The Dynamic Range Effect on Protein Quantitation in Polyacrylamide Gels and on Western Blots

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Introduction

SDS-PAGE and western blotting are staple technologies in most laboratories working with proteins, and the list of detection reagents and imaging technologies to quantitate the associated data has been growing rapidly. However, for most laboratories, Coomassie gel staining using traditional reagent recipes and chemiluminescent western blots using HRP-conjugated secondary antibodies have remained the tools of choice for protein research. Furthermore, images of gels have been produced using a wide variety of equipment, from basic digital cameras to office scanners, and western blots are still being imaged using film. These traditional technologies have been used mostly for qualitative analyses to demonstrate the presence or absence of proteins in samples and their level of purity relative to other proteins.

In the post–human genome era, proteomics is taking on a prominent role in most basic research labs, and the demand for accurate, quantitative data from protein gels and western blots is rapidly increasing. This has prompted an interest in new reagents, instruments, and software that will allow researchers to quantitate relative band density between samples but also to push the limits of sensitivity to detect elusive low-abundance proteins in their samples. The biotechnology community has responded with a variety of instruments, software, and reagents for accurate and sensitive quantitative protein research. However, with so many available technologies, the job of selecting the right combination to assure quality results can be time consuming.

This tech note examines traditional techniques and some new detection reagents coupled with instrument technologies that are optimized to produce sensitive, quantitative image data from stained protein gels and western blots. The pitfalls and advantages of each technology are shown using a consistent set of samples, along with the key steps that should be taken for experiments that require accurate, quantitative data for publication.

The results demonstrate the importance of determining the linear range of detection for the gel/membrane/antibody combination by performing a dilution series of a representative experimental sample. This will assure an appropriate working concentration with accurate relative densitometries of the protein bands.

Methods

Protein Sample Preparation and Separation

A mixture of maltose binding protein (MBP) and tagged MBP, purified from the Profinity eXact™ lyophilized lysate was prepared to load as a standard dilution series from 1,500 ng to 0.6 ng on Criterion™ 4–15% gradient gels (Bio-Rad Laboratories, Inc.). All sample wells were loaded with 20 µl of protein in Laemmli buffer with separation using the Criterion™ Dodeca™ cell (Bio-Rad) for 1 hr at 200 V.

Coomassie Staining

Gels were fixed and then stained in Bio-Safe™ Coomassie stain (Bio-Rad) for approximately 2 hr at room temperature with destaining in water.

Western Blots

For blotting procedure details, see Bio-Rad bulletin 5723. In brief, protein gels were incubated for 5 min in western transfer buffer and blotted to low-fluorescence FluoroTrans membrane (Pall Corporation) using Criterion staining trays (Bio-Rad). Membranes were blocked in 4% milk in TBST (Tris buffered saline containing 0.2% Tween-20) for 1 hr, washed four times in TBST, and incubated overnight at 4°C in 12 ml of TBST with the Profinity eXact monoclonal antibody (mAb) (Bio-Rad) (1/1,000) or rabbit anti-MBP (Bio-Rad) (1/1,000).

For chemiluminescent detection, selected membranes were washed four times in TBST and incubated with goat anti-mouse HRP (1/20,000) for 1 hr in TBST, washed four times in TBST, and incubated for 5 min in Immun-Star™ Western C™ chemiluminescent substrate (Bio-Rad) prior to image acquisition.

For multifluorescent detection, selected blots that were first incubated with the Profinity eXact mAb were washed four times in TBST and then incubated with the rabbit anti-MBP polyclonal

antibody for 3 hr in TBST. Membranes were washed in TBST and sequentially incubated for 1 hr with the Qdot 625 goat anti-rabbit antibody conjugate (Invitrogen Corporation) in TBST with 1% milk, followed by three washes and a 1 hr incubation with the Qdot 525 goat anti-mouse antibody conjugate (Invitrogen) in TBST with 1% milk, followed by four washes in TBST.

Detection

The chemiluminescent blots were imaged on the Molecular Imager® ChemiDoc™ XRS system (Bio-Rad) with no filter. The multifluorescent blots were imaged on the ChemiDoc XRS system with excitation in the UV range and emission was captured using the SYBR® Green and SYPRO Ruby 62 mm standard filters for the Qdot 525 and Qdot 625 conjugates, respectively. All blots and gels were imaged using the chemi high sensitivity setting. For the Coomassie-stained gels, a Molecular Imager® GS-800™ calibrated densitometer (Bio-Rad) was used, and Quantity One® 1-D analysis software (Bio-Rad) was used for image acquisition.

Band Analysis

The band analysis tool of Quantity One software was used for background subtraction and to determine the density of the bands in all gels and blots.

Results

Sensitivity and Dynamic Range for Coomassie-Stained Polyacrylamide Gels

It is generally accepted that the limit of detection (LOD) for Coomassie R-250 staining ranges between 36 and 47 ng with a dynamic range of one order of magnitude. Bio-Safe Coomassie stain, which uses Coomassie G-250, provides a less toxic solution for protein gels by allowing destaining in water (see Bio-Rad bulletin 2423). Moreover, Bio-Safe Coomassie stain provides a much lower LOD of 8–28 ng than does the traditionally used Coomassie Brilliant Blue R-250 (Figure 1).

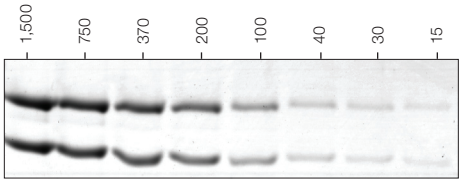

Fig. 1. Dynamic range of Coomassie staining. Dilution series of MBP (lower band) and Profinity eXact fusion-tagged MBP samples (upper band) were separated on a Criterion gel and stained with Bio-Safe Coomassie stain. Quantity of protein loaded in each lane is indicated in nanograms.

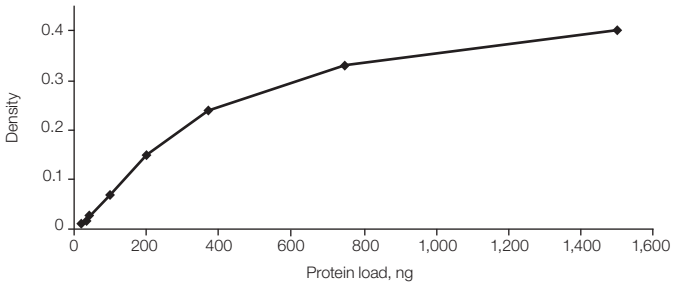

Fig. 2. Densitometry plot of the Profinity eXact fusion-tagged MBP bands in Figure 1.

The relative density of the bands increased linearly between 15 ng and 200 ng (Figure 2), demonstrating a linear dynamic range of about one order of magnitude (tenfold) for relative quantitation.

Chemiluminescent vs. Fluorescent Western Blots

The dilution series of the MBP and Profinity eXact fusion-tagged MBP samples were blotted to directly compare the LOD and linear dynamic range of chemiluminescence and fluorescence. Both the chemiluminescent and the fluorescent blots used the same primary antibody (Profinity eXact mAb). Higher LOD and linear dynamic range were observed with the fluorescent secondary antibody conjugate (Figures 3 and 4).

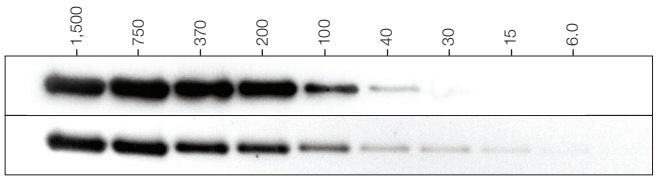

Fig. 3. Comparison of the dynamic range of chemiluminescent and fluorescent detection. Serial dilutions of Profinity eXact fusion-tagged MBP were blotted and detected using goat anti-mouse HRP (top panel) and Qdot 525 goat anti-mouse secondary antibody conjugates (bottom panel). The primary antibody was raised against the Profinity eXact affinity tag. Quantity of protein loaded in each lane is indicated in nanograms.

For the Qdot 525 secondary antibody conjugate, the density of the signal values increased linearly between 15 ng and 200 ng (Figure 4A) with good correlation between the difference in density vs. protein load (Figure 4B).

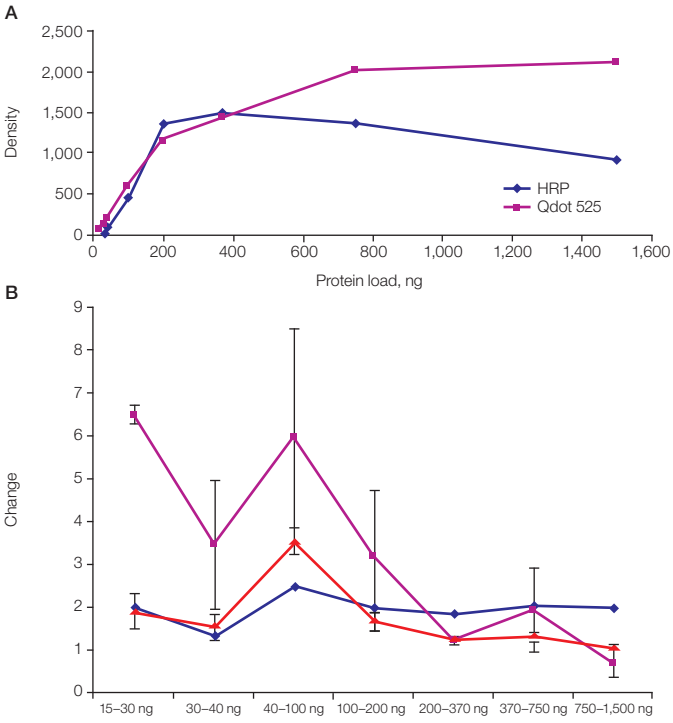

Fig. 4. Correlation between relative signal intensity and protein concentration. A, intensity plot of the protein bands shown in Figure 3; **B**, comparison of change in protein concentration (◆) and corresponding chemiluminescent (■) and fluorescent signal intensity (▲) over a range of protein quantities. Error bars represent the standard deviation.

In contrast, the chemiluminescent blot produced a linear increase in density between 30 ng and 200 ng (Figure 4A) but with poor correlation between the change in density vs. protein concentration at lower concentrations (Figure 4B).

Multifluorescent Western Blots

The LOD of the multifluorescence western blot generated with the anti-rabbit MBP and the Profinity eXact mAb coupled with the Qdot 525 goat anti-mouse and Qdot 625 goat anti-rabbit secondary antibody conjugates were compared (Figure 5). Images of each Qdot fluorophore were taken successively by switching the filter positions from SYBR® Green to SYPRO Ruby. The membranes were kept wet to maximize the fluorescent signal. Quantity One image analysis software was then used to overlay the images. For Qdot 525 and Qdot 625 fluorophores, the signal intensity values increased linearly between 15 ng and 200 ng and between 1.5 ng and 40 ng, respectively (Figure 6), with good correlation between the relative increase in density vs. protein load.

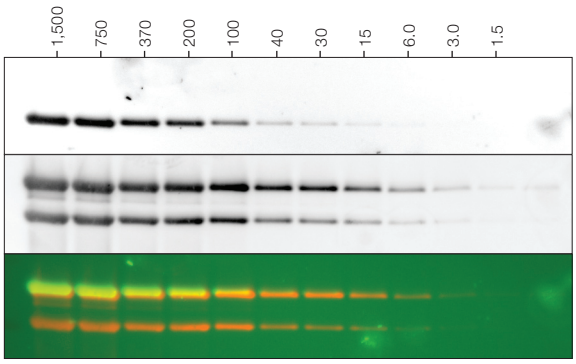

Fig. 5. Multifluorescent western blot detection of MBP, and Profinity eXact fusion-tagged MBP bands using Qdot 625 and Qdot 525 conjugates. Fluorescent Qdot 525 (top panel) and Qdot 625 (middle panel) secondary antibody conjugates were used to probe a blot of MBP and Profinity eXact fusion-tagged MBP. The blot was imaged using the ChemiDoc XRS imager with UV excitation. Separate images were taken by switching between the SYBR® Green (Qdot 525) and SYPRO Ruby (Qdot 625) filters (bottom panel). Quantity of protein loaded in each lane is indicated in nanograms.

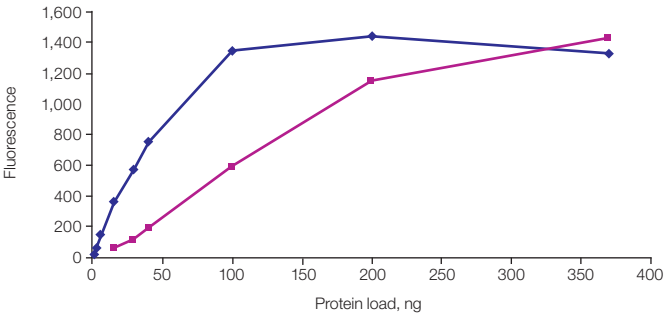

Fig. 6. Comparison of the dynamic range of QDot 525 and QDot 625 antibody conjugates. The intensities of the Qdot 525 (■) and Qdot 626 (◆) fluorescent signals obtained in Figure 5 were measured using Quantity One software.

Conclusions

SDS-PAGE Dynamic Range for Quantitation of Proteins

All protein dyes bind to specific amino acids. Since proteins have different compositions of amino acids, the intensity of the coloration of individual proteins will vary, resulting in different saturating concentrations and LOD for each protein. Furthermore, staining of a protein band in a gel can reach saturation above a critical protein amount. This effect is the same for any protein, gel, and dye combination, and therefore, if relative quantitation between bands is required, a dilution series of one of the experimental samples should be performed to determine the linear range of protein concentrations that can be used for reliable quantitation. Bio-Safe Coomassie stain from Bio-Rad offers a good alternative to traditional Coomassie R-250 stains for increased sensitivity and dynamic range with reduced toxicity. Bio-Safe Coomassie stain used with a GS-800 calibrated densitometer forms an excellent combination of equipment and reagent for accurate quantitation of protein band density or spot detection on 2-D gels. For larger dynamic ranges, fluorescent dyes such as Flamingo™ fluorescent gel stain (Bio-Rad) are required, along with an imager that will excite and detect fluorescence at the appropriate wavelengths. The ChemiDoc XRS system from Bio-Rad is very well suited for this application.

Chemiluminescent and Fluorescent Western Blotting

The sensitivity and linear dynamic range for western blots are dependent on three key factors.

One factor is the binding capacity and specificity of the primary antibody. A primary antibody with low specificity will result in detection of multiple proteins, and low binding capacity will result in poor detection and LOD.

Another factor is the type of secondary antibody used (chemiluminescent or fluorescent). Chemiluminescent detection uses HRP-conjugated secondary antibodies. Detection is done with an instrument that can detect the low emission of light or by exposure to light-sensitive film. Fluorescent detection takes advantage of a large variety of fluorophores conjugated to secondary antibodies. Many fluorophores are excited with UV radiation and emit at specific and distinct wavelengths, making them amenable to simultaneous use to produce multifluorescent blots. Fluorescent probes also have a much higher and more stable intensity of signal than does chemiluminescence. Fluorescent probes do not require any substrate, and they can be visualized on a variety of imagers.

A third factor is the binding capacity of the membrane used for blotting. Proteins tend to form layers on the membrane surface when applied at high concentrations, and they ultimately saturate the membrane. Higher loads therefore do not result in higher activity due to steric hindrance or other phenomena and result in a plateau of signal intensity.

Chemiluminescence

Chemiluminescent detection has been the method of choice for western blotting due to its sensitivity and because it does not require sophisticated detection intruments. Chemiluminescent detection relies on an enzymatic reaction that produces light that can be captured on regular photographic film. However, this reaction will decrease in intensity as the enhanced chemiluminescent substrate is depleted. The time dependence of the signal intensity compromises accurate quantification. To assure accurate quantitative data, it is important that the amount of substrate over each band is in excess and that the timing for detection is such that no significant reagent depletion over each band occurs. It is also important to avoid the extreme situation known as burn through, in which most of the detection reagent is depleted over the most concentrated protein bands. This can cause poor correlation between protein amounts and measured band density, as was observed in this study.

Fluorescence

Emission of fluorescence from a fluorophore is constant over time. Proteins can therefore be accurately quantified because the signal generated by the different amounts of proteins on the membranes is measured in a static state. This makes fluorescent detection more precise and accurate than chemiluminescence for measuring relative signal intensities, but it does not eliminate the dynamic range effect.

Each of the Qdot fluorophores is excited optimally in the UV range and emits at specific wavelengths with narrow band width and no photobleaching. This allows multiple detections on a single blot when primary and Qdot secondary antibody conjugate combinations with no cross-reactivity and with distinct emission spectra are chosen. In this study, a multifluorescent western blot was performed with Qdot 525 goat anti-mouse and Qdot 625 goat anti-rabbit conjugates. The linear range of quantitation and associated correlation of band intensity was between one and two orders of magnitude for the Qdot conjugates with good correlation between the relative increase in density and protein concentration.

Finally, the key point for any technology using antibodies for signal detection is that the LOD and dynamic range are entirely dependent on the binding capacity (Kd) of the primary antibody used and the intensity of signal output from the secondary detection antibody. Antibodies from different sources can have widely different binding capacities, which must be taken into consideration when performing quantitative western blots. It was observed in this study that the binding capacity of the Profinity eXact mAb was lower than that of the rabbit anti-MBP Ab, which gave an apparent lower LOD and higher dynamic range for Qdot 625 conjugates.

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