APPLICATION NOTE

Optimization of Assay Conditions Using LumiGLO Reserve™ vs. LumiGLO® Chemiluminescent Substrates in Western Blotting

KPL Research & Development

With the development of chemiluminescent substrates, assay sensitivity limits have been greatly improved over those obtained with traditional colorimetric substrates. The results have been shown most strikingly with membrane-based immunoblotting assays. Now with second and third generations of improved chemiluminescent substrates on the market that offer the highest sensitivity to date, researchers are able to obtain results not possible prior to the availability of these products. However, with improved sensitivity comes the invariable increase in the potential for background. For this reason, it is imperative that Western blot assays are optimized to maximize the full potential of these chemiluminescent substrates for sensitive detection.

There are many assay variables that may contribute to background, thus reducing the signal to noise ratio. These variables include antibody conjugate activity and use dilution, exposure time to film, block solutions, membrane types, and choice of chemiluminescent substrates. By optimizing the various assay parameters, signal to noise may be maximized and assay consistency maintained.

KPL has developed a second-generation chemiluminescent substrate, LumiGLO Reserve™, for those applications where the highest sensitivity is required in order to detect low abundant proteins otherwise not detectable with standard detection reagents. While LumiGLO Reserve has been designed to deliver greater signal with less background as compared to other competitive substrates, optimization of the assay remains critical to its overall performance. This application note demonstrates the differences in the use of the traditional LumiGLO® Chemiluminescent Substrate and the higher sensitivity LumiGLO Reserve™ on Western blots - how the assays are optimized and the results expected with each.

MATERIALS AND METHODS

Non-reduced purified Mouse IgG (KPL) samples were prepared and run on precast 4 - 20% Criterion™ polyacrylamide gels (BioRad Laboratories) and transferred to Immun-Blot® PVDF (BioRad) membranes. Samples were loaded in triplicate on the gel to make replicate blots. The membranes were blocked in 1X Detector™ Block with 1% Detector Block Powder (KPL) for 1 hour at room temperature. Goat anti-Mouse (H+L) HRP (KPL) was prepared in 1X Detector Block without Detector Block Powder at three concentrations: 100 ng/mL, 10 ng/mL, and 1 ng/mL (1:1000, 1:10,000, and 1:100,000 dilution of a 0.1 mg/mL stock). While still in the block solution, the membranes were cut into 3 identical blots and were subsequently transferred to each of the diluted conjugate solutions. The membranes were incubated in the diluted conjugate solutions with gentle agitation for 1 hour at room temperature. The conjugate solutions were then decanted and the membranes were washed in 1X Wash Solution (KPL) 3 times for 5 minutes each, followed by a final 10-minute wash at room temperature with constant agitation. LumiGLO and LumiGLO Reserve Substrates (KPL) were prepared per their respective instructions and added to the membranes for 1 minute. The membranes were blotted onto filter paper to remove excess substrate and transferred to Hybridization Bags (KPL) for membrane detection. Membranes were exposed to BioMax™ Light film (Kodak) for 30 seconds, 2 minutes, and 10 minutes prior to development of the film in a processor.

RESULTS AND DISCUSSION

In the development of LumiGLO Reserve, it became apparent that its performance in immunoblotting applications was more greatly impacted by modest changes in assay conditions than the traditional

LumiGLO Chemiluminescent Substrate. The results of this study help illustrate the significance of assay optimization to the overall success of the assay. As shown in the following data, the antibody concentration and the film exposure can be balanced to appropriately manage the intense signal produced by more sensitive substrates like LumiGLO Reserve, thus yielding the greatest signal to noise.

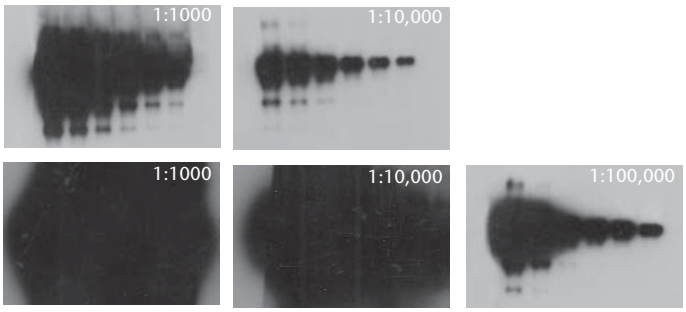

Figure 1: LumiGLO (top) vs. LumiGLO Reserve (bottom) with a 10-minute exposure to film. Non-reduced Mouse IgG was transferred to the blots at 25 ng, 12.5, 6.25, 3.125, 1.56, 0.78 ng. Conjugates were diluted to concentrations of 1:1,000, 1:10,000 and 1:100,000 as indicated. No data are shown for blots detected with anti-mouse HRP at 1:100,000 and LumiGLO since no detection was possible at this antibody use dilution.

ANTIBODY USE DILUTION

In Figure 1, LumiGLO and LumiGLO Reserve are compared for sensitivity and signal to noise when used with a series of antibody titers. LumiGLO shows specific, clean detection at both 1:1,000 and 1:10,000 conjugate dilutions with a 10 minute exposure (Figure 1). All Mouse IgG samples are represented by intense bands from 25 ng to 780 pg with little non-specific background. While the 1:1,000 dilution shows greater sensitivity than the 1:10,000, the more concentrated antibody provides excessive signal that hinders proper analysis of the protein. When a 10-minute exposure is an acceptable time frame for the assay, the 1:10,000 dilution would be the optimal condition for use with LumiGLO.

Because LumiGLO Reserve offers significantly greater sensitivity than LumiGLO, a 10-minute exposure of the blots treated with either the 1:1,000 or 1:10,000 conjugate dilutions yield unacceptably high signal and background. Instead, an antibody use dilution of 1:100,000 is more suitable for detection of the amounts of protein available in this system. Sensitivity is similar to that of the 1:10,000 dilution conjugate detected with LumiGLO and it is clear that even lower levels of detection are possible with the LumiGLO Reserve.

It is important to note that with the use of high intensity substrates such as LumiGLO Reserve, subtle differences in enzyme conjugate activity can translate to large differences in signal and background. Each new lot of conjugate should be optimized individually.

FILM EXPOSURE

Alternatively, the exposure time of the identical membranes was reduced from 10 minutes to 2 minutes to control signal to noise (Figure 2). At 2 minutes, the LumiGLO membrane with a 1:1,000 dilution now delivers desirable results, while the sensitivity has been somewhat inhibited in the blot where the conjugate was used at a concentration of 1:10,000. The background from the blot reacted with LumiGLO Reserve is eliminated with the conjugate at 1:10,000, showing still much stronger signal than either LumiGLO blots. The sharpness of the bands is improved without impact on detection limit when the exposure time is further reduced to 30 seconds (data not shown). On the other hand, the use of LumiGLO Reserve on the membrane with the 1:1,000 conjugate dilution continues to provide too much background at this exposure time. In fact, the signal remains too strong and diffuse at even a 30-second exposure that it does not allow adequate analysis of the protein in nanogram to high picogram range. For this particular study, LumiGLO Reserve in combination with the antibody at 1:100,000 provides suitable high-end detection of the protein with the added benefit of antibody conservation.

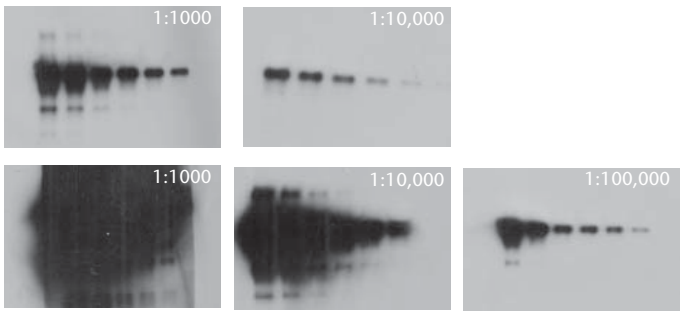

Figure 2: LumiGLO (top) and LumiGLO Reserve (bottom) membranes exposed to film for 2 minutes. The same series of Mouse IgG blots used in Figure 1 were exposed to a second set of film and the exposure time shortened to 2 minutes.

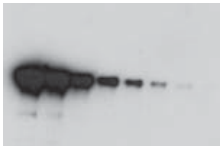

Figure 3: Detection of low picogram concentrations of Mouse IgG using LumiGLO Reserve at the optimized conjugate dilution. Non-reduced Mouse IgG was transferred to the blots at 500 pg, 250, 125, 62.5, 31.25, 15.6, 7.8, 3.9 pg. Sample was detected using anti-mouse HRP antibody (1:10,000) and LumiGLO Reserve and exposed to film for 5 minutes.

LOW LEVEL PROTEIN DETECTION

A similar study was performed to investigate the impact of optimization on the detection of protein in lower concentrations using LumiGLO Reserve. When the 1:10,000 dilution of conjugate is utilized in an assay detecting limited quantities of material, sensitivity can be obtained to the low picogram level (Figure 3).

MEMBRANE AND BLOCK

Other considerations when designing a Western blot assay include which type of membrane (PVDF or nitrocellulose) and what blocking protein to use. In general, both membranes provide high binding to retain the protein and have been successfully used when detecting with either LumiGLO or LumiGLO Reserve. However, PVDF may be more difficult to consistently block (data not shown). If background persists when using PVDF, changing to nitrocellulose is recommended to alleviate the issue.

Attention should also be given to the choice of blocking solution. Non-fat milk has traditionally been the choice of block in Western blotting, but with this choice comes limitations. While it is an inexpensive option, protein content often varies lot to lot. The high concentration of protein can also interfere with specific signal decreasing sensitivity. Due to the inherent presence of biotin, milk should be avoided as a blocking agent if a biotin/streptavidin system for detection is used. [Note: For more detail, see KPL's Application Note - *A Comparison of Blocking Solutions Used in Western Blotting.*] KPL's Detector™ Block was used in this study to maximize signal to noise and sensitivity although milk, casein, and BSA are all compatible with both LumiGLO and LumiGLO Reserve detection. Use of these blocks will require conjugate optimization for their particular assays.

CONCLUSION

Overall, optimization of each assay should consider the conjugate use dilution and exposure time to film. Reducing exposure time to film may compensate for intense signal from stronger conjugates. However, further dilution of the conjugate or loading less of the target may be required for best signal to noise. Optimization of an assay to a two-minute exposure to film will allow the end user a greater amount of flexibility for assay to assay use of the conjugate. This will allow shorter exposures for targets in abundance but also provide low background so that longer exposures may be obtained for greater sensitivity of proteins expressed in much lower amounts.

After assay optimization, Western detection is consistent, sensitive, and without background. For instance, using previously optimized primary and secondary antibodies, a Western blot of titrated control protein and a nuclear lysate is shown in Figure 5, demonstrating c-myc expression in a HeLa nuclear lysate. Sensitivity of LumiGLO Reserve is far superior to either of the other detection reagents when the assays are optimized. One can achieve unsurpassed sensitivity with these new and improved substrates, but optimization of each system will be crucial to the outcome.

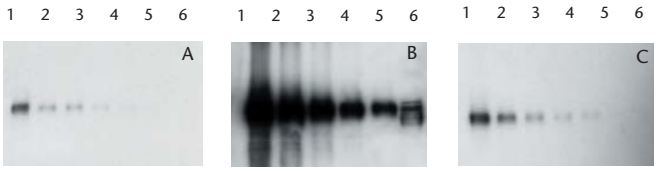

Figure 5: Relative expression of transcription factor, c-myc, using different chemiluminescent substrates. Five two-fold serial dilutions of purified c-myc (Santa Cruz Biotech) (25 ng - 1.56 ng, lanes 1-5) were compared to a 64 µg total protein HeLa nuclear lysate (Protein One) (lane 6). Following separation on a 4-20% PAGE gel and transfer to PVDF, protein was detected using rabbit anti-c-myc antibody (Santa Cruz Biotech) (1:200) and anti-rabbit HRP conjugate (KPL) (1:10,000). Detection conditions were identical with the exception of substrate. While the c-myc lysate sample was not detectable with A) LumiGLO or C) ECL Plus™ (GE Healthcare) after 10 minutes, the sample was easily detected with B) LumiGLO Reserve after just a 2-minute film exposure.

Related Products:

Description	Size	Catalog No.
LumiGLO Reserve™ Chemiluminescent Substrate Kit *Kit contents: LumiGLO Reserve Substrate Solutions A and B, and Wash Solution Concentrate*	2400 cm^2 600 cm^2	54-71-00 54-71-01
LumiGLO® Chemiluminescent Substrate	2 X 120 mL 6 X 120 mL 2 X 30 mL	54-61-00 54-61-01 54-61-02
Protein Detector™ LumiGLO Reserve Western Blot Kit *Kit contents: LumiGLO Reserve Substrate Solutions A and B, and Wash Solution Concentrate, Detector Block, and HRP-labeled Anti-Rabbit and Anti-Mouse Conjugate*	2400 cm^2	54-13-50
Protein Detector LumiGLO Western Blot Kit *Kit contents: LumiGLO Substrate Solutions A and B, and Wash Solution Concentrate, Detector Block, and HRP-labeled Anti-Rabbit and Anti-Mouse Conjugate*	2500 cm^2	54-12-50
Detector Block (5X)	240 mL	71-83-00
HRP-labeled Goat Anti-Mouse IgG(H+L) HSA, liq	1.0 mL	474-1806
HRP-labeled Goat Anti-Rabbit IgG (H+L), liq	1.0 mL	474-1506
Wash Solution Concentrate	4 X 200 mL	50-63-00

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

SeraCare LIFE SCIENCES
www.seracare.com
800.676.1881
508.244.6400

KPL *Power Your Immunoassays*
www.kpl.com
800.638.3167
301.948.7755