digital imaging competitive with film, especially when the extra time required for developing film is considered. Digital imaging of chemiluminescent Western blots with the FluorChem HD2 or FluorChem Q provides the accurate and sensitive solution required for quantitative Western blotting.

References

(1) Patton, W.F. 2000. A thousand points of light: The application of fluorescence detection technologies to two-dimensional gel electrophoresis and proteomics. Electrophoresis. 21:1123-1144.

(2) Armband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/, 1997-2008.

Acknowledgments

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The FluorChem Q Imaging System provides the sensitivity of chemiluminescence and the quantitative power of fluorescence in one easy-to-use instrument. Designed with fast lens technology, the peltier cooled camera captures high-resolution images with a linear dynamic range that out performs film, and with speeds 10X faster than a laser scanner. Equipped with three integrated excitation sources for multicolor westerns, the FluorChem Q is compatible with Cy dyes, Alexa dyes, Qdots, and chemiluminescent western blotting kits. Additionally, the FluorChem Q provides the flexibility to image fluorescently stained DNA gels, as well as Coomassie and silver stained protein gels.

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