APPLICATION NOTE

Optimization of SignaLOCK™ HRP ChemiWestern Kit (Imager) for Femtogram Detection

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INTRODUCTION

Chemiluminescent Western blotting is a highly sensitive immunoassay application that is commonly used to observe proteins of interest at picogram to femtogram levels. When detecting proteins at low concentration, assay optimization is vital to obtaining quality Western blots.

Optimizing Western blots takes time. Frequently, in the pursuit of higher signal, the optimization focus has been on the substrate and not on the antibody or blocker. Indeed, while second and third generation substrates have greatly improved sensitivity, non-fat dry milk, a blocker known to inhibit signal[1], continues to be used. Finding an appropriate balance between blocking solutions, antibody concentrations, and high-sensitivity substrates is critical for successful, sensitive chemiluminescent Western blots.

To answer this problem, KPL has developed SignaLOCK HRP ChemiWestern Kit (Imager), a high sensitivity chemiluminescent kit. The kit contains SignaLOCK Blocking Solution, a non-protein Western blot blocker, LumiGLO Ultra™ Chemiluminescent Substrate, a next-generation substrate, and background reducing 20X Wash Solution Concentrate. Together these components work in concert to reduce non-specific binding, amplify signal, and provide the highest Western blot sensitivity possible. This application note demonstrates proper optimization techniques to achieve femtogram detection limits using the SignaLOCK HRP ChemiWestern Kit (Imager).

DIRECT ASSAY DETECTION PROTOCOL

1. A 3-fold serial dilution of 1 µg/10 µL Human IgG was separated via SDS-PAGE, transferred to nitrocellulose, and blocked overnight at 4°C in the following solutions: 10X Phosphate-Buffered Saline (KPL Catalog No. 51-13-01), Milk Diluent/Block Solution Concentrate (KPL Catalog No. 50-82-01), 5X Detector Block, a fish-gelatin based blocker (KPL Catalog No. 71-83-00), and 5X SignaLOCK Blocking Solution, a proprietary non-protein blocker (KPL Catalog No. 54-54-00). Each blocking solution concentrate was diluted to 1X in reagent quality water prior to blocking.
2. Western blots were washed 3 times for 5 minutes with 20X Wash Solution Concentrate (KPL Catalog No. 54-54-00), diluted 1:20 in reagent quality water.
3. Horseradish Peroxidase (HRP)-labeled Goat Anti-Human IgG (γ) (KPL Catalog No. 474-1002) was diluted 1:300,000 in the corresponding blocking solution and incubated for 30 minutes at room temperature on a platform shaker.
4. Western blots were washed 1 time for 15 minutes, followed by 3 times for 5 minutes in Wash Solution Concentrate, and 1 time for 5 minutes in reagent quality water.
5. LumiGLO Ultra Chemiluminescent Substrate (KPL Catalog No. 54-54-00) was prepared and added to each Western blot for 1 minute without shaking. Western blots were blotted dry and placed in the imager for a series of additive exposures.

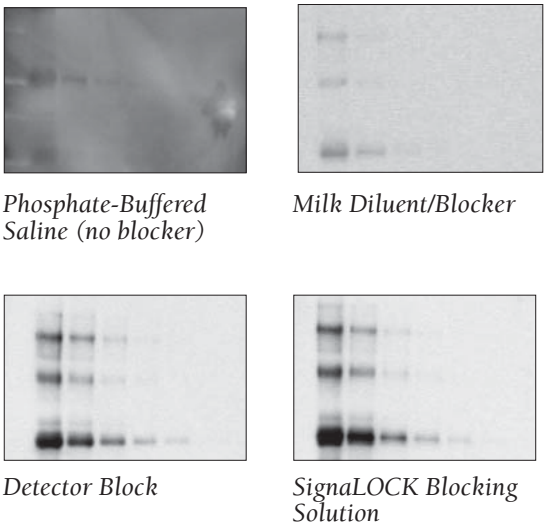

Figure 1. Sensitivity of protein detection using the SignaLOCK HRP ChemiWestern Kit with different blocking solutions: A 3-fold serial dilution of 1 µg/10 µL Human IgG was separated via SDS-PAGE, transferred to nitrocellulose, probed with Goat Anti-Human IgG (γ), and subsequently detected with LumiGLO Ultra, the substrate from the kit. After a 5-minute exposure the sensitivity achieved with Milk, Detector Block, and SignaLOCK Blocking Solution were 111, 12, and 12 ng, respectively. PBS achieved no observable bands.

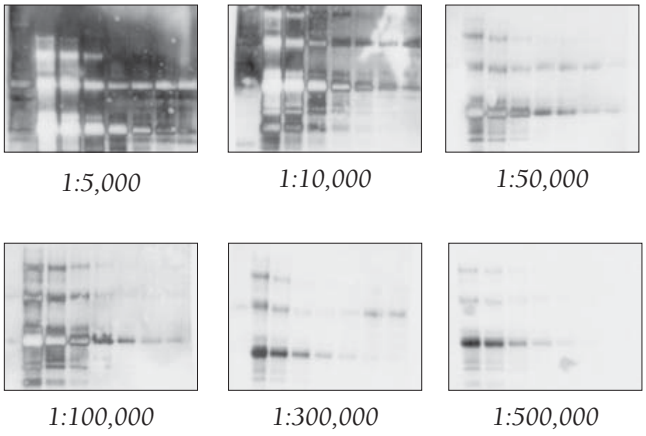

Figure 2. Optimization of conjugated antibody for a direct assay using SignaLOCK HRP ChemiWestern Kit (Imager): A 3-fold serial dilution of 1 µg/10 µL Human IgG was separated via SDS-PAGE, transferred to nitrocellulose, blocked with SignaLOCK Blocking Solution, probed with Goat Anti-Human IgG (γ), and subsequently detected with LumiGLO Ultra. HRP-labeled Goat Anti-Human IgG (γ) at 1 mg/mL was diluted in SignaLOCK Blocker to the dilutions shown above. The Western blots were imaged for 5 minutes.

INDIRECT ASSAY DETECTION PROTOCOL

1. A 3-fold serial dilution of 1 ng/10 µL Lysozyme was separated via SDS-PAGE, transferred to nitrocellulose, and blocked overnight at 4°C in 1X SignaLOCK Blocking Solution.

2. Purified Rabbit anti-Lysozyme was diluted in 1X SignaLOCK Blocking Solution to a 1:10,000 dilution and incubated for 30 minutes at room temperature on a platform shaker.

3. Western blots were washed 3 times for 5 minutes each with 1X Wash Solution Concentrate.

4. HRP-labeled Goat Anti-Rabbit (H+L) (KPL Catalog No. 074-1506) was diluted in 1X SignaLOCK Blocking Solution and incubated for 30 minutes at room temperature on a rotator.

5. Western blots were washed 1 time for 15 minutes, followed by 3 times for 5 minutes in Wash Solution Concentrate, and 1 time for 5 minutes in reagent quality water.

6. LumiGLO Ultra Chemiluminescent Substrate was prepared and added to each blot for 1 minute without shaking. Western blots were blotted dry and placed in the imager for a series of additive exposures.

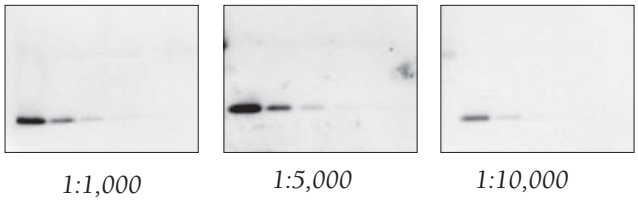

Figure 3. Optimization of primary antibody for indirect assay using SignaLOCK HRP ChemiWestern Kit (Imager): A 3-fold serial dilution of 1 ng/10 µL Lysozyme was separated via SDS-PAGE, transferred to nitrocellulose, and blocked overnight at 4°C in 1X SignaLOCK Blocking Solution. Purified Rabbit anti-Lysozyme was diluted from a 1 mg/mL stock in 1X SignaLOCK. Blots probed with a 1:300,000 dilution of HRP-labeled Goat Anti-Rabbit (H+L) diluted in 1X SignaLOCK Blocker. After a 5-minute exposure, the amounts of primary antibody resulted in a detection limit of 37, 37, and 111 pg for 1:1,000, 1:5,000, and 1:10,000, respectively.

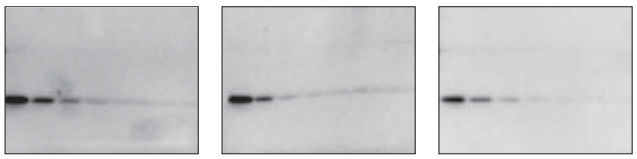

Figure 4. Optimization of conjugated antibody for an indirect assay SignaLOCK HRP ChemiWestern Kit (Imager): A 3-fold serial dilution of 1 ng/10 µL Lysozyme was separated via SDS-PAGE, transferred to nitrocellulose, and blocked overnight at 4°C in 1X SignaLOCK Blocker. Purified Rabbit anti-Lysozyme was diluted from a 1 mg/mL stock in 1X SignaLOCK Blocker to a 1:10,000 dilution. HRP-labeled Goat Anti-Rabbit (H+L) was diluted in 1X SignaLOCK Blocker to the dilutions shown above. After a 5-minute exposure, the 1:75,000 conjugate dilution resulted in 4 pg detection, while the 1:25,000 and 1:50,000 conjugate dilutions resulted in 460 fg detection.

RESULTS

Using the substrate and wash solution from SignaLOCK HRP ChemiWestern Kit (Imager) with Milk, Detector Block, and SignaLOCK Blocking Solution in an imager generated a sensitivity of 111, 12, 12 ng, respectively (Figure 1). In an indirect assay using the substrate and wash solution from the kit, optimizing both the primary and secondary antibody showed the optimal primary antibody concentration was 1:5,000, while the optimal secondary antibody concentration was 1:25,000 (Figures 3 and 4). These should be used only as guidelines for assay development with the SignaLOCK HRP ChemiWestern Kit (Imager), each assay will require individual optimization.

SUMMARY

Assay optimization is vital to obtaining effective Western blotting results. In this Application Note, we show that having an optimized kit enables improved band sharpness, band clarity, and intense contrast as a result of minimal background signal.

The first protocol showed that the optimized components of the SignaLOCK HRP ChemiWestern Kit provided the best signal intensity and band quality (Figure 1). In the second protocol, we showed this highly effective kit enabled the systematic selection of optimal primary and secondary antibody, permitting femtogram levels of protein detection (Figures 3 and 4).

Image systems help reduce the need for exposure time optimization by obtaining multiple images at a variety of time points. Through optimization as outlined, one can achieve excellent contrast of signal to background when using powerful chemiluminescent substrates making analysis of imager data easier.

REFERENCES

1. Connelly, A. and Mahy, H. A Comparison of Blocking Solutions Used in Western Blotting. Gaithersburg, MD: Kirkegaard and Perry Laboratories (US).

PRODUCTS

Description	*Size*	*Catalog No.*
SignaLOCK HRP ChemiWestern Kit (Imager)	1000 cm^2	54-54-00
SignaLOCK HRP ChemiWestern Kit (Film)	1000 cm^2	54-53-00
SignaLOCK AP ChemiWestern Kit (Film/Imager)	1000 cm^2	54-56-00
5X SignaLOCK Blocking Solution	125 mL	50-58-00
LumiGLO Ultra Chemiluminescent Substrate	50 mL	54-51-00

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