blotting

Molecular Weight Estimation and Quantitation of Protein Samples Using Precision Plus Protein™ WesternC™ Standards, the Immun-Star™ WesternC™ Chemiluminescent Detection Kit, and the Molecular Imager® ChemiDoc™ XRS Imaging System

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Introduction

Western blotting, also referred to as immunoblotting, is a widely used and sensitive technique for the detection and characterization of proteins. This technique takes advantage of the specificity of antibody recognition toward an antigenic sequence in the protein of interest (Burnette 1981, Towbin et al. 1979). In a typical western blotting protocol, solubilized protein is separated by SDS-PAGE and electrophoretically transferred to a membrane, such as nitrocellulose or PVDF (Figure 1). The transferred protein is irreversibly bound to the surface of the membrane, allowing access to the specific antigens recognized by monoclonal or polyclonal antibodies, and to the detection reagents used to develop the blotted membrane (blot). After the remaining binding sites are blocked with a detergent or protein reagent, the blot is probed with the primary antibody and then washed. The antibody-antigen complexes are subsequently identified by horseradish peroxidase (HRP) or alkaline phosphatase (AP) coupled to the secondary anti-IgG antibody. A variety of colorimetric and chemiluminescent detection substrates are available to visualize the blot, and film or CCD-based digital imaging systems are then used to document the detected signal. Achieving the desired results from a western blotting experiment can depend on a number of critical factors at each step of the process.

Among the various protocols available, the choice of detection substrates and documentation methods can have the greatest impact on the quality of the data obtained from a western blot. Chemiluminescent detection using a digital imaging system offers a number of important advantages over film-based or colorimetric procedures. In general, chemiluminescent substrates offer greater sensitivity over traditional colorimetric methods (Sandhu et al. 1991). Furthermore, a chemiluminescent signal can be recorded using a digital imaging system, and varying the exposure time is one method that can be used to optimize the signal intensity and the ratio of signal to noise on the blot.
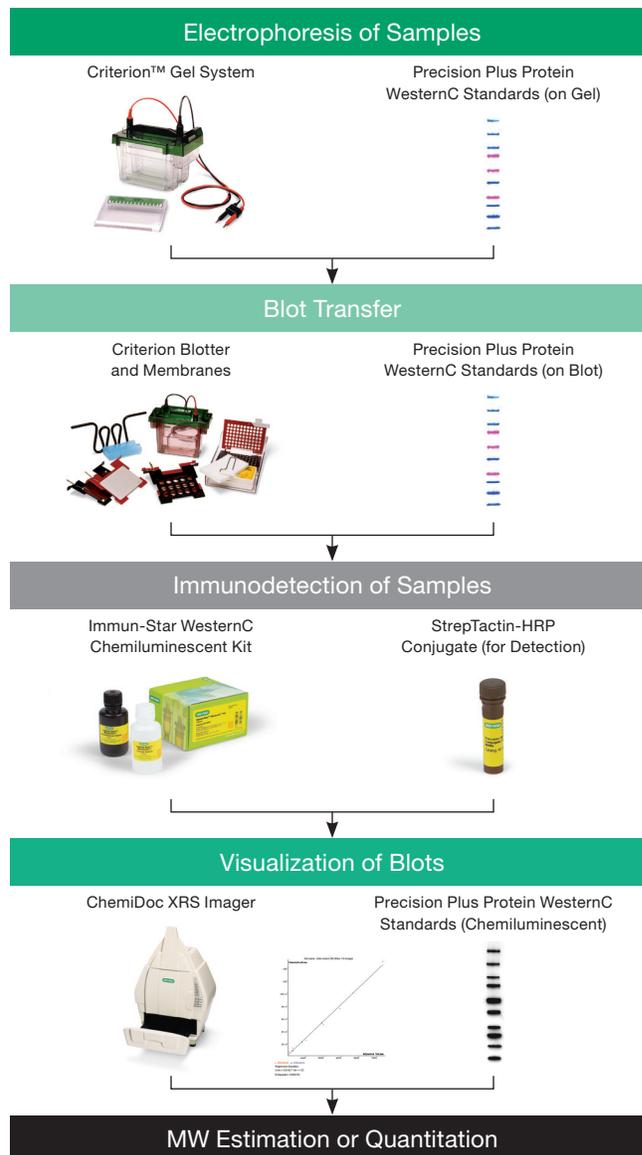

Fig. 1. Western blotting workflow from SDS-PAGE to visualization and analysis. The appearance of Precision Plus Protein WesternC standards at each step in generating a chemiluminescent western blot is shown on the right. Key equipment and reagents are shown on the left.

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A common objective of western blotting experiments is to obtain quantitative information about a protein of interest, such as estimating molecular weight (MW) or the concentration of a protein in a complex sample. To this end, chemiluminescent images captured on digital documentation systems possess a wider dynamic range and are easier to quantitate by densitometry compared to colorimetric detection or film-based techniques.

Protein standards are a key component in the analysis of a western blotting experiment. Standards used for western blots can provide reliable MW estimation and can also serve as an internal control for monitoring the progress of the electrophoresis run and the efficiency of the blot transfer step. A number of protein MW markers are available, including prestained standards, unstained standards, and standards for chemiluminescent detection. To ensure accurate MW estimation of a protein on a western blot, standards must contain enough sharp, distinct bands of known MW that are visible following development of the chemiluminescent signal. Additionally, the ability to visualize protein standards (for example, by using prestained standards) during the electrophoresis run and directly on the blot following the transfer step is also advantageous. Prestained protein standards also allow the efficiency of the protein transfer step to be determined prior to continuing with a western blotting experiment, potentially saving valuable time, labor, and materials.

Precision Plus Protein* WesternC standards are recombinant protein markers optimized for direct visualization on gels and transferred blots, as well as for parallel detection in chemiluminescent western blotting applications (Figure 1). The standards consist of a combination of ten dual color bands, 10–250 kD, that are prestained blue or pink for easy band referencing, protein transfer confirmation, and blot orientation. An integrated *Strep*-tag sequence permits detection of all ten bands on film or CCD-based digital imaging systems when developed with chemiluminescent substrates and a StrepTactin-HRP conjugate. The WesternC standards are carefully engineered to generate sharp bands with balanced prestained and chemiluminescent intensities on PVDF and nitrocellulose membranes and can be used for accurate estimation of MW directly from blots.

The Immun-Star WesternC detection kit provides a sensitive chemiluminescent substrate for HRP detection with digital imaging systems. The WesternC substrate is compatible with most HRP-conjugated secondary antibodies and is specifically designed to generate rapid, intense light emission of extended duration, which is optimal for use with CCD-based imagers. Digital CCD-based imagers, such as the ChemiDoc XRS system, offer the advantages of instant image manipulation, greater resolution, and a larger dynamic range than film; however, CCD-based imagers perform optimally with a substrate that produces a strong signal of sufficient duration. The WesternC detection kit produces an intense chemiluminescent signal capable of detecting femtogram levels of protein for up to 24 hr on PVDF or nitrocellulose membranes and allows the capture of digital images that can be analyzed qualitatively and quantitatively.

This study demonstrates the performance and reliability of the Precision Plus Protein WesternC standards, Immun-Star WesternC chemiluminescent detection kit, and ChemiDoc XRS imaging system when used to estimate the MW and amount (in ng) of protein samples in a western blotting experiment. In one experiment, the WesternC standards were used to construct a standard curve of log MW versus relative migration distance (R_f) from blots, and the accuracy and linearity of the markers were checked against a control protein of known MW. The MW of a test protein was then calculated by interpolation of the standard curve plot based on the R_f value of the test sample. In another experiment, a dilution series of a purified control protein of known concentration was used to construct a standard curve of signal volume versus protein amount, and the accuracy of the plot was checked at three separate unknown amounts on a blot. Using the same methodology, a purified test protein was used to construct a standard curve, and the amounts of test protein in a dilution series of a crude sample were calculated from the curve.

Methods
Sample Preparation
As a control, purified actin from bovine muscle (Sigma A3653) was prepared in a TE buffer, pH 8.0, to a stock concentration of 1 mg/ml. A purified 27 kD protein was prepared by immobilized metal affinity chromatography (IMAC) from a crude *E. coli* lysate sample obtained from bacterial cells expressing a recombinant 27 kD protein. Crude *E. coli* lysate test sample was prepared by mechanical disruption of the cells at a 1:10 (w/v) ratio in 50 mM potassium phosphate, 300 mM sodium chloride buffer, pH 8.0, then clarification by centrifugation. Purified 27 kD protein was eluted from the IMAC column and prepared in a 50 mM potassium phosphate, 300 mM sodium chloride, 20 mM imidazole buffer, pH 8.0, to a stock concentration of 1 mg/ml. Concentrations of the actin control and the 27 kD protein stock solutions were calculated and adjusted to 1 mg/ml using absorbance measurements at 280 nm. Dilutions of the purified actin and the 27 kD protein were prepared in Laemmli

sample buffer (Bio-Rad catalog #161-0737) from the 1 mg/ml stock solutions to final protein amounts of 200, 150, 100, 75, 50, 25, 12.5, 6.2, 3.1, and 1.5 ng per 5 µl gel sample load. For the quantitation experiments (unknown protein samples #1, #2, and #3), dilutions of actin and the crude *E. coli* lysate containing the 27 kD protein were prepared in Laemmli sample buffer to final target protein amounts of 50, 25, and 12.5 ng per 5 µl gel sample load. The actual protein amount for the 27 kD protein in the unknown samples was estimated using densitometry of Coomassie Blue-stained gels. Known amounts of the purified 27 kD protein and serial dilutions of the crude *E. coli* lysate used in this study were separated by SDS-PAGE and compared to generate an estimate of target protein contained within the lysate. For mass spectrometric analysis, samples were desalted into water using Micro Bio-Spin™ columns with Bio-Gel® P-6 gel. MW of the purified sample proteins was confirmed by MALDI-TOF analysis on an Applied Biosystems 4700 proteomics analyzer. The manufacturer's reported MW for bovine actin is 42–43 kD.

Gel Electrophoresis and Staining

Laemmli gel samples were heated at 95°C for 5 min, and 5 µl of sample and Precision Plus Protein WesternC standards (catalog #161-0376) were run in duplicate on Criterion 4–20% Tris-HCl precast gels in a Criterion cell at 200 V for 60 min. Following electrophoresis, one gel was blotted and the other gel was stained with Bio-Safe™ Coomassie Blue G-250 stain according to standard procedures for fixing, staining, and destaining. Stained gels were imaged on a Molecular Imager® GS-800™ calibrated densitometer using Quantity One® 1-D analysis software.

Western Blotting and Immunodetection

Following separation by SDS-PAGE, gels were transferred to Criterion 0.45 µm nitrocellulose/filter paper sandwiches using a Criterion blotter with plate electrodes at 100 V for 30 min. Immunodetection was carried out as described in the manual included with the Immun-Star WesternC chemiluminescent detection kit, at room temperature with agitation. Tris-buffered saline with 0.05% Tween 20 (TTBS) was used as a diluent and for all wash steps. Briefly, membranes were blocked for 1 hr in TTBS with 3% BSA (Fraction V, EMD 2930) and rinsed 3 times for 5 min. For actin immunodetection, membranes were incubated with primary antibody using a 1:5,000 dilution of mouse monoclonal anti-actin (Sigma A4700) for 1 hr. For immunodetection of the 27 kD test protein, membranes were incubated with primary antibody using a 1:5,000 dilution of purified rabbit polyclonal for 1 hr. Membranes were washed 5 times for 5 min and incubated with secondary antibody using a 1:50,000 dilution of goat anti-mouse (GAM)-HRP conjugate for actin (catalog #170-5047) or goat anti-rabbit (GAR)-HRP conjugate for the 27 kD protein (catalog #170-5046) for 1 hr. During the secondary antibody step, the membranes were

also incubated using a 1:10,000 dilution of StrepTactin-HRP conjugate for immunodetection of the Precision Plus Protein WesternC standards. Membranes were washed 6 times for 5 min and were then incubated with the Immun-Star WesternC chemiluminescent detection kit reagents at 0.1 ml/cm² membrane for 5 min.

Image Capture and Quantitation

Membranes were visualized on a ChemiDoc XRS system and analyzed using Quantity One software. Images were captured on the Chemiluminescence setting, and blot exposure times were adjusted to minimize the number of saturated pixels present in the bands used for quantitation. For MW analysis, R_f of the bands was measured using Quantity One software on the images obtained from the blots. Data were exported to Microsoft Excel software to calculate a standard curve for the protein standards as described in the Results and Discussion section and Bio-Rad bulletin 3133 (2004). To quantitate the amount of protein present on the blot, signal volumes for actin and the 27 kD protein were measured with Quantity One software using volume analysis. Data were exported to Microsoft Excel to calculate a standard curve for the dilution series as described in the Results and Discussion section.

Results and Discussion

MW Analysis

A well-characterized protein of known MW, bovine actin (~42 kD), and a 27 kD protein were separated by SDS-PAGE alongside the Precision Plus Protein WesternC standards, transferred to blots, and visualized by chemiluminescent detection (Figure 2). Electrophoretic R_f of the standards, actin, and the 27 kD protein are presented in Table 1. R_f values for the standards were plotted versus log MW to generate a standard curve for the Precision Plus Protein WesternC standards. A linear fit to the data points was calculated, and the corresponding R^2 values for the standards on each blot are shown in Figure 3. The accuracy of the calculated MW for the sample proteins is dependent on the linearity of the standard curve generated for the markers, represented by the R^2 value. The closer the R^2 value is to 1.0, the better the fit of the data points to a trend line. The strong linear relationship ($R^2 > 0.99$) between the standard proteins' MW and R_f values demonstrates a high degree of reliability in predicting the MW of the sample proteins. The standard curves in Figure 3 were used to calculate the MW of actin and the 27 kD protein; the results are shown in Table 2. The differences between the calculated and the mass spectrometry-determined MW were within 3% for both proteins (Table 2). The degree of accuracy of the calculated MW for each sample confirms that the Precision Plus Protein WesternC standards provide a robust and direct method for protein MW estimation from western blots.
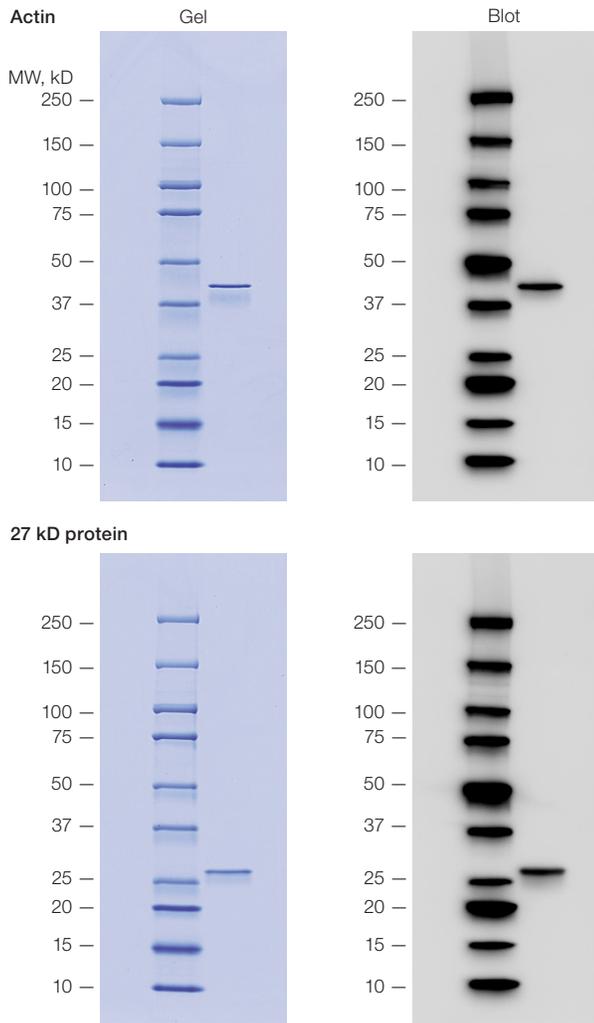
Actin Gel Blot

MW, kD
250 —
150 —
100 —
75 —
50 —
37 —
25 —
20 —
15 —
10 —

250 —
150 —
100 —
75 —
50 —
37 —
25 —
20 —
15 —
10 —

27 kD protein

250 —
150 —
100 —
75 —
50 —
37 —
25 —
20 —
15 —
10 —

250 —
150 —
100 —
75 —
50 —
37 —
25 —
20 —
15 —
10 —

Fig. 2. SDS-PAGE and western blot analysis with Precision Plus Protein WesternC standards. Lane 1, WesternC standards; lane 2, sample: 400 ng of purified bovine actin or purified 27 kD recombinant protein. Left panels, Coomassie Blue-stained gels; right panels, chemiluminescent blots.

Table 1. Measurement of R_f values for Precision Plus Protein WesternC standards, actin (~42 kD), and the 27 kD protein from western blots. Each blot was independently analyzed.

Actin Blot		27 kD Protein Blot	
MW of Standards and Protein Sample	R_f Value	MW of Standards and Protein Sample	R_f Value
250	0.187	250	0.205
150	0.265	150	0.283
100	0.343	100	0.365
75	0.402	75	0.423
50	0.492	50	0.511
37	0.572	37	0.588
25	0.668	25	0.680
20	0.717	20	0.726
15	0.792	15	0.795
10	0.861	10	0.868
Actin	0.539	27 kD protein	0.656

Actin

$R^2 = 0.9953$

log MW
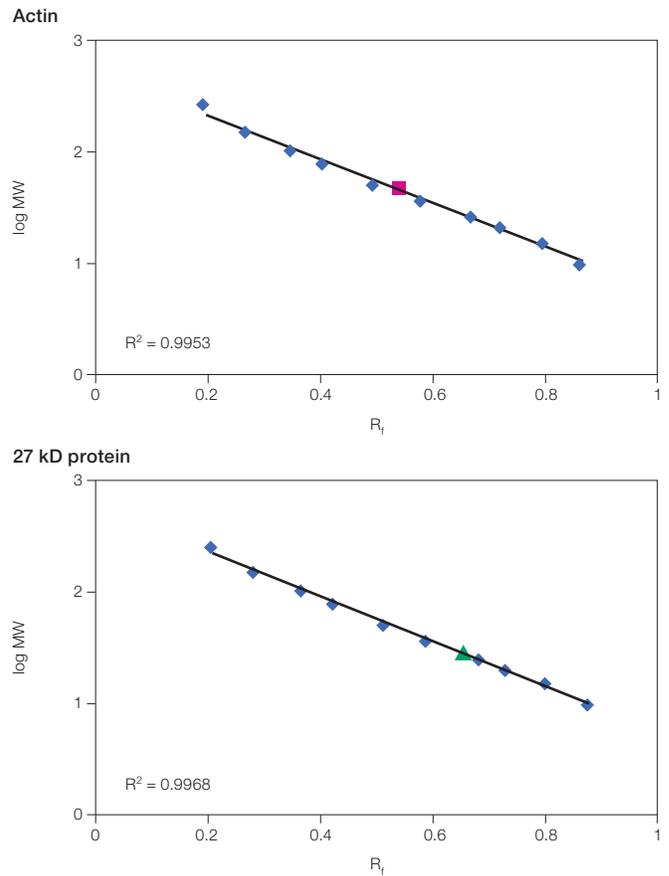
R_f

27 kD protein

$R^2 = 0.9968$

log MW

R_f

Fig. 3. Determining the MW of a protein using Precision Plus Protein WesternC standards. Standard curves of log MW versus R_f were generated using Precision Plus Protein WesternC standards from chemiluminescent-detected western blots. Linear fits to the standards' data points (10–250 kD, ◆) are shown. Curve for actin (■) blot, y = –1.97x + 2.70, curve for the 27 kD protein (▲) blot, y = –2.02x + 2.76. The high R^2 values (>0.99) obtained from the standard curves demonstrate the exceptional linearity of the Precision Plus Protein WesternC standards.

Table 2. Comparison of actual versus calculated MW for actin and the 27 kD protein from western blots using Precision Plus Protein WesternC standards.

Protein Sample	Actual MW (Mass Spectrometry)	Calculated MW (WesternC Standards)	Difference, %
Actin	42.49	43.77	3.0
27 kD protein	27.78	27.15	2.3

Actin

200 150 100 75 50 25 12 6 3 1 ng   #1 #2 #3

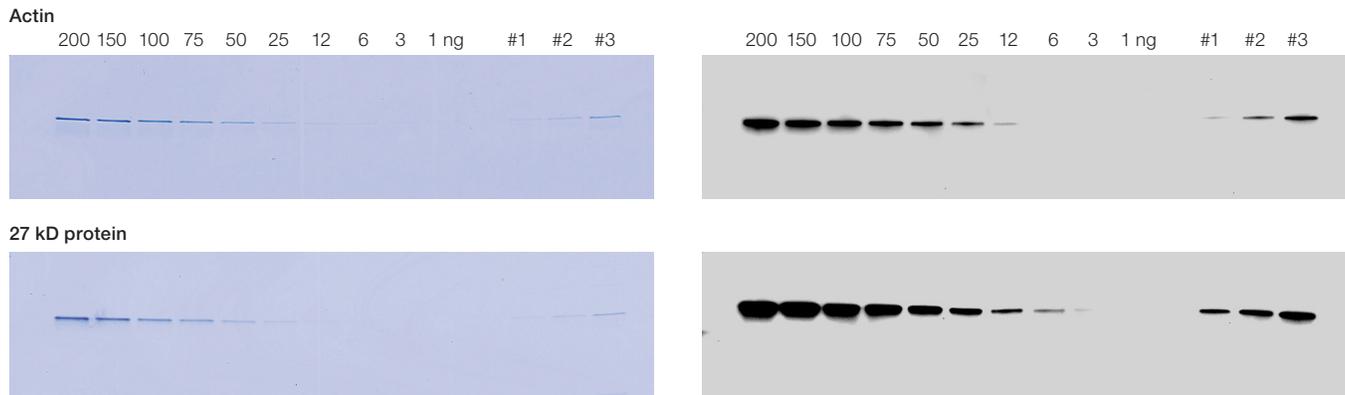200 150 100 75 50 25 12 6 3 1 ng   #1 #2 #3

27 kD protein

Fig. 4. Dilution series and three unknown amounts of actin and the 27 kD protein detected on chemiluminescent western blots. Samples were purified bovine actin or purified 27 kD protein and 27 kD protein from *E. coli* lysate. The amounts of protein loaded for the unknown samples were: #1, 12.5 ng; #2, 25 ng; #3, 50 ng. Left panels, Coomassie Blue-stained gels; right panels, chemiluminescent blots.

Protein Quantitation

A dilution series and three unknown dilutions of bovine actin and a 27 kD protein were separated by SDS-PAGE, transferred to blots, and subsequently visualized by chemiluminescent detection following the protocol for the Immun-Star WesternC chemiluminescent detection kit (Figure 4). Signal volumes for actin and the 27 kD protein bands were measured, and local background correction was applied for each band by normalizing to an adjacent region on the blot. The background-adjusted signal volume of each band in the dilution series was plotted against amount of protein (except for 200 ng) to generate a standard curve. A linear fit to the data points was calculated, and the corresponding R^2 value for actin and the 27 kD protein are presented in Figure 5. The high R^2 values ($R^2 > 0.99$) from the standard curves generated for both samples indicate a strong linear relationship between signal volume and amount of protein over a wide dynamic range. Based on the standard curve plot from the actin dilution series, the amounts of purified actin in three dilutions were calculated using the background-adjusted volumes for each band (Table 3). The results obtained from this analysis confirm that this is a reliable method to obtain quantitative information, such as protein amount, from a western blot.
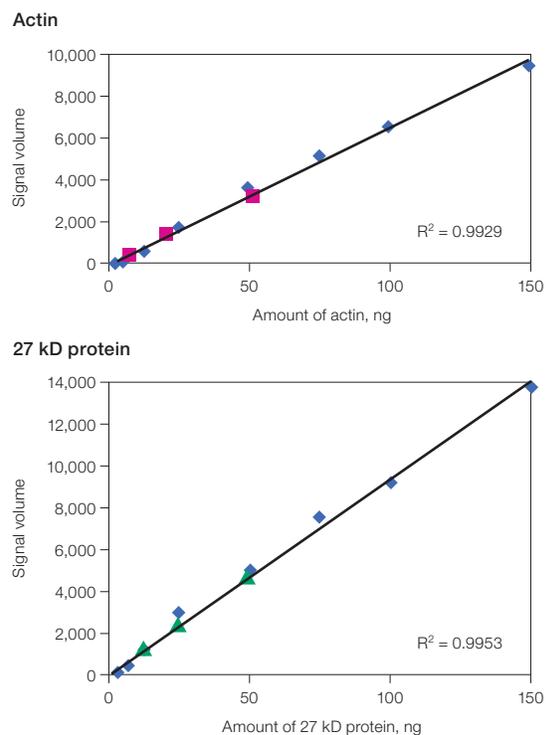
Actin

27 kD protein

Fig. 5. Determining the linearity of protein quantitation from chemiluminescent western blots using the Immun-Star WesternC kit and ChemiDoc XRS system. Standard curves of signal volume versus protein amount (in ng) were generated using data from the dilution series of actin and the 27 kD protein. Linear fits to the dilution series' data points (◆) are shown with the corresponding R^2 values. Upper panel, curve for purified actin dilution series (150–3.1 ng) and the unknown dilutions of actin (■), $y = 64.84x + 13.66$. Lower panel, curve for purified 27 kD protein dilution series (150–1.5 ng) and the unknown dilutions of 27 kD protein from an *E. coli* lysate (▲); $y = 92.56x + 231.96$. The strong linear relationship ($R^2 > 0.99$) between signal volume and protein amount from the dilution series demonstrates the reliability of this method for estimating the amount of protein from western blots.

Using the same methodology, three unknown amounts of a 27 kD protein in an *E. coli* lysate sample were calculated using the standard curve derived from the purified form of the protein and the background-adjusted volumes for the unknown bands (Table 3). In this example, the accuracy of the calculated amount of the 27 kD protein in the crude lysate was within approximately 2% of the estimated amount of protein. Based on the results from both proteins examined using this method, it can be concluded that the accuracy of protein quantitation is highest when the unknown samples fall within the middle of the standard curve for the dilution series. Taken together, these results demonstrate that chemiluminescent data obtained from the Immun-Star WesternC kit and the ChemiDoc XRS imager can be used to obtain reliable quantitative sample information directly from western blots.

Table 3. Comparison of actual versus calculated protein amounts for actin and the 27 kD protein from western blots using the Immun-Star WesternC chemiluminescent detection kit.

Protein Sample	Adjusted Signal Volume	Actual Protein Amount, ng	Calculated Protein Amount, ng
Actin			
Unknown #1	480	12.5	7.2
Unknown #2	1,367	25.0	20.9
Unknown #3	3,349	50.0	51.4
27 kD Protein			
Unknown #1	1,401	12.5	12.6
Unknown #2	2,572	25.0	25.3
Unknown #3	4,794	50.0	49.3

Conclusions

The performance of the Precision Plus Protein WesternC standards and the Immun-Star WesternC chemiluminescent detection kit have been tested in two typical western blotting applications. Estimation of MW from western blots is a common technique used to characterize proteins and depends upon reliable markers to obtain accurate measurements. Precision Plus Protein WesternC standards have ten sharp, prestained bands across a broad MW range that are visible at all steps of a western blotting experiment. In this study, the standards provided an accurate method of MW estimation for two independent protein samples on chemiluminescent western blots. While convenient and accurate MW estimation from blots is the principal benefit of the Precision Plus Protein WesternC standards, the benefits of direct band visualization during all steps of the process is a clear advantage over unstained protein markers (see Figure 1).

In addition to MW analysis, it is also possible to quantitate the amount of a target protein in a western blotting experiment using a known, purified control protein to construct a standard curve. This technique can be particularly useful when trying to estimate the amount of a protein expressed in a complex sample, such as a cell lysate. Chemiluminescent detection is a highly sensitive and well-proven method for visualizing proteins on western blots that can take advantage of the quantitative capabilities inherent in digital imaging systems. The Immun-Star WesternC chemiluminescent detection kit is optimized to produce an intense signal of prolonged duration suitable for the requirements of CCD-based imagers. As demonstrated in this study, methods using the WesternC chemiluminescent substrate and the ChemiDoc XRS imager to measure signal volume yielded reliable estimates of target protein quantity for both a purified sample and a crude lysate. In summary, the Precision Plus Protein WesternC standards and Immun-Star WesternC chemiluminescent detection kit provide a convenient and accurate method for generating high-quality, quantitative results from western blotting experiments.

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