APPLICATION NOTE

Optimization of Western Blotting Assays Using PhosphaGLO™ and PhosphaGLO Reserve™ AP Substrates

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Chemiluminescence is a valuable tool for visualizing specific proteins. As the reagents for detection improve, the lower limit for protein detection has improved as well. However, with increased sensitivity, there is also a risk of unacceptable non-specific backgrounds in membrane-based assays such as Western blots. For this reason, optimization of variables such as exposure time, conjugate concentration, blocking time, blocking solutions, and type of membrane is central to developing a successful chemiluminescent Western blot assay.

KPL has developed two chemiluminescent Western blotting substrates that may be used with alkaline phosphatase (AP)-labeled antibodies. PhosphaGLO™ AP Substrate is designed for general Western blotting applications, whereas PhosphaGLO Reserve™ AP Substrate is recommended for even greater sensitivity. Both of these substrates may be used in most standard Western blotting protocols, but variables should be critically considered and properly optimized to obtain the best possible results. A good basic procedure can be found in the product data sheets. This application note will discuss ways to increase true signal over non-specific noise to give the most sensitive detection against a clearer background.

MATERIALS AND METHODS

A 250-pg sample of purified non-reduced Mouse IgG was serially diluted 1:2, run on pre-cast 4 - 20% Criterion™ polyacrylamide gels (Bio-Rad Laboratories), and transferred to either Immun-Blot® Nitrocellulose or PVDF (Bio-Rad Laboratories) membranes. Replicate samples were loaded in order to make multiple blots.

Following blotting, the membranes were blocked in 1X Detector™ Block overnight at 4° C without agitation. Alkaline phosphatase-labeled goat anti-mouse IgG (H+L) was prepared in 1X Detector Block at a concentration of 10 ng/mL (1:10,000 dilution of a 0.1 mg/mL stock). The blots were then transferred to the conjugate solution and incubated for 1 hour at room temperature with shaking. The conjugate solution was decanted and the membranes washed in 1X Wash Solution 3 times for 5 minutes each, followed by a 10-minute wash. Finally, the membranes were rinsed 2 times for 2 minutes each in 1X Assay Buffer.

PhosphaGLO AP Substrate and PhosphaGLO Reserve AP Substrate were warmed to room temperature prior to use and then added to the appropriate membranes for 1 minute. The membranes were blotted on filter paper to remove excess substrate, placed in Hybridization Bags, exposed to Biomax™ Light film (Kodak) for the times specified in the figures. The films were then developed in a film processor.

RESULTS AND DISCUSSION

Simple changes in protocol can drastically improve signal while decreasing background in Western blots detected with PhosphaGLO or PhosphaGLO Reserve. The results described below will illustrate the impact of these changes to the assay.

OPTIMIZING DETECTION

CHOICE OF MEMBRANE

The most basic choice in designing a Western blot is the type of membrane used. Both nitrocellulose (NT) and PVDF are appropriate for most applications. Each has strengths and weaknesses, so the choice may vary with application or may need to be determined empirically. PVDF is tougher but tends to be more difficult to wet because of the hydrophobicity of the membrane. In some applications PVDF is also more difficult to block consistently. If films show inconsistent background when run with PVDF, then NT should be tried. NT is a more brittle material, which can be problematic when extra handling is required such as subdividing blots for comparative assays. Conversely, optimization of exposure times tends to be easier when using NT.

As you can see from Figure 1, PVDF shows an increased sensitivity compared to NT in equivalent Western blots transferred and developed with PhosphaGLO. The same type of results are seen with PhosphaGLO Reserve (not shown).

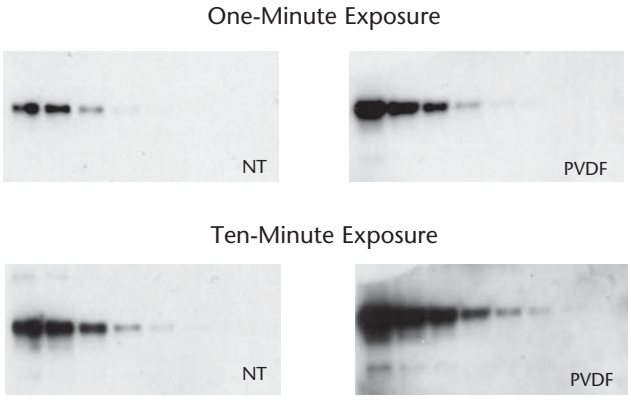

Figure 1. Two polyacrylamide gels were run with 1:2 serial dilutions of Mouse IgG. One was transferred onto nitrocellulose and the other was transferred onto PVDF. Protein bands on the blots were then detected using KPL reagents and treated with PhosphaGLO AP Substrate. One- and ten-minute exposures were then obtained.

FILM EXPOSURE

One of the simplest ways to control blot appearance is to vary the amount of time the film is exposed to the chemiluminescent signal (Figures 1 & 2). With shorter times (one minute), distinct bands and clearer background were obtained as compared to five- and ten-minute exposures. Longer exposures (five and ten minutes) resulted in a darker background but provided more sensitive detection of the target. Care must be taken with long exposures because in a grossly overexposed blot, the background signal will overwhelm and obscure specific bands. A two-minute exposure time is usually a good starting point for optimization.

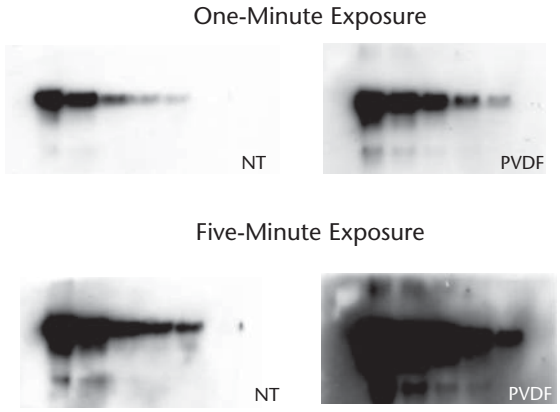

Figure 2. The same conditions as Figure 1 but the detection substrate was PhosphaGLO Reserve AP Substrate with one- and five-minute exposures.

SUBSTRATE

PhosphaGLO Reserve was formulated to detect lower concentrations of target than PhosphaGLO. As expected, when equivalent gels are run (Figure 1 & 2), PhosphaGLO Reserve provided more sensitive detection. A more sensitive reagent will also allow for shorter exposures times. Regardless of the choice of substrate, PhosphaGLO or PhosphaGLO Reserve may be used on either PVDF or nitrocellulose membrane without the bother and cost of the additional chemicals needed for other AP chemiluminescent substrates.

Another advantage of using PhosphaGLO or PhosphaGLO Reserve over other substrates is the prolonged chemiluminescent signal generated. Both substrates can emit signal for a minimum of five days after detection. This enables repeated exposures for optimum visualization of results. As seen in Figure 3, a two-minute exposure performed six days after the initial addition of substrate resulted in blots similar to those developed on day one.

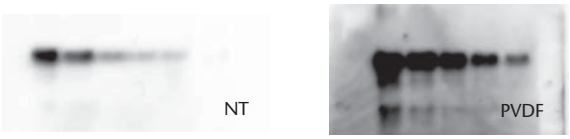

Figure 3. The same blots from Figure 2 were exposed to film for two minutes six days after the blots were treated with PhosphaGLO Reserve AP Substrate.

When striving for optimum sensitivity, PVDF used with PhosphaGLO Reserve may be the combination of choice. When sensitivity is not an issue, PhosphaGLO and NT may be preferable for ease of optimization.

OPTIMIZING EXPERIMENTAL PARAMETERS

ANTIBODY DILUTION

Antibody concentration is an important factor in producing the best results with Western blotting. The primary antibody should be diluted to the concentration recommended by the manufacturer or empirically determined by the researcher. Precious primary antibodies can be diluted below maximal concentration where sensitivity is not an issue. Slight differences in the affinity of the secondary antibody for different primary antibodies may indicate that the concentration of the secondary antibody should be adjusted using the manufacturer's recommendation as a starting point. High levels of secondary antibody may produce unnecessarily high background whereas use of a very dilute antibody may decrease the lower limit of detectable target. An optimization experiment can be set up where duplicate dilutions of a known quantity of target are run in different lanes on a gel and transferred to a membrane. The membrane is then blocked. Each set can be treated with a series of 1:10 dilutions of antibody then exposed to film for different lengths of time to determine optimum results. Dilution of the antibody conjugate will often result in an increase in the signal-to-background ratio when using chemiluminescent detection. This is illustrated by the results depicted in Figure 4 using PhosphaGLO Reserve. With a 1:100,000 dilution of the antibody conjugate, the background was significantly reduced when compared to the 1:10,000 dilution for this particular antibody. Decreasing the amount of antibody conjugate not only decreases the background on blots but also allows for conservation of secondary antibody.

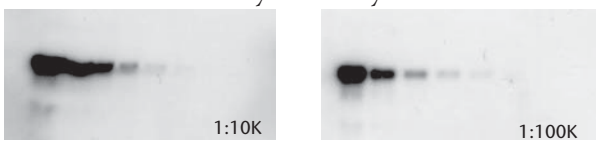

Figure 4. Same conditions as Figure 1 except that following treatment with 1X Detector Block, one blot was detected with a 1:10,000 dilution of AP Goat anti-Mouse IgG (H+L), whereas the other was detected using a 1:100,000 dilution of the conjugate. The blots were then treated with PhosphaGLO Reserve AP Substrate and exposed to film for five minutes.

BLOCKING SOLUTIONS

The choice of blocking solution is another key consideration when designing a Western blot assay using chemiluminescent reagents. Although PhosphaGLO and PhosphaGLO Reserve may be used with a variety of blocking proteins, including milk, BSA and casein, the best results were achieved with Detector Block (data not shown). Due to their undefined and inconsistent composition, more optimization is required and more variation is seen when using these other blocking solutions. For more information on how the use of blocking solutions may affect different assay systems, please see KPL Application Note - A Comparison of Blocking Solutions Used in Western Blotting.

CONCLUSION

Logically optimizing Western blots developed with PhosphaGLO and PhospaGLO Reserve produces optimum results as summarized in the chart below. For standard Western blot procedures, begin with NT or PVDF membranes and PhosphaGLO Substrate. For the ultimate sensitivity, begin with a PVDF membrane and PhosphaGLO Reserve Substrate. Multiple film exposures may be tried to obtain the best results for each system. Optimizing the amount of secondary antibody should be tested to increase sensitivity or decrease background as needed. Overall, thorough assay optimization is required to maximize signal-to-noise in Western blot assays when using chemiluminescent substrates such as PhosphaGLO or PhosphaGLO Reserve. If trouble persists, an excellent troubleshooting guide is included with the product data sheets. Whether this optimization is accomplished through film exposure time, conjugate concentration, type of membrane, substrate, or blocking agent, the Western blot assay will provide the researcher with easily visible and sensitive results.

Optimizing Signal-to-Noise

Desired Result	Membrane	Film Exposure	Substrate	Secondary Ab	Blocking
Increased Signal	PVDF	Increase	PhosphaGLO Reserve	Increase concentration	Not applicable
Reduced Background	NT	Decrease	PhosphaGLO	Decrease concentration	Detector Block

Related Products and Literature

Products

Description	Size	Catalog No.
Anti-Human IgG (H+L) antibody, Phosphatase labeled	1.0 mL	475-1006
Anti-Rabbit IgG (H+L) antibody, Phosphatase labeled	1.0 mL	475-1506
Anti-Mouse IgG (H+L) antibody, Human Serum Adsorbed and Phosphatase labeled	1.0 mL	475-1806
Streptavidin, Phosphatase labeled	1.0 mL	475-3000
AP Stabilizer	200 mL	55-15-00
Detector Block (5X)	240 mL	71-83-00
10% BSA Diluent/Blocking Solution	200 mL	50-61-00
Milk Diluent/Blocking Concentrate Kit	200 mL	50-82-01
Wash Solution Concentrate Kit	800 mL	50-63-00
Phosphatase Assay Buffer (10X)	200 mL	50-63-14
PhosphaGLO Reserve AP Substrate	30 mL	55-60-01
PhosphaGLO Reserve AP Substrate	100 mL	55-60-02
PhosphaGLO AP Substrate	30 mL	55-60-03
PhosphaGLO AP Substrate	100 mL	55-60-04
Hybridization Bags	50 bags	60-00-51

Literature

PhosphaGLO Data Sheet
PhosphaGLO Reserve Data Sheet
A Comparison of Blocking Solutions Used in Western Blotting.

To order or for more information on KPL's full line of protein detection products, contact us at 800.638.3167 / 301.948.7755, or visit us at www.kpl.com

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