Imaging of Chemiluminescent Western Blots: Comparison of Digital Imaging and X-ray Film

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Introduction

Western blotting is a widely used procedure that uses antibodies to detect and quantify a specific protein in a complex mixture (Towbin et al. 1979). Proteins separated by polyacrylamide gel electrophoresis are transferred onto a nitrocellulose or PVDF membrane before probing with a specific antibody. The membrane-bound antibody-protein complex is detected by a secondary antibody conjugated to horseradish peroxidase (HRP) or alkaline phosphatase (AP), in conjuction with a substrate that produces a luminescent signal.

Chemiluminescence has become a popular method for sensitive western blotting detection. The advantages of chemiluminescence over radioactive probes or colorimetric assays include high sensitivity, large dynamic range, rapid results, low cost, and safety (Towbin et al. 1979; Kricka 1991; Rongen et al. 1994; Beck et al. 1990). Traditionally, the chemiluminescent signal is detected and recorded by exposure of the membrane to a light-sensitive film (X-ray film). The recent development of digital imaging systems that use charge-coupled device (CCD) cameras to capture the luminescent signals on western blots has provided new options for the detection and quantitation of signals.

This study compares the performance of X-ray film with that of Bio-Rad's digital imaging systems for the visualization and quantitation of chemiluminescent signals on western blots.

Methods

Reagents and equipment were from Bio-Rad Laboratories, Inc., unless otherwise specified. Human serum was purchased from Bioreclamation, Inc. Detection reagents were goat anti-rabbit and goat anti-mouse (GAM) HRP-conjugated antibodies. All samples were run on 10% Tris-HCl Criterion™ gels using a Criterion electrophoresis cell. Samples were diluted in Laemmli sample buffer containing 5% β-mercaptoethanol and heated at 95°C for 5 min prior to loading on gels. Electrophoresis was performed at 200 volts for 55 min. Gels were blotted onto Pall Fluorotrans PVDF membranes using a Criterion blotter. The membranes were blocked for 1 hr at room temperature with agitation in Tris-buffered saline + 0.05% Tween (TBST) containing 3% nonfat dry milk. They were then rinsed in TBST prior to incubation for 1 hr at room temperature in primary antibody diluted 1/1,000 in TBST + 3% nonfat dry milk. The membranes were washed 3 times for 10 min in TBST and secondary antibody incubations (1/50,000 dilutions in TBST + 3% nonfat dry milk) were performed at room temperature for 1 hr with agitation. After incubation, the membranes were washed 6 times in 100 ml of TBST and then immersed in chemiluminescent substrate.

The decay of signal over time was performed using serial dilution of a *E. coli* cell lysate containing a 51 kD polyhistidine-tagged recombinant protein run on a 4–20% linear gradient Criterion gel. Blots were probed with mouse anti-polyhistidine-tag primary antibody followed by incubation with GAM-HRP secondary antibody and StrepTactin HRP (1:10,000) for 1 hr each. The blots were then cut into two sections, each containing an identical dilution series of protein. The sections of membrane were incubated in chemiluminescent substrates.

Chemiluminescent detection was performed using the Immun-Star™ WesternC™ chemiluminescent kit and the Molecular Imager® VersaDoc™ MP 4000 and 5000 systems, as well as the Molecular Imager ChemiDoc™ XRS system. Kodak BioMax light film was used for X-ray film exposure. The film was developed in an automated processor, and the signal was quantitated by scanning with the Molecular Imager GS-800™ calibrated densitometer followed by analysis of the digital image. For digital acquisition of chemiluminescent signals by the CCD camera, the wrapped blot was placed on the imaging surface, then imaged, and the data were analyzed using Quantity One® 1-D analysis software.

Results and Discussion

Decay of Signal Intensity Over Time

Chemiluminescence is a chemical reaction that generates energy released in the form of light. The reaction occurs in the presence of an enzyme such as HRP that catalyzes luminol oxidation in the presence of hydrogen peroxide. The reaction forms an excited state of the substrate that emits light as it decays to the ground state (Beck et al. 1990). The intensity of the chemiluminescent reaction fades exponentially with time (Figure 1), but the luminescence will last for several hours if the blot does not dry out. The Immun-Star WesternC substrate has a half-life of approximately 80 min, whereas ECL Plus substrate has a half-life of about 30 min.

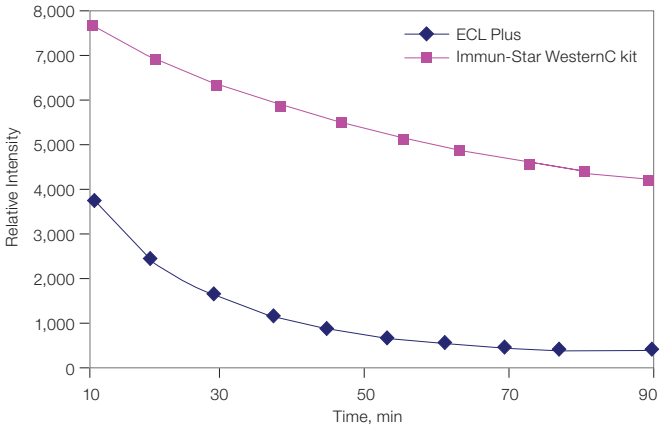

Fig. 1. Relative intensity of luminescent signal over time. Decay of luminescence intensity for Immun-Star WesternC substrate or ECL Plus was assessed using blots of a serial dilution of an *E. coli* cell lysate containing a 51 kD polyhistidine-tagged recombinant protein. The membranes were imaged using the Molecular Imager ChemiDoc XRS system. The chemiluminescence application (no binning) was selected in Quantity One software. Sequential images of 30 sec integration were obtained at 10 min intervals over a 100 min period.

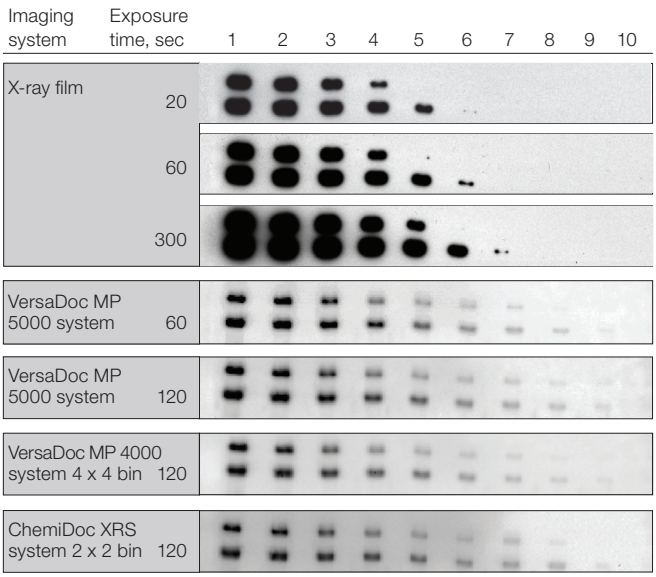

Fig. 2. Comparison of X-ray film and digital imaging system performances in western blot detection. Blots of twofold serial dilutions of human serum were probed with rabbit anti-human transferrin polyclonal antibodies. A 1/1,000 dilution of human serum was used to make the twofold serial dilutions.

CCD vs. X-ray Film: Dynamic Range and Limit of Detection

Dynamic range and limit of detection (LOD) are two essential criteria for assessing the performance of signal detection systems. The dynamic ranges and LOD were determined for the Molecular Imager VersaDoc MP 5000, VersaDoc MP 4000, and ChemiDoc XRS systems using their respective standard settings in Quantity One software. For the VersaDoc MP imaging systems, a NIKKOR 50 mm lens (Nikon) set at an aperture of f/1.4 was used.

To determine dynamic ranges and limits of detection, blots of serial dilutions of human serum were probed with rabbit anti-human transferrin antibody as described in the Methods section. The probed blots were wrapped in clear plastic and imaged for 60 and 120 sec. For the X-ray film exposure, the blots were placed in a cassette and pressed against the film for 20, 60, and 300 sec. The developed film was scanned using a Molecular Imager GS-800 densitometer. The data were analyzed using the Volume Analysis application in Quantity One software.

The results presented in Figure 2 show a limited dynamic range over a given exposure time for X-ray film compared with the Molecular Imager systems. Low signal intensities require long exposure time, while high signal intensities rapidly reach saturation. Therefore, to gather maximum information from a blot that has both intense and weak signals, multiple exposures must be performed (Figure 2). In addition signals in close proximity merge together with overexposure. In contrast, the tested Molecular Imager systems were capable of capturing data over a broad range of intensities in a single exposure time with a significantly lower LOD (Figure 2). Furthermore, plots of signal intensity relative to the amount of serum loaded on the gel show that X-ray film yields a sigmoidal curve with a narrow linear response, whereas the data captured by the digital imagers show a broader linear response (Figure 3).

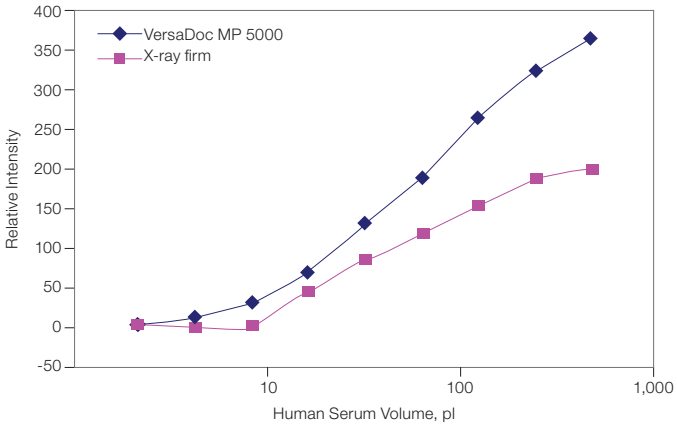

Fig. 3. Dynamic range of CCD and X-ray film Serial dilutions of human serum (starting at 500 pl) were probed with rabbit anti-human transferrin antibodies (see Figure 2). Blots were imaged using a Molecular Imager VersaDoc MP 5000 system with an exposure time of 120 sec and then were exposed to X-ray film for 120 sec followed by scanning using a densitometer. The intensity of the signal did not decrease significantly within this time frame (data not shown).

Visual Assessment vs. Digital Quantitation

The use of X-ray film has traditionally been associated with a visual assessment of relative band intensities. This approach is adequate in situations where a comparison of band intensities is sufficient to draw a conclusion and when the compared signal intensities differ significantly. However, this type of analysis has severe limitations and is not useful for precise signal quantitation. Signal quantitation on X-ray film can be achieved by scanning the X-ray film image and measuring the density of the bands, but accurate measurement of band density depends on the sensitivity of

the film, linear response range, and exposure time. As seen in Figures 2 and 3, signal saturation can be reached quite rapidly, resulting in dark dense bands on the X-ray film. Once saturation has been reached, the change in signal intensity cannot be measured.

Visual analysis of western blot images obtained with X-ray film clearly shows that the signal on X-ray film rapidly exposes the film to saturation, giving strong dark bands and the perception that X-ray film is more sensitive than is digital imaging. However, as shown in Figure 2, the lower LOD of the film is high compared with that of digital imaging. Weak signals need extensive exposure time to be detected and quantitated when X-ray film is used. It should be noted that digital imaging systems, because of limitations on integration time, are not as sensitive as X-ray films that are exposed for extended periods of time.

A CCD-captured image, on the other hand, with its greater dynamic range, will typically have data distributed over a much broader range (Figure 3). Moderate signals, which would have reached saturation (black) on film, may still be in the middle (gray) range of the dynamic range of the digital imager. A digital imager typically records from 4,096 to 65,536 different levels of intensities. When prepared for publication, blot images are generally displayed over 256 levels of gray. The most intense data will be at level 255 and data of half intensity will be at level 128. For purposes of publication, an image closer in appearance to the more familiar X-ray film can be achieved by transforming the image using image analysis software (Quantity One 1-D analysis software) to present midrange bands closer to black (Figure 4). However, these changes are purely aesthetic, and these images should not be used to draw conclusions from the data.

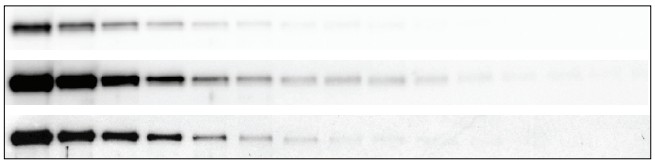

Fig. 4. Transformation of digital image to mimic X-ray film. Duplicate chemiluminescent western blots were imaged for 30 sec on a VersaDoc MP 4000 system or exposed to X-ray film for 60 sec. The top image is the VersaDoc MP 4000 system image with the presentation evenly distributed over the entire range of possible intensities. The middle image is the same file with the presentation adjusted to show a linear gradient of gray up to 20% of the intensity range and all greater values expressed as black. The bottom image is a densitometer scan of the X-ray film.

Cost Savings With Digital Imaging

Digital image capture requires a significant initial investment, but because of lower operation costs, over time it is a less expensive alternative. The costs associated with the use of expensive X-ray film, purchase and maintenance of the film processor, and the chemicals needed to process the film are avoided by using a digital imager. In addition, if quantitation of the chemiluminescent signal is desired, a densitometer is also required, which adds significantly to the cost of western blot analysis using X-ray film. The labor required for the extra steps of multiple film exposures, film development, and densitometry scans should also be factored into the total cost of using X-ray film.

Conclusion

Digital imaging systems provide a convenient and practical solution for imaging and performing quantitative analysis of chemiluminescent blots. The Molecular Imager VersaDoc MP and ChemiDoc XRS systems provide better linearity between amount of protein and signal intensity over a broader dynamic range, with higher sensitivity than does traditional X-ray film for short exposure times. In addition, imaging systems are less expensive over time.

Compared with X-ray film, successful imaging of chemiluminescent blots using a digital imaging system is fast, accurate, and reproducible.

References

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