Focal Points

Application Note FP-111

UVP BioImaging Systems

Optimizing Western Blots for Chemiluminescent Detection with Cooled CCD Camera Imaging Systems

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Abstract

Parameters affecting the quality of Western blots were examined with the aim of demonstrating the impact of these variables. Using Chaperonin 60 from *E. coli* as a model, SDS-PAGE separated protein was blotted and assessed for the effects of membrane type, blocking reagent, antibody conjugate concentration, chemiluminescent substrate, and imaging method on the quality of the blot. For Chaperonin 60, optimal blotting conditions include a nitrocellulose membrane, powdered skim milk for blocking, an anti-GroEL peroxidase conjugate (Sigma) at a concentration of 1:10,000, BioWest™ Extended Duration peroxidase substrate (UVP BioImaging Systems), and imaging with a digital, high resolution, cooled CCD camera system (The BioChemi™ System, UVP). Variables affecting the quality of a Western blot are very dependent upon the biochemistry of the antigen under examination and antibodies used for detection. These variables need to be empirically matched with membrane type and blocking reagent.

Introduction

The detection, characterization, and quantitation of proteins are daily objectives in life science and biomedical laboratories. Though these analyses can be conducted in a variety of ways, many techniques continue to apply antibodies raised against the protein of interest. Of the different immunoassays available, the immunoblot is widely used as it not only can detect a protein, but also provides insight to its size and configuration (i.e., subunits, disulfide bonding). Coupled with densitometry, immunoblotting can also be used to quantify the amount of protein in a blot.

Since its development in the 1970s, the immunoblot, or what is now commonly called the Western blot, has evolved (Burnette, 1981; Towbin et al., 1979). Many of the changes are subtle, such as the use of membranes other than nitrocellulose, while others are revolutionary, including the use of chemiluminescence for detection. These changes have made Western blotting a much simpler technique to master as compared to fifteen years ago. However, a successful series of reproducible Western blots is still a significant achievement for an aspiring researcher.

The difficulties often experienced with Western blotting are inherent to the technique since it is actually a series of linked techniques all of which can be difficult to master. Not only do the mechanics of these techniques require a certain degree of art, but polyacrylamide gel electrophoresis, electroblotting, and enzyme immunoassays are sensitive to variations in reagents and dependent on the nature of the biomolecules being examined. Quality control criteria can help to alleviate variations in reagents, but the researcher has little influence over the nature of biomolecules. Hence, with a set of difficult techniques there is greater room for error.

Over the past twenty years, the Western blot has evolved from a relatively difficult technique to master to one that can be routinely performed with good results. By today's standards, Western blots were labor-intensive acts that often yielded inconsistent results. This may partly be attributed to the fact that many of the luxuries available to researchers today did not exist. Pre-cast gels, pre-made buffers, purchased antibody conjugates and prepared substrates are commonplace today, but these components simply did not exist and were made by the researcher. For example, original blots made use of manually poured gels that required as much art as science. The variability introduced by manually pouring gels is enormous. Compounding this variability was the use of antibodies labeled with radioactive iodine, which by its nature changes with time. Consequently, the development of enzyme-antibody conjugates and chemiluminescent substrates greatly improved blotting consistency without sacrificing sensitivity (Heinick et al., 1992). In short, the consistency introduced by pre-formulated, stable products has greatly improved the quality of Western blots.

The means by which blots are imaged is also changing and resulting in improved quality. An increasingly popular device that is simplifying blot detection is the cooled CCD camera. CCD is an acronym for charged-coupled device and is a means by which light emitted from a blot is collected and counted. CCD cameras are cooled to enable long-term exposures without producing "noisy" images. These exposure times can vary between 15 seconds and one hour depending on the camera, optics, substrate and blotting technique. Where film captures an image by grains of silver in the emulsion reacting with light, a CCD camera turns a photon into an electrical signal. The lens of a CCD camera focuses light onto a chip that typically has over one million individual wells (photodiodes or pixels) designed to absorb photons. Scientific grade CCD chip sensors manufactured for today's systems have wells with between 10,000 and 50,000 electrons. When a photon strikes the well, an electron is stimulated, converted into an electrical current and digitized with a specific gray scale value. The complete array of all these gray scale values is then used to create the digital image.

The goal of this paper is to examine parameters that affect Western blotting, with specific emphasis on using chemiluminescent detection with cooled CCD cameras. To accomplish this, we used the *Escherichia coli* Chaperonin 60 protein as a target in Western blotting. Chaperonin assists in the proper folding of proteins in cultures of *E. coli* grown at elevated temperatures (Lorimer, 1996). It is readily available commercially and easily isolated from active bacterial cultures. Blots of this protein will be subjected to variations in blocking, substrate types, antibody concentrations, and membrane types. These blots will be imaged with both film and a cooled CCD camera system.

Though experience dictates that methods used by one researcher may not work for another, we hope to at least highlight parameters we feel deserve attention and will be useful to the novice. For more experienced researchers, we hope that our discussion on cooled CCD imaging for chemiluminescent Western blot detection provides useful insight.

Materials and Methods

Protein samples and antibodies Chaperonin 60, GroEL was purchased from Sigma (C7688) or isolated from *E. coli* strain TOP10 (Invitrogen). Anti-GroEL peroxidase conjugate (anti-chaperonin peroxidase conjugate), prepared against Chaperonin 60 in rabbit, was purchased from Sigma (A8705).

Preparation of protein samples Chaperonin 60 (1 mg) was dissolved in 1 ml sterile PBS to a concentration of 1 ug/ul. All subsequent dilutions of chaperonin were in sterile PBS. Laemmli sample buffer (BioRad, 161-0737) was prepared by mixing 950 ul of stock buffer with 50 ul of beta-mercaptoethanol. For electrophoresis, 4 ul of protein sample was mixed with 8 ul of sample buffer and boiled for 4 min. Samples were then cooled and centrifuged for two minutes in order to retrieve any condensation that may have collected on the lids. Samples were then loaded onto the gel.

Electrophoresis procedures The Bio-Rad Mini-PROTEAN II electrophoresis unit was used for these experiments. Samples were separated on pre-cast 4-20% polyacrylamide gradient gels (Bio-Rad, 161-1177). Tris-Glycine buffer was diluted to 1X from a 10X concentrate (Bio-Rad, 161-1177). After loading the samples, the gel was run for 40 minutes at 200 volts.

Preparation of solutions for blotting Transfer buffer (25 mM Tris base, 192 mM glycine, 5% v/v methanol) was prepared by dissolving 3 g of Tris base and 14.4 g of glycine in 1 liter of de-ionized water. To 950 ml of this buffer, 50 ml of methanol (VWR, EM-MX 04751) were added. The buffer was then refrigerated. It is important that the buffer temperature is 4°C at the start of the transfer. Blocking solution was prepared by diluting 10X blocking solution (Roche Diagnostics, 1585614) to 1X in PBS (Sigma, P3813). Wash buffer was prepared by diluting Tween 20 (Sigma, P1379) in PBS to a final concentration of 0.05%.

Preparation of substrates The ECL substrate (Amersham Pharmacia, RPN2108) was prepared by mixing an equal volume of detection solution 1 with detection solution 2. The ECL Plus substrate (Amersham Pharmacia, RPN2132) was prepared by mixing detection reagents A and B in a ration of 40:1. The BioWest Extended Duration peroxidase substrate (UVP) was prepared by mixing equal parts of Luminol/ Enhancer Solution and Stable Peroxide solution. The final volume required was 0.125 ml/cm^2 per membrane. All substrates were prepared immediately before use.

Preparation for blotting A 7.5 cm x 8 cm nitrocellulose membrane (Invitrogen, LC2001) was soaked in transfer buffer for 30 minutes at room temperature. This was done to insure proper binding. For PVDF, the membrane was first wetted in methanol and then soaked in transfer buffer. Following electrophoresis, the gel was rinsed in transfer buffer prior to blotting in order to remove salts and detergents that can increase conductivity of the transfer buffer and heat generated during the transfer. Equilibration also allows the gel to adjust to its final size prior to electrophoretic transfer.

Electrophoretic transfer procedures The Mini Trans-Blot Electrophoretic Transfer Cell from Bio-Rad was used for the transfer. Transfer took place for one hour at 100 volts. Following the transfer, the membrane was blocked with 30 ml blocking solution for 1 hour at room temperature atop an orbital shaker set at 100 rpm. After blocking, anti-GroEL peroxidase conjugate was added to the blocking solution at a dilution of 1:10,000 unless otherwise stated. The conjugate was allowed to bind for 1 hour at room temperature with shaking. The membrane was transferred to a new container and washed in PBS-Tween for five minutes with agitation. This wash step was repeated three more times.

Detection equipment The BioChemi System (UVP) installed with a digital, high resolution, cooled CCD camera was used in conjunction with LabWorks Image Acquisition and Analysis Software. Through LabWorks, the exposure time was standardized to 30 seconds at full aperture. A live preview was used to position the blot prior to imaging. No filter was used during the imaging.

Detection procedure The membrane was transferred to a new container and soaked in the appropriate substrate for five minutes with agitation. Excess buffer was drained, and the membrane was placed in an acetate sheet protector. Any bubbles between the membrane and the acetate sheet were removed. The blot was placed inside the Epi Chemi II Darkroom (UVP) and imaged. The blots were also captured on X-Omat AR film (Kodak) with an exposure time of 30 seconds.

Results and Discussion

The objective of this paper is to examine several major factors within the control of the researcher that can be manipulated to generate better and more consistent Western blots. As stated above, many of the reagents used in all steps can increase the variability of blotting. Consequently, all available pre-made reagents were purchased. Manufactured reagents, such as pre-cast gels and pre-made buffers, are normally produced with stringent specifications for acceptance. Therefore, the focus of this analysis is on parameters other than reagents.

Examination of Antibody Concentration A blot with samples run in triplicate sets was cut in thirds and incubated with three different concentrations of anti-chaperonin peroxidase conjugate, namely 1:1000, 1:10,000 and 1:20,000. Figure 1 illustrates the affect of these concentrations on the blot. With the cooled CCD camera system, all three antibody concentrations produced clear bands, although band intensity did decrease with dilution. The 1:1000 gave good intensities, however some background could be observed around the edge of the bands. The 1:10,000 dilution proved to be a good intermediate concentration in that it generated both good signal and low background. Consequently, this intermediate concentration was used in this study. The results produced by the cooled CCD camera were striking compared to the results captured on film. Very significant background is seen on film when the antibody is used at higher concentrations. It is very evident that film is more negatively affected by background than the BioChemi System. This will be examined below.

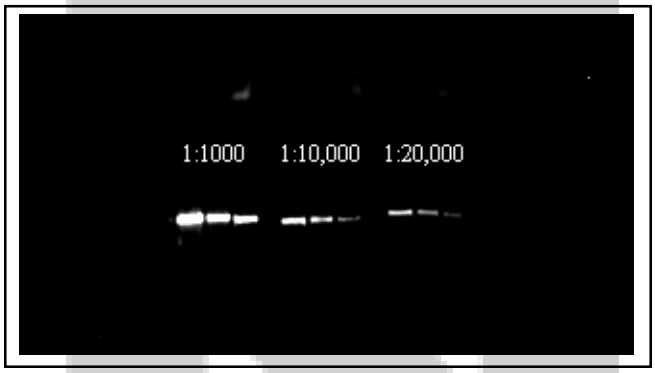

Figure 1. *The effect of anti-chaperonin peroxidase conjugate on signal intensity. Chaperonin loaded in triplicate at 40, 20, and 10 ng/well (left to right), blotted to nitrocellulose, and treated with antibody conjugate as noted. The 1:1000 gave enhanced signal and good intensity at the lower chaperonin concentration, however background on film was relatively high (not shown). The middle dilution of 1:10,000 proved to be a good concentration for both cooled CCD camera and film detection.*

Though this experiment is simple, in practice a dot blot can be used as effectively without the need for electrophoresis and electroblotting. Antigen solution can be spotted on small strips of membrane, dried, blocked, and incubated with different antibody concentrations in test tubes or small petri dishes. These test strips can then be imaged and used to rapidly assess optimal antibody concentrations. Though the anti-chaperonin peroxidase conjugate represents a primary antibody detection system, this optimization can also be performed with secondary antibodies as well.

Antibodies are perhaps the single greatest source of variation between blots targeting different antigens. By their nature, antibodies have variation in chemical composition and affinity for the antigen. Variation in amino acid sequence in polyclonal antibodies, in addition to the microheterogenicity of glycosyl groups, can cause antibody solutions to passively bind to membranes differently between sera preparations. Hence the concentration of antibody used in one blot may cause high background in another.

Differences in antibody affinity will also affect the quality and information gained from a blot. It is well known that antisera produced from different animals, or antisera from different bleedings of one animal, can differ significantly in its affinity for an antigen. Differences are also evident for antibodies generated from different hybridoma clones. Consequently, the best working concentration for each antibody must be determined by comparing signal of the bands to the background. Once a working concentration is selected, then the optimal incubation time should be determined as well.

Since antibody-to-antibody variation can be extreme, these reagents are often the limiting factor in a blot. Good antibodies were once difficult to obtain and the selection for the more esoteric antigens was limited. Indeed the task of purifying a protein for use as an immunogen was laborious. However, raising good antibodies has been greatly simplified for proteins in which the corresponding genes have been cloned and sequenced. Raising antibodies against peptides synthesized from sequence data is more efficient than purifying a protein to near homogeneity. Therefore, if a particular antibody is not producing satisfactory blots, it is relatively easy and cost effective to have a new antibody produced. This, of course, is dependent upon the existence of sequence data.

Comparison of Nitrocellulose and PVDF Membranes To demonstrate the effect of membrane type on the blotting of chaperonin, a gel run with a duplicate set of samples was split and transferred onto nitrocellulose and PVDF membranes. The bands, which were generated using UVP's BioWest Extended Duration substrate, are visible on each blot. However, depending on the imaging method, results differed. With the BioChemi System, both blots were comparable. Images obtained on film differed significantly (Figure 2) with greater band intensities from nitrocellulose than the PVDF membrane. This result may differ for other proteins, but the correct membrane type should be determined empirically, and imaged with a cooled CCD camera system for consistency in the results.

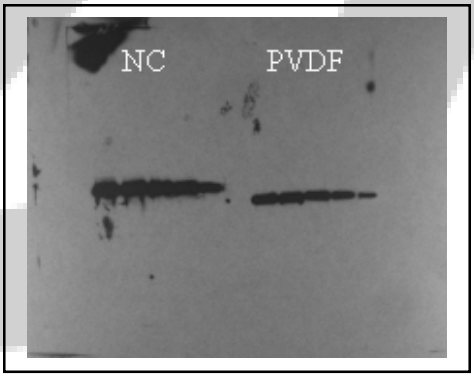

Figure 2. *Film image of Western blot of Chaperonin 60 on nitrocellulose and PVDF membranes. Chaperonin was serially diluted (160, 80, 40, 20, and 10 ng/well from left to right) in duplicate and separated by PAGE. Transfer was on two side-by-side membranes, and all subsequent methods were constant. The intensity of bands from the nitrocellulose is substantially greater than PVDF, however background appears slightly higher as well.*

Several membrane types are available for Western blotting. The first Western blots made use of nitrocellulose membranes, but have since been joined by PVDF and nylon. Each of these membranes has a different surface chemistry and therefore binds proteins differently. The decision of which membrane to use in blotting is often one of personal preference, however each has its advantages. For instance, nitrocellulose is the most widely used and best documented while nylon membranes are exceptionally durable and have been shown to generate sharper bands. Techniques for eluting proteins from PVDF allow this membrane to serve as a purification tool.

Veterans of blotting recognize that batch-to-batch variation occurs when membranes are manufactured. This variation can dramatically affect the quality of blots. Consequently, it is very common to test different manufacturing lots of a membrane prior to making large purchases. It is common practice to stockpile a specific manufacturing lot of membrane once it has proven effective in blotting.

Comparison of BSA, Blocking Reagent, and Powdered Skim Milk for Blocking As with other components of the blot, the choice of blocking solution can also affect the quality of a blot. To demonstrate this difference, a single membrane was used to blot three sample sets of chaperonin and molecular weight markers. This membrane was then cut in thirds and treated with 3% BSA in PBS, 1X Blocking Reagent (Roche Biochemicals) diluted in PBS, and 3% powdered skim milk (store brand) in PBS. Following the addition of anti-chaperonin peroxidase conjugate and washing, the blot was treated with BioWest substrate. Figure 3 illustrates the effect of the blocking reagents on the blot.

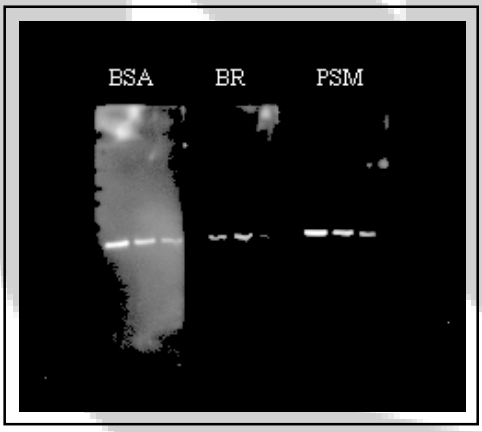

Figure 3. *Comparison of chaperonin Western blots blocked with BSA, Blocking Reagent, and powdered skim milk. Chaperonin was loaded in triplicate at 40, 20, and 10 ng/well, separated, and transferred to nitrocellulose. The membrane was cut in thirds and blocked with 3% BSA, 1X Blocking Reagent (Roche Biochemical), and 3% powdered skim milk (store brand). Powdered skim milk generated the strongest signals while significant background is seen with BSA blocking.*

The three blocking reagents each impacted the quality of the blot. The BSA blocked membrane generated significant background as can be seen with the non-specific signal generated across the membrane. In contrast, the Roche Blocking Reagent and powdered milk did not have significant noise, however the band intensity from the Blocking Reagent blot was lower than the membrane blocked with powered skim milk.

As with other reagents, the choice of blocking reagent should be tested for a particular blot. As non-specific binding of antibody creates background, the protein used for blocking may need to be changed depending on the nature of the antibody. Furthermore, BSA is often used as a carrier for haptens during immunization procedures. With polyclonal antibody preparations, anti-BSA antibodies may well be present in the preparation, and these could specifically bind to BSA blocked membranes.

Comparison of Chemiluminescent Substrates Three peroxidase specific chemiluminescent substrates were compared. Three parallel blots were used to assess BioWest (UVP), ECL (Amersham Pharmacia Biotech), and ECL Plus (Amersham Pharmacia Biotech). Each substrate was prepared by the manufacturer's instructions and applied to the membranes. The blots were placed in the BioChemi System and exposed for 30 seconds. The blots were imaged automatically at 0, 15, 30, 60, and 150 minutes, using the Sequential Integration feature of LabWorks 4.0. Sequential Integration, different from the Dynamic Integration feature of LabWorks, enables the user to pre-set multiple exposure times and walk away from the system to work on something else. In addition, the DVP™ (Digital Video Playback) feature records the sequence of acquired images and allows the user to playback the recording to select the best images for documentation, analysis or archiving. We found these very useful imaging tools for chemiluminescent detection with a cooled CCD camera.

The signals generated from the three substrates differed significantly with BioWest generating a stronger signal than ECL Plus that was in turn stronger than ECL. The results are illustrated in Figure 4.

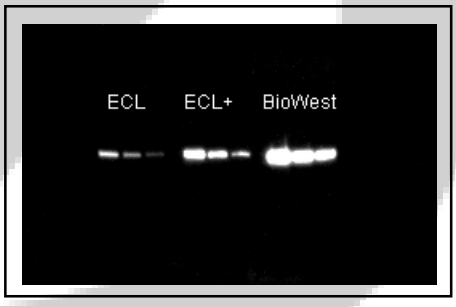

Figure 4. Comparison of BioWest, ECL, and ECL Plus chemiluminescent peroxidase substrates. Three concentrations of Chaperonin 60 (40, 20, and 10 ng/well) were loaded in triplicate on a 4-20% polyacrylamide gel, separated, and transferred to nitrocellulose. Prior to the addition of substrate, the membrane was cut into thirds and each of the chemiluminescent substrates was applied separately. The BioWest substrate generated a signal 3.5 times greater than ECL Plus and 14 times greater than ECL. This membrane was subsequently imaged for 150 minutes to assess the duration of the signal.

Not only did the choice of commercial substrate have a significant impact on the sensitivity of the Western blot, but the duration of the signal as well. The BioWest Extended Duration substrate consistently generated strong signals over

the serial dilution of chaperonin as compared to ECL Plus. The ECL substrate lost intensity very rapidly as compared to the BioWest and ECL Plus substrates. The bands for this time course assay were digitized and plotted against time to illustrate the duration of signal intensity (Figure 5).

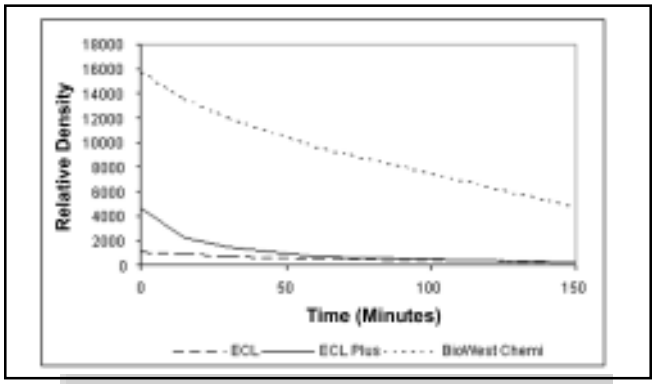

Figure 5. *Time course analysis of band intensities for ECL, ECL Plus, and BioWest substrates. Parallel blots of Chaperonin 60 were imaged at 0, 15, 30, 60, and 150 minutes and then measured for relative density. Initial intensity of the BioWest substrate was 3.4 times greater than ECL Plus and 14 times greater than ECL. In addition, the signal duration of BioWest greatly exceeded the other substrates.*

Chemiluminescence has evolved into an extremely sensitive method for detecting proteins. Original methods for Western blotting involved labeling antibodies with radioactive ^{125}I. When used as a secondary antibody, such blots had relatively good sensitivity and could detect as little as 5 ng of protein (Sambrook et al., 1989). However, radioactive labels have many limitations, including the hidden costs of using radioactivity, the unavoidable decay of the label and the resulting need to re-synthesize the antibody conjugate which itself is variable. These limitations provided the incentive for the development of enzymatic substrates that could compete with the sensitivity of ^{125}I. In 1983, the first chemiluminescent substrate was reported and since that time the reagent systems have been dramatically improved (Kricka, 1991). The BioWest chemiluminescent substrate used with the BioChemi System actually enables protein detection down to the femtogram level of material.

Comparison of Film and Cooled CCD Cameras All the blots were imaged with both film and a digital, high resolution cooled CCD camera. Both methods provide useful results, however the CCD camera is less affected by background variations. Whether it is due to handling of the film or the membrane, background on film images is routinely higher than blots imaged with a cooled CCD camera. This is most dramatically illustrated within the blocking reagent experiment (Figure 6).

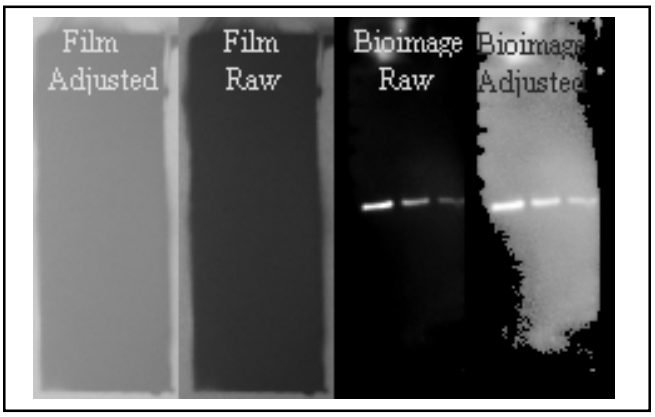

Figure 6. *Comparison of images captured on film and a cooled CCD camera. BSA blocked nitrocellulose was shown to cause high background in the chaperonin Western blot. When a BSA blocked membrane was exposed to film, significant background was detected. Upon acquiring that film image with a CCD camera, enhancements could not resolve bands within the background. However, BSA blocked membranes directly imaged by the BioChemi System captured the bands with exceptional signal and without background problems.*

Though film is the traditional means by which to detect bands from a Western blot, newer technologies that make use of CCD cameras and analytical software easily surpass imaging with film. Additionally, film processing, like any laboratory manipulation, is sensitive to variation in reagents, temperature, and the film itself.

As noted, a major difference between film and imaging with a CCD camera is the latter's capability to adjust for background noise. Using CCD cameras cooled over 40°C from ambient temperature reduces the background noise per well to as few as 5-20 electrons (i.e., the baseline). Any electrical impulse above the baseline is signal. What is reported from the CCD, however, is not a count but rather a ratio of counts to the noise (i.e., signal to noise ratio, or SNR). The maximum signal to noise ratio of any camera is most accurately represented as the maximum number of photons a well can adsorb divided by the total noise from the camera. The resulting SNR is also the maximum number of gray scales the camera is capable of resolving and is referred to as the camera's dynamic range. By dividing the log of the SNR by 0.30 (the log of 2), you can calculate the actual bit performance of the camera (Figure 7).

$$\text{SNR} = \frac{\text{Full Well Electrons}}{\text{Noise}}$$
$$= \text{Maximum Levels of Gray Scale from CCD}$$
$$= 2^{(\text{CCD Bits})}$$
$$= \text{CCD Dynamic Range}$$

Figure 7. *CCD Camera Calculations*

The digital, high resolution, cooled CCD camera of the BioChemi System utilizes Deep Pixel Technology, or DPT™, to create "super pixels". These super pixels contain 400,000 full well electrons. Because this camera is also deeply cooled, the noise is maintained at only 7 electrons. The resulting signal to noise ratio is an impressive 57,143 gray scales. This approaches true 16-bit performance and is the reason why the BioChemi System is able to surpass the sensitivity of film in detection of chemiluminescent Western blots.

Summary

The variable biochemistry of proteins makes the use of a single protocol for Western blotting impractical. For each protein, the optimal concentration of polyacrylamide, membrane type, blocking reagent, antibody concentration, wash conditions, and visualization method must be determined. With the Chaperonin 60 protein of *E. coli*, we found that commonly used conditions produced a good blot, but optimizing methods could generate stronger and longer lasting signals. By using the UVP BioChemi System in combination with the BioWest Extended Duration peroxidase substrate we generated extremely intense band signals, drastically lowering the detection limit for the protein. This substrate also generated very long lasting signals, which enabled us to acquire multiple images over an extended period of time. In addition, the digital images captured from the cooled CCD camera were easily background corrected, enhanced for publication and quantified.

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