

Western blot analysis: Comparison of film and the C-DiGit[®] Blot Scanner

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Enhanced chemiluminescence (ECL) has been used since the 1970s to detect target proteins on Western blots.¹ For detection, horseradish peroxidase (HRP) enzyme is conjugated to a secondary antibody. The enzyme oxidizes the luminol-based chemiluminescent substrate, causing it to transiently produce light at ~428 nm. Chemiluminescent Western blots are typically documented by exposure to x-ray film, which darkens in response to the emitted light. Signal intensity is determined by the number of HRP molecules reacting with the substrate at a given time.

Chemiluminescent Westerns are often analyzed by visual comparison, to confirm the presence or absence of a band or to compare bands of substantially different intensities. For more detailed analysis, film images may be digitized and the relative band intensities determined by densitometry. Film is frequently used for signal capture, but its response to light is non-linear. This limits the usefulness of film for quantitative analysis of chemiluminescent Western blots.

Two critical variables affect the accuracy of Western blot analysis: detection chemistry and signal capture method. This study compares the performance of two signal capture methods: film exposure, and the C-DiGit Chemiluminescent Western Blot Scanner. A light-generating LED imaging target was used to eliminate detection chemistry as a source of variability.

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1. Variables that affect analysis of chemiluminescent Westerns

1.1 Detection chemistry and enzyme/substrate kinetics

In chemiluminescent detection, the HRP enzyme causes oxidation of the luminol-based chemiluminescent substrate, creating an excited-state product. As it decays to a lower energy state, this product transiently produces light.¹ Sensitivity is excellent, but the enzymatic nature of signal generation introduces two potential sources of error:

- Signal intensity is dynamic and transient. Intensity peaks shortly after substrate is applied to the blot, then fades exponentially with time as the chemical reaction slows. Persistence of signal depends on the chemiluminescent substrate used. Extended-stability substrates are available, but are typically more expensive than substrates that fade quickly.
- Substrate availability and exhaustion are also concerns.¹ Distribution and accessibility of the substrate are never uniform across the entire blot. Strong bands with high local concentrations of HRP enzyme may cause rapid local depletion of substrate, causing central “white-outs” where light is no longer being generated. Pooled substrate and bubbles also affect substrate availability and signal generation. Because signal intensity is governed by enzyme/substrate kinetics, intensity may not be proportional to the amount of target present, or its proportionality may change over time.

These limitations are intrinsic to chemiluminescent detection, regardless of the signal capture method used (film exposure, CCD imaging, C-DiGit Blot Scanner, etc.).

1.2 Signal capture and densitometry

Chemiluminescent signals are often captured by exposure to x-ray film. This method is very sensitive, because long exposures can be used to detect low-intensity signals. **However, the optical density (OD) of the developed film is *not* a direct function of light generation by the chemiluminescent substrate. It is an indirect function that depends on the non-linear response of film to light.**² When densitometry is used to quantify signals, accuracy is dependent on the sensitivity, linear response range, and exposure time of the film.

- Multiple film exposures of different lengths are typically required to capture the desired image on film. Exposures must be long enough to detect the desired target, but short enough to limit undesired background and “blow-out” of strong bands (spreading and diffusion of bands that obscures other signals). A single exposure can only provide accurate detection across a narrow linear response range.² Boundaries of the linear response range are different for every exposure, making it difficult to compare band intensities from different exposures.
- Reciprocity failure (discussed below) may cause both faint and strong bands to be under-represented on film, and saturation of bands prevents measurement of further change in signal intensity.³ These issues further complicate comparison of band intensities.

Digital imaging with the C-DiGit Blot Scanner bypasses these limitations, and offers a much wider linear response range than film. Because photographic emulsions are not used, the non-linear response of film no longer affects the accuracy of Western blot results.

Table 1. Detection chemistry and signal capture variables affect the accuracy of Western blot quantification. The C-DiGit Blot Scanner bypasses inaccuracy caused by signal capture with film.

	Film + chemiluminescence	C-DiGit Scanner + chemiluminescence
Detection chemistry	<p>LIMITED</p> <ul style="list-style-type: none"> • Enzyme/substrate kinetics • Substrate exhaustion issues • Signal instability • Single color only 	<p>LIMITED</p> <ul style="list-style-type: none"> • Enzyme/substrate kinetics • Substrate exhaustion issues • Signal instability • Single color only
Signal capture	<p>LIMITED</p> <ul style="list-style-type: none"> • Film saturates quickly • Dependent on exposure time • Strong bands blur together • Densitometry underestimates strong and faint signals • No digital image generated • Requires multiple exposures 	<p>ROBUST</p> <ul style="list-style-type: none"> • Digital signal capture • Unlikely to reach saturation • Strong and faint bands clearly resolved • Easy to adjust brightness and contrast • Only one image acquisition required

2. Comparison: Signal quantification with film and the C-DiGit Blot Scanner

To further examine signal capture issues related to film exposure, we used a model system that eliminates variability caused by Western blot detection chemistry ([LINK to photochemistry paper?](#)).

2.1 Experimental design: LED light manifold

In these experiments, the enzyme/substrate reaction was replaced with a light-generating performance test target (PTT) manifold.⁴ The PTT emits calibrated light across a wide range of intensities, using an LED backlight panel with regulated brightness levels that decrease in two-fold steps (Fig. 1). The PTT is useful for direct testing of imaging response, because its consistent and reproducible light production is unaffected by timing or enzyme/substrate concerns.

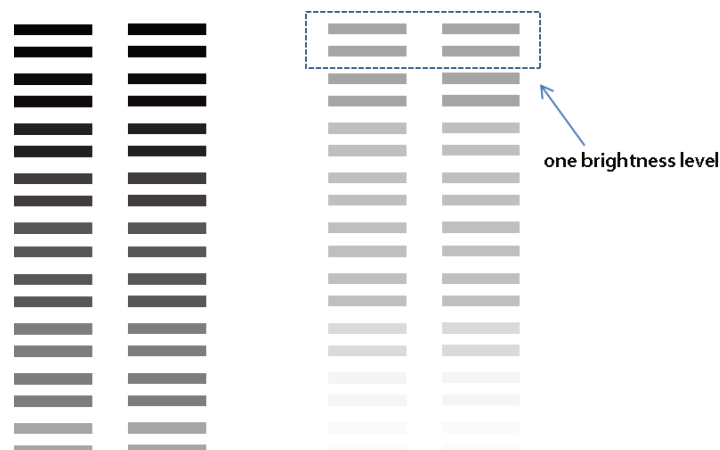


Figure 1. PTT light manifold. The LED backlight panel emits regulated levels of brightness, decreasing in two-fold steps. There are four duplicate slots for each brightness level, and a total of 18 brightness levels. Each slot is 8 mm x 1 mm. Slots are spaced 1 mm apart within each brightness level, and 1.5 mm from one level to another.

2.2 Film exposure and densitometry

The PTT manifold was used to generate a series of film exposures that were analyzed by densitometry.⁴ A non-linear response to strong signals was clearly observed (a phenomenon known as high-intensity reciprocity failure; described in Section 3). As strong signals caused film to become progressively more exposed, the optical density of bands began to plateau. Near the saturation point, bands no longer showed tonal variations on the developed films. Overexposure caused strong and moderate bands to appear similarly dark and dense (Fig. 2).

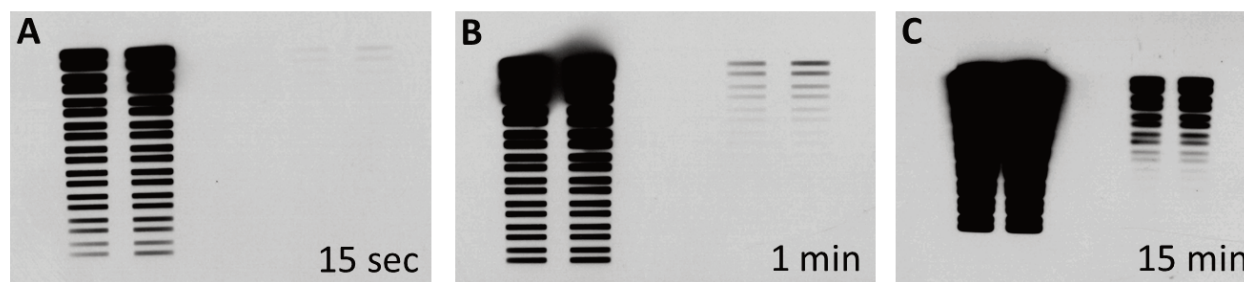


Figure 2. On film, strong signals quickly became saturated. A) 15-second film exposure of PTT light manifold. Strong and moderate signals appeared similarly dark and dense. The strongest signals (top) were saturated, and had begun to blur and spread. Weaker signals (right) were not visible in a short film exposure. B) 1-minute film exposure. All strong bands appeared dark and dense. The strongest signals (top left) showed significant blurring. Weaker signals (right) began to be detected. C) 15-minute film exposure. This long exposure allowed weaker signals to be detected (right), but even these signals began to saturate and blur. Strong signals (left) were saturated and completely blurred together.

Quantification of signals from the PTT manifold is shown in Fig. 3. All exposures demonstrated plateau and saturation of strong signals, even if they were very short (1 - 5 seconds). Each exposure displayed a narrow range of linear response (4- to 8-fold), but the boundaries of that range were different for every exposure. This illustrates the importance of standard curves for analysis of protein levels with film and densitometry.

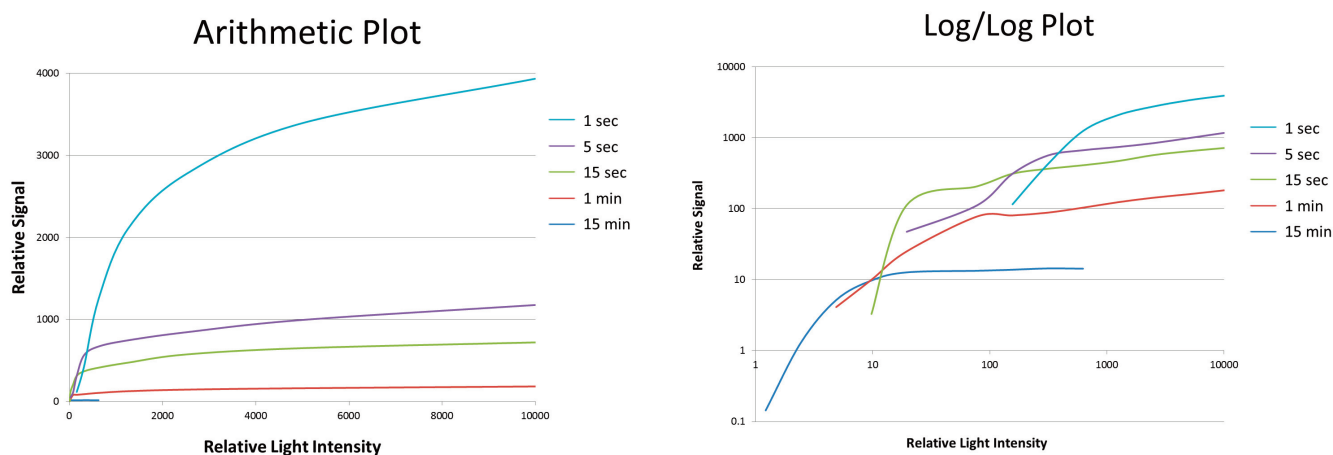


Figure 3. Quantification demonstrates the narrow linear response range of film. The PTT manifold was used to perform a series of film exposures (1 sec, 5 sec, 15 sec, 1 min, and 15 min). Films were analyzed by densitometry, and data were expressed relative to the strongest intensity detected. At all exposure times, strong signals quickly reached a plateau. The log/log plot clearly shows the narrow, variable linear response range of film for all exposure times. Light intensity values that fell within the linear range were different for each exposure.

2.3 Imaging with the C-DiGit Blot Scanner

The same experiment was performed with the C-DiGit Blot Scanner. A single image was captured to record signals from the PTT.⁴ Adjustment of image display settings showed that both strong and weak signals were clearly documented in this single image file (Fig. 4). Even the strongest signals (which were a black blur in the 15-minute film exposure; Fig. 2C), could be individually detected and separated (Fig. 4A).

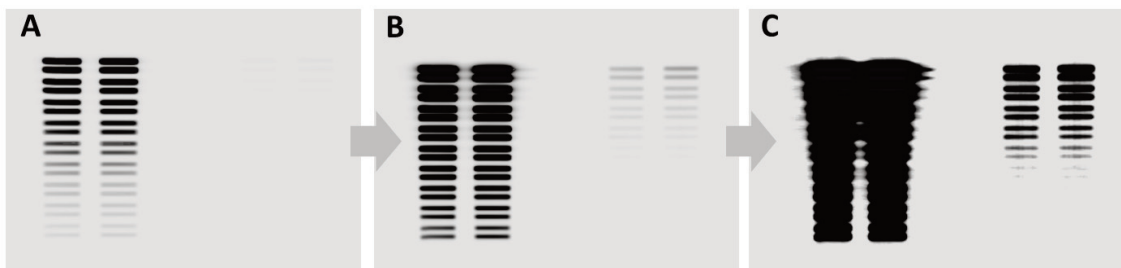


Figure 4. Strong and faint signals were detected in a single C-DiGit image. Image display adjustments were used to render images visually similar to the film exposures shown in Fig. 2, all from the single image file. Both strong and faint signals were clearly viewed. Image display settings adjust how the digital data are mapped to the pixels on the computer screen, but do not affect raw data or signal intensity values.

When PTT signals were quantified from the C-DiGit digital image, results were very different from film. Stronger signals did not plateau, and faint signals were detected with equivalent sensitivity to film. This generated a very wide linear response range. Signals were linear across 13 brightness levels, with a correlation coefficient (R^2) > 0.99 across this range. C-DiGit and film results are plotted together in Fig. 5, demonstrating the wider linear response range of C-DiGit technology.

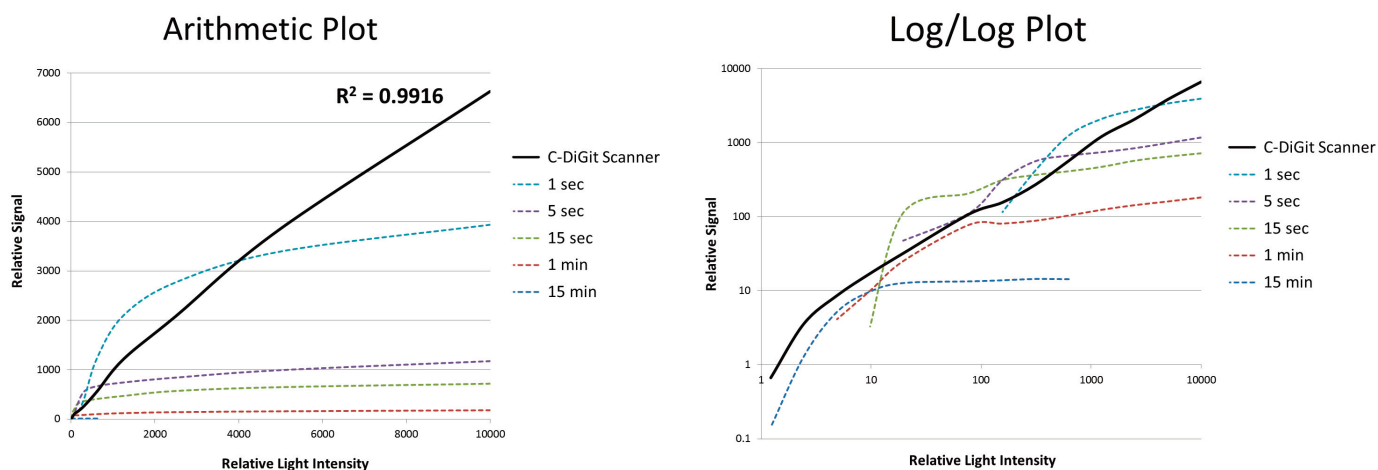


Figure 5. Linear response range of the C-DiGit Blot Scanner is much wider than film. The PTT light manifold was imaged with the C-DiGit Blot Scanner. Signals were then quantified from a single C-DiGit image (Fig. 4), and plotted as a solid black line. Arithmetic and log/log plots are shown. The single C-DiGit digital image yielded a very wide linear response range. Signals were linear across 13 brightness levels (correlation coefficient $R^2 = 0.9916$). The film exposure and densitometry results (Fig. 3) are included on these graphs for comparison. At all exposure times, strong signals quickly reached a plateau when detected with film. The linear response range of film was narrow, and the boundaries of that range were different for each exposure.

3. Signal capture: linearity and limitations of film response

The limitations of film arise from the characteristics of the photographic emulsion and its non-linear response to light^{2,3} (add LINK to photochemistry paper?).

The response of film to light is governed by the **reciprocity law**. This law states that film response is proportional to light intensity and duration of exposure. It is an inverse relationship: bright light of short duration can produce the same response as dim light of longer duration. However, when signals are very strong or very faint, this relationship falls apart and “reciprocity failure” occurs – film response is no longer proportional to light intensity and duration.^{3,5}

3.1 Faint signals: Low-intensity reciprocity failure

When light intensity is very low, film is less responsive and becomes disproportionately insensitive. Multiple photons must be absorbed by the photographic emulsion to form a stable latent image. If signals are faint and long exposures are required, a stable latent image may not form.³ This phenomenon is clearly seen in dilution series of samples (Fig. 6A), where it causes faint signals to drop off abruptly on film rather than decreasing gradually. The C-DiGit image of the same blot (Fig. 6B) shows the gradual decrease in signal that is expected for serial dilutions. Similar results were observed with the LED light manifold. (Fig. 7).

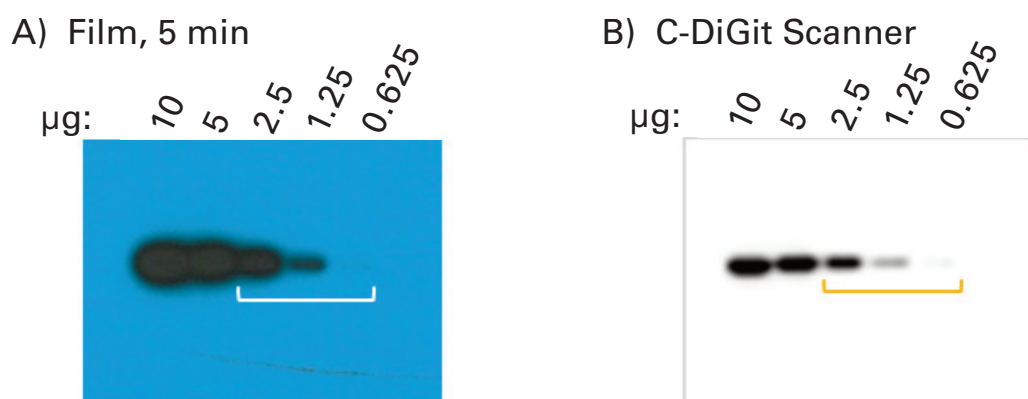


Figure 6. C-DiGit imaging reveals the expected gradual decrease in signal intensity across two-fold serial dilutions of sample. ERK2 was detected in two-fold serial dilutions of NIH-3T3 cell lysates. An experimental chemiluminescent substrate was used. A) 5-min film exposure. Fainter signals did not show a proportional decrease across the blot. B) C-DiGit image of the same blot. Signal decrease across the blot was gradual. The limit of detection for both blots was 0.625 µg.

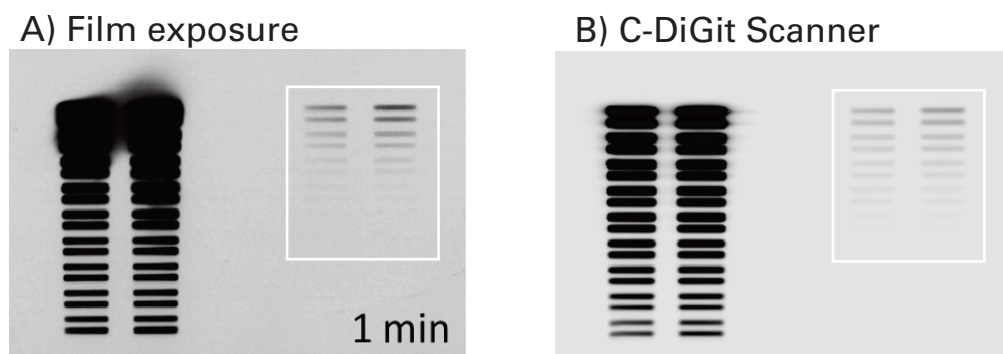


Figure 7. Unlike film, the C-DiGit image demonstrates a gradual decrease in signal. The PTT light manifold was imaged with film or the C-DiGit Blot Scanner (see Figs. 2 and 4). A) In this film exposure (1 min), faint signals dropped off quickly (boxed area) due to low-intensity reciprocity failure. B) In the C-DiGit image, signal decrease was more gradual (boxed area).

3.2 Strong signals: High-intensity reciprocity failure

The non-linear response of film to strong signals is caused by the statistics of silver grain activation.^{2,3} Two factors are involved.

- Strong signals cause saturation of film – a point when all silver grains have been activated and no more signal can be recorded, regardless of light intensity.
- Film response begins to plateau well before saturation is reached.

As the film becomes more exposed and more silver grains are activated, each new photon of light is statistically less likely to strike an unactivated silver grain.^{2,3} As a result, strong signals are under-represented and appear similarly dark (Fig. 8A). A logarithmic, non-linear increase in optical density occurs prior to saturation. The linear range of detection is very narrow (4- to 8-fold for the experiment shown in Fig. 8), and boundaries of the linear response range are different in every exposure. Imaging of the LED light manifold with film also showed loss of tonal variation in strong signals (Fig. 9).

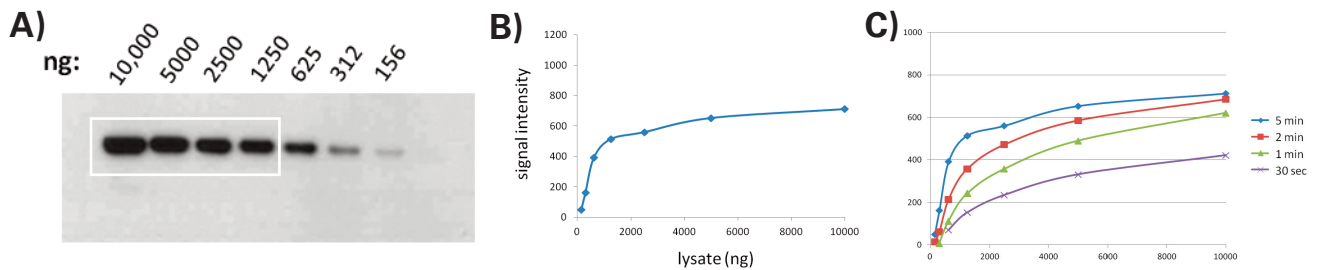


Figure 8. On film, strong signals plateau and become saturated. A) Akt was detected in serial dilutions of NIH-3T3 cell lysate, using SuperSignal® West Dura substrate and 5-min film exposure. Strong signals were underestimated; they reached a plateau and failed to show tonal variation (box). B) Densitometry clearly showed the lack of linear response at higher protein concentrations. The linear range in this experiment spanned only 3-4 dilutions (4- to 8-fold). C) Densitometry results for multiple film exposures show that strong signals reached a plateau at all exposure times. At longer exposure times, more bands were affected.

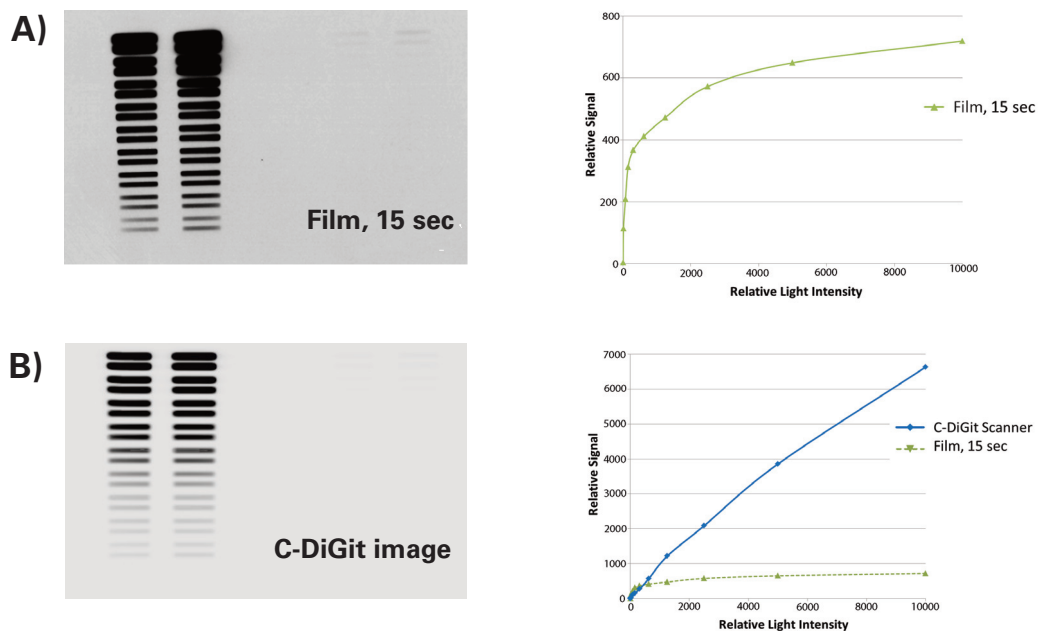


Figure 9. Unlike film, C-DiGit imaging captures the tonal variations in strong signals. A) In this film exposure (15 sec), strong signals appear similar dark. Densitometry results show a dramatic plateau in film response, due to high-intensity reciprocity failure. B) In the C-DiGit image, the expected tonal variations are seen. The data remain linear, and strong signals do not show a plateau effect.

The inability of film to accurately represent both faint and strong signals severely limits its linear response range. In these carefully controlled experiments with the PTT light manifold, which eliminates variability caused by detection chemistry, the linear response range of film was unpredictable and very limited. In contrast, C-DiGit imaging displayed a much wider linear response range.

4. Conclusions

Exposure to film is a sensitive and widely-used method for documentation of chemiluminescent Western blots. However, the inherent non-linear response of film to faint and strong signals (known as reciprocity failure) may affect data interpretation and quantification.^{2,3} In addition, methods used to digitize films and perform quantification of bands can be a significant source of variability.⁷

The experiments described here, using the PTT light manifold, demonstrate the narrow linear response range of film. When light of various intensities was imaged, the detail and nuance of these signals could not be adequately captured by film. However, the C-DiGit Blot Scanner displayed a much wider linear response range for the same intensities of light, and captured more tonal variations.

These data indicate that the C-DiGit Blot Scanner may be a more appropriate detection method for accurate quantification of protein levels on chemiluminescent Western blots. This may be particularly important when both strong and faint signals are detected on the same blot.

References

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