# KPL SignaLOCK™ ChemiWestern Kits

(Film and Imager Analysis)

| Products | Catalog No. |
|---|---|
| KPL SignaLOCK™ HRP ChemiWestern Kit (Film) | 5410-0010 (54-53-00) |
| KPL SignaLOCK™ HRP ChemiWestern Kit (Imager) | 5410-0011 (54-54-00) |
| KPL SignaLOCK™ AP ChemiWestern Kit (Film/Imager) | 5410-0012 (54-56-00) |

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## Introduction

Western blotting is a highly specific method for analysis of proteins. Use of an alkaline phosphatase (AP) or horseradish peroxidase (HRP)-linked affinity purified antibody together with a highly sensitive chemiluminescent substrate provides an excellent method for detection and characterization of samples bound to membranes through Western or dot blotting. Proteins are immobilized on membranes by way of either electrophoresis and subsequent transfer or dot blotting. For dot blots, proteins are spotted directly to the membrane. For a Western blot, proteins are separated by SDS-polyacrylamide gel electrophoresis (or a comparable method) and transferred to the membrane through either electrophoretic or passive transfer. Following attachment to membrane, a primary antibody is used to selectively bind the protein of interest. Alternatively, a known protein is bound to the membrane for screening of specific monoclonal antibodies or serum samples. An enzyme-labeled secondary antibody directed against the species of origin of the primary antibody or antiserum is then applied. This antibody is coupled to an enzyme (either AP or HRP) which work to destabilize the acridane present in each substrate. This reaction allows for the emittance of light. The resulting signal can be measured using a camera or X-ray film.

## Principle of KPL SignaLOCK™ ChemiWestern Kits

SeraCare offers a line of elite chemiluminescent substrates, KPL LumiGLO Reserve™, KPL LumiGLO Ultra™ and KPL PhosphaGLO Reserve™ that have been paired with a superior blocker, KPL SignaLOCK to achieve the ultimate in Western blot sensitivity. Femtogram detection is readily achievable with these kits. They offer improvements in the way of signal intensity – greater than 20-fold more sensitive than LumiGLO® and other competitive substrates. These products are specifically designed for the detection of proteins that are (1) difficult to detect because they are in such low quantities; or (2) from samples that are precious and, therefore, desired to be conserved. In addition, these kits provide the benefit of strong signal with the use of reduced amounts of antibodies and antibody conjugates.

Kits have been carefully optimized to allow for ease of selection for either imager or film detection. KPL SignaLOCK HRP ChemiWestern Kit (Film) contains KPL LumiGLO Reserve, a known high-performance substrate, which will accomplish femtogram detection on film. KPL SignaLOCK HRP ChemiWestern Kit (Imager) utilizes KPL LumiGLO Ultra, a novel substrate which allows for rapid, sensitive detection on imagers. KPL SignaLOCK AP ChemiWestern Kit (Film/Imagers) allows for versatile detection on both film and imagers with the use of KPL PhosphaGLO Reserve. All three kits utilize KPL SignaLOCK Blocking Solution, a non-protein blocker that eliminates cross-reactivity issues as well as enhancing signal visibility. For added convenience, KPL Wash Solution is also included to further minimize background issues.

These combinations deliver rapid and accurate identification of proteins for your more challenging detection work where one is forced to manage signal:noise contrast when pushing the detection limits of Western blot to clearly detect low abundance, short half-life, poorly expressed or otherwise small samples of protein.

## MATERIALS AND EQUIPMENT

**KPL SignaLOCK™** HRP ChemiWestern Kit (Film)
KPL LumiGLO Reserve™ Chemiluminescent Substrate A
KPL LumiGLO Reserve Chemiluminescent Substrate B
KPL 5X SignaLOCK™ Blocking Solution
KPL 20X Wash Solution

**KPL SignaLOCK™** HRP ChemiWestern Kit (Imager)
KPL LumiGLO Ultra™ Chemiluminescent Substrate A
KPL LumiGLO Ultra Chemiluminescent Substrate B
KPL 5X SignaLOCK™ Blocking Solution
KPL 20X Wash Solution

**KPL SignaLOCK™** AP ChemiWestern Kit (Film/Imager)
KPL PhosphaGLO Reserve Chemiluminescent Substrate
KPL 5X SignaLOCK™ Blocking Solution
KPL 20X Wash Solution Concentrate

Sufficient reagents are provided to test approximately 1000 $cm^2$ of membrane (ten 10 cm x 10 cm mini-blots) when using recommended minimal volumes. Reagents are stable for a minimum of one year when stored at 2–8°C. KPL SignaLOCK Blocking Solution is stable for a minimum of one year when stored at 2–25°C.

**PRODUCT SAFETY AND HANDLING**
See SDS (Material Safety Data Sheet) for this product.

**REQUIRED SUPPLIES AND EQUIPMENT FOR WESTERN BLOTTING NOT INCLUDED**

- Primary and secondary antibodies (purified)
- Nitrocellulose or PVDF Membrane (Nylon is not recommended)
- Platform shaker or rocker
- Blotting or Whatman paper
- Electrophoresis equipment
- Polyacrylamide gels
- Protein standards
- X-ray film (double emulsion) or CCD Imager
- Developing chemicals/equipment
- Incubation trays or tubes (Note: Gel box containers are not suitable for incubating membranes).

**WARNINGS AND PRECAUTIONS**

- Read ALL instructions thoroughly before using the kit.
- Always wear protective gloves and a lab coat for personal protection, as well as protection of the membrane and immunoassay reagents from contaminants such as skin oils or proteins.
- Caution should be used when preparing or handling polyacrylamide gels; monomeric acrylamide is a neurotoxin.
- For proper analysis of results, always include positive and negative controls, blanks and/or protein standards as appropriate.
- Prior to application of the kit reagents, the protein of interest must be immobilized onto the test membrane. Nitrocellulose and polyvinylidene difluoride (PVDF) membranes have both been determined to be suitable for use with these reagents.

**WESTERN BLOT DETECTION AT A GLANCE**
Total Time: 4 hours
**Polyacrylamide Gel Electrophoresis**
⇓
**Immobilize Protein on Membrane**
⇓
**Block Membrane**
1 hour or overnight
⇓
**Incubate Primary Antibody**
30 minutes - 1 hour
⇓
**Wash Membrane**
3 x 5 minutes per wash
⇓
**Incubate Conjugate**
30 minutes - 1 hour
⇓
**Wash Membrane**
1X Wash Solution, 4 x 5 minutes per wash
Reagent Quality Water, 1 x 5 minutes
⇓
**Incubate with Substrate**
1 minute
⇓
**Expose to Film or Imager**
10 seconds - 10 minutes

**NOTES FOR OPTIMAL KIT PERFORMANCE**

- To achieve optimal results, please blot the membrane completely dry with filter paper before image analysis.
- Different kits require different ratios of substrate. Special instructions for mixing of substrates are highlighted as **IMPORTANT: Substrate Instructions**.
- It is imperative to optimize primary and conjugate dilutions for your Western blot assay. Slight differences in activity can result in overwhelming background when employing powerful chemiluminescent substrates for detection. Primary and secondary antibodies should be purified to ensure optimal results.

**Polyacrylamide Gel Electrophoresis and Western Blotting**

| STEPS | CRITICAL POINTS |
|---|---|
| 1. Prepare samples by diluting to desired concentration with sample diluent. If using a reducing agent (ex. – β- mercaptoethanol), incubate samples at 100°C for 3 minutes prior to electrophoresis. | *If this kit is being used for the first time, reduced volumes of target may be loaded to achieve comparable detection to routine HRP-chemiluminescent substrates.* |
| 2. Electrophorese samples and transfer by standard methods. | *Both nitrocellulose and PVDF membranes may be used. Nitrocellulose is preferred.* |
| 3. Mark the orientation of the protein samples on the membrane prior to detection. | *The membrane may be cut into strips at this time if desired. Alternatively, it may be more convenient to cut strips after the entire membrane has been blocked.* |

**Detection of Western Blots**

There are many protocols available for the detection of Western blots. For optimal signal to noise and sensitivity, the following protocol and reagents are recommended.

| STEPS | CRITICAL POINTS |
|---|---|
| 1. Block the membrane by immersing in 1X KPL SignaLOCK using a minimum of 0.2 mL/cm$^2$ of membrane. Block at room temperature for 1 hour with gentle rocking or shaking, or stationary at 2–8°C overnight. If blocking overnight, container should be covered. | *Dilute KPL SignaLOCK 1:5 in reagent quality water. Make sure to use a container of proper size that allows the block solution to freely float over the membrane.* |
| 2. Remove blocking solution. Incubate membrane with purified primary antibody diluted 1:1,000 – 1:10,000 from a 1 mg/mL stock for 30 minutes - 1 hour. This antibody should be diluted in fresh blocking solution. | *It is recommended that serial dilutions through a dot blot are performed to determine the optimal working dilution of the primary antibody.* |
| 3. Remove primary antibody in block solution. Wash the membrane in a generous amount of 1X KPL Wash Solution (at least 25 mL for a 100 cm$^2$ membrane). Wash membrane 3 times for 5 minutes per wash with agitation. | *Dilute 20X KPL Wash Solution 1:20 in reagent quality water. This solution will provide optimal signal to noise.* |
| 4. Dilute appropriate enzyme-labeled secondary antibody 1/25,000 – 1/250,000 of a 1.0 mg/mL stock in freshly prepared 1X KPL SignaLOCK using a minimum of 0.2 mL/cm$^2$ of membrane. | *The optimal dilution may vary for different lots of conjugate. It is imperative that you titrate the conjugate to determine the optimal working dilution. These dilutions hold true for either AP or HRP.* |
| 5. Incubate blot with diluted conjugate for 30 minutes - 1 hour at room temperature with shaking. | |
| 6. Remove the substrate from refrigeration. During the conjugate incubation step, prepare the substrate. | *Allow substrate to warm to room temperature prior to use. Minimize exposure to light.* |

| STEPS | CRITICAL POINTS |
|---|---|
| **IMPORTANT: Substrate Instructions** | |
| **KPL SignaLOCK HRP ChemiWestern Kit (Film) and KPL SignaLOCK HRP ChemiWestern Kit (Imager)** | ***For substrate components labeled KPL LumiGLO Reserve: mix one part Solution A with two parts Solution B. Protect mixed solution from light until use.***<br><br>***For substrate components labeled KPL LumiGLO Ultra: mix equal parts Solution A with Solution B. Protect mixed solution from light until use.*** |
| **KPL SignaLOCK AP ChemiWestern Kit (Film/Imager)** | ***For substrate labeled KPL PhosphaGLO Reserve: Pipette requisite amount into a tube and allow to equilibrate.*** |
| 7. Wash the membrane in a generous amount of 1X KPL Wash Solution (at least 25 mL for a 100 $cm^2$ membrane). Wash membrane 4 times for 5 minutes per wash with agitation.<br><br>8. Rinse the membrane once for 5 minutes in reagent quality water with agitation.<br><br>9. Pour off the water from the blot and place the membrane on a sheet protector or a dry tray. Membrane should be slightly damp. | |
| 10. Pipette 0.05 mL/$cm^2$ of the previously prepared substrate onto the membrane. Incubate ***without*** rocking for 1 minute. | *Example: For a 10 x 10 cm blot, use 5 mL of substrate. The surface tension of the substrate will keep it on the surface of the membrane.* |
| **IMPORTANT: Substrate Instructions** | |
| **If using substrates labeled KPL LumiGLO Ultra or KPL LumiGLO Reserve:** | **Incubate the membrane for 1 minute.** |
| **If using substrate labeled KPL PhosphaGLO Reserve:** | **Incubate the membrane for 5 minutes.** |
| 11. Blot the membrane thoroughly dry with filter paper. | *Excess substrate on the blot will contribute to background.* |
| 12. **If using film:** Lift the membrane with clean forceps and seal in clear plastic. Expose to X-ray film for 10 seconds to 10 minutes. Adjust exposure time as needed. | *Take caution to ensure the surface of the membrane to which the assay reagents were applied is facing the film. Do not allow the film to get wet, nor move during exposure. Optimal exposure time should be determined by the signal to noise ratio and the amount of conjugate used. When using greater amounts of conjugate, 10 seconds may provide acceptable results.* |
| 13. **If using an imager:** Lift the membrane with clean forceps and place in the imaging system. Collect images for 5 seconds – 30 minutes. Peak signal intensity for KPL LumiGLO Reserve is at ~ 5 min, while peak signal intensity for KPL LumiGLO Ultra is at ~ 10 minutes. Peak signal intensity for KPL PhosphaGLO Reserve is at ~ 10 minutes. | *Follow the manufacturer's recommendations regarding the set up and operation of the imager.* |

## STRIPPING AND REPROBING MEMBRANES

After performing protein transfer, detection with substrate and film exposure, membranes may be stripped and reprobed with new primary and secondary antibodies.

1. Strip antibodies by incubating blot for 30 - 90 minutes at 70°C in erasure buffer: 2% SDS (w/v), 62.5 mM Tris-HCl (pH 6.8 at 20°C), 100 mM β-mercaptoethanol.
2. Wash 2 times, for 10 minutes each, in TBS: 10 mM Tris-HCl (pH 7.4 at 20°C), 150 mM NaCl.
3. Block for 2.5 hours in 1X KPL SignaLOCK Block (or equivalent block).
4. Repeat detection procedure.

## TROUBLESHOOTING GUIDE FOR CHEMILUMINESCENT DETECTION OF WESTERN BLOTS

### PROBLEM 1: NO SIGNAL

| Possible Cause | Corrective Measure |
|---|---|
| • Inactive enzyme conjugate. | Verify enzyme activity by mixing 10 µL of diluted conjugate with 1 mL of substrate (in a dark room, the substrate should glow). |
| • No binding of conjugate to the primary antibody. | Check that the specificity of the conjugate is correct for the primary antibody (i.e. do not use an anti-rabbit HRP with a mouse primary antibody). |
| • No transfer of target to membrane | Use a protein stain on unblocked membrane to verify attachment of target protein or use a pre-stained protein marker to monitor transfer. |
| • Detection of non-blotted side of membrane | Check that correct orientation of the membrane was maintained during the assay and during film exposure. |
| • Inhibition of horseradish peroxidase | Be sure that no buffers containing sodium azide were used; azide will inhibit horseradish peroxidase activity. |
| • Missed step in procedure | Be sure all steps of the procedure were followed correctly. |

### PROBLEM 2: WEAK SIGNAL

| Possible Cause | Corrective Measure |
|---|---|
| • Insufficient amount of antibody | Optimize antibody concentrations. Affinity of the primary antibody may change after proteins are denatured through SDS-PAGE. |
| • Amount of protein loaded/blotted is too low | Increase the amount of protein loaded onto the gel. |
| • Insufficient incubation of primary antibody to target | Increase the incubation times for weak primary antibodies. |
| • Insufficient exposure time | Increase the time of exposure to film, or increase image capture time. |
| • Excessive washing beyond recommended procedure | Be sure the procedure was followed as written. |

**PROBLEM 3: EXCESSIVE SIGNAL, NONSPECIFIC BANDS OR GENERAL BACKGROUND**

| Possible Cause | Corrective Measure |
|---|---|
| • Excessive antibody used for detection | Optimize conjugate concentration. Reduce antibody concentrations; optimal conjugate dilution should be 1/10,000 – 1/100,000 of a 0.1 mg/mL stock.<br><br>OR<br><br>Decrease the amount of primary antibody. |
| • Overexposure of blot | Expose or image the membrane to for a shorter period of time. |
| • Excessive protein loaded on the gel | Decrease the amount of protein loaded onto the gel. |
| • Insufficient blocking or washing, causing non-specific reaction | Increase blocking time and extend washing time or increase number of washes. |
| • Endogenous peroxidase in the sample (HRP only) | Test by incubating the blocked membrane in HRP substrate (without antibodies). After film exposure, if signal is obtained, blocking reagents such as 3% $H_2O_2$ in 100% MeOH may be required to remove the endogenous peroxidase activity. |
| • Extra bands not due to blocker. | Add 5% serum of animal from secondary antibody to conjugate incubation step. |

**PROBLEM 4: POORLY DEFINED OR "FUZZY" BANDS OR DOTS**

| Possible Cause | Corrective Measure |
|---|---|
| • Poor transfer of protein to membrane | Follow manufacturer's recommended procedure or contact the manufacturer for additional support regarding the blotting apparatus. |
| • Excessive substrate | Remove excess substrate before exposure of the membrane to film. |
| • Ghost images from shifted position of film during development | Avoid movement of film over membrane during exposure period. |
| • Inadequate handling of membranes | Certain membranes require special handling (ex. – PVDF must be pre-wet with 100% methanol before use. Check with the membrane vendor for correct procedures. |

Please contact SeraCare Technical Services at **800-638-3167** (USA) or **301-540-8200** for assistance in facilitating the smoothest transition possible to KPL SignaLOCK kits.

| **RELATED PRODUCTS** | **CAT. NO.** |
|---|---|
| KPL LumiGLO Ultra Western Blotting Substrate | 5430-0039 (54-51-00) |
| KPL LumiGLO Reserve Western Blotting Substrate | 5430-0051 (54-71-02) |
| KPL PhosphaGLO Reserve Western Blotting Substrate | 5430-0052 (55-60-01) |
| KPL 5X SignaLOCK Blocking Solution | 5440-0001 (50-58-00) |
| KPL 20X Wash Solution | 5150-0008 (50-63-00) |

Note: The recommendations of this bulletin are provided solely for the benefit of users who need practical guidance on immunoassay procedures. Due to the fact that experimental conditions for the use of the suggested products are beyond the control of SeraCare Life Sciences, Inc., it is impossible for SeraCare to implicitly guarantee the performance of the mentioned products for any and all assay procedures. Users who need additional information are encouraged to call Technical Services at 800/638-3167 or 301/591-8200 for assistance.