# KPL PhosphaGLO™ AP Substrate

| Catalog No. | Size |
|---|---|
| 5430-0054 (55-60-03) | 30 mL |
| 5430-0055 (55-60-04) | 100 mL |

## DESCRIPTION

KPL PhosphaGLO AP Substrate contains a dioxetane-based chemiluminescent substrate designed for use with phosphatase-labeled (AP) reporter molecules. KPL PhosphaGLO offers improvements in the way of signal intensity over traditional substrates, both chromogenic and chemiluminescent. This product is designed for the detection of proteins in both Western blotting and ELISA.

KPL PhosphaGLO AP Substrate is provided as a stable one-component solution. The product provides rapid and accurate identification of proteins in routine assays.

Two sizes are available. Results can be obtained on X-ray film or a chemiluminescent imager to provide a permanent record. A luminometer should be used for ELISA.

**NOTE**: This product may arrive frozen.

## CONTENTS

5430-0054 (55-60-03) contains:
1 x 30 mL KPL PhosphaGLO AP Chemiluminescent Substrate

5430-0055 (55-60-04) contains:
1 x 100 mL KPL PhosphaGLO AP Chemiluminescent Substrate

## STORAGE/STABILITY

Store this product at 2 – 8 °C. If it arrives frozen, allow it to thaw prior to use. KPL PhosphaGLO Solution should remain stored in its original container and protected from light.

The product is stable for a minimum of two years from date of receipt when stored under proper conditions.

## KPL PHOSPHAGLO CHEMILUMINESCENT SUBSTRATE USER'S GUIDE

- ⇒ KPL PhosphaGLO can be used with nitrocellulose and PVDF membranes. For best results, nitrocellulose is recommended.
- ⇒ The KPL PhosphaGLO working solution should be protected from light and warmed to room temperature prior to its use.
- ⇒ KPL PhosphaGLO is a very sensitive substrate. Insufficient washing of membranes or contamination of substrate with AP will result in non-specific background.
- ⇒ Because of KPL PhosphaGLO's sensitivity, the AP conjugate should be titrated to give the best results.
- ⇒ Do not allow KPL PhosphaGLO to contact the film. If this occurs, KPL PhosphaGLO solution will cause dark spots to appear on the film.
- ⇒ KPL PhosphaGLO emits light over the course of 5-7 days. Because of its high light intensity, images may be captured over the course of many different intervals. Initially, perform a 1 minute and a 10 minute exposure.

## APPLICATIONS

KPL PhosphaGLO AP Substrate has been optimized for Western blotting and dot blotting applications. It is also suitable for use in microwell applications such as ELISA. The following is a recommended procedure for Western blot detection.

## WESTERN BLOT DETECTION

### Suggested Reagents/Equipment Not Included

1. Primary antibody
2. AP-labeled secondary
3. Nitrocellulose or PVDF membrane
4. Blocking Solution (See RELATED PRODUCTS)
5. X-ray film (double emulsion) or CCD Imager
6. Platform shaker or rocker
7. Developing chemicals/equipment
8. Incubation trays or tubes
9. Wash solution (see RELATED PRODUCTS)
10. Assay buffer (see RELATED PRODUCTS)

## CONJUGATE OPTIMIZATION PRIOR TO DETECTION

- ⇒ Before beginning the assay, the optimal conjugate dilution should be determined. The use of highly sensitive chemiluminescent substrates on Western blots can cause high background if the conjugate concentration is not optimized. Each lot of conjugate should be optimized, as slight differences in activity can produce major differences in background.
- ⇒ Recommended conjugate dilutions should be tested at a range from 1/10,000 to 1/100,000 of a 0.1 mg/mL stock.

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**WESTERN BLOT DETECTION AT A GLANCE**

Total time: 4 hours

Polyacrylamide Gel Electrophoresis
⇓
Immobilize Protein on Membrane
Block Membrane
1 hour or overnight
⇓
Incubate with Primary Antibody
1 hour
⇓
Wash Membrane
3 x 5 minutes
1 x 10 minutes
⇓
Incubate with Conjugate
30 minutes - 1 hour
⇓
Wash Membrane
3 x 5 minutes
1 x 10 minutes
⇓
Rinse with Assay buffer
2 x 2 minutes
⇓
Incubate with PhosphaGLO Substrate
1 minute
⇓
Expose to Film
10 seconds - 10 minutes

| STEPS | | CRITICAL POINTS |
|---|---|---|
| 1. | Block the membrane by immersing in block solution (1X Detector Block is recommended) using a minimum of 0.2 mL/cm2 of membrane. Block at room temperature for 1 hour with gentle rocking or shaking, or stationary at 2 – 8 ˚C overnight. | *Example: for a 10 x 10 cm blot, use 20 mL of block. Make sure to use a container of proper size that allows the block solution to float freely over the membrane.* |
| 2. | Incubate membrane with primary antibody or serum sample for at least 1 hour. This antibody should be added directly to the Block Solution that was used for blocking (Step 1). | *Test serial dilutions through a dot blot to determine the optimal working dilution. Alternatively, use the recommended concentration determined by the primary antibody supplier.* |
| 3. | Wash the membrane in a generous amount of 1X Wash Solution (at least 25 mL for a 100 cm2 membrane). Wash membrane 3 times for 5 minutes each, followed by one 10-minute wash. | *1X TBS-TWEEN may also be used.* |

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| STEPS | | CRITICAL POINTS |
|---|---|---|
| 4. | Dilute appropriate conjugate 1/10,000 – 1/100,000 (of a 0.1 mg/mL stock) in freshly prepared conjugate diluent using a minimum of 0.2 mL/cm2 of membrane. | *Example: 2 µL conjugate + 20 mL diluent. Suggested diluents include KPL Detector Block and TBS-TWEEN. The optimal dilution may vary for different lots of conjugate. Titrate the conjugate to determine the optimal working dilution.* |
| 5. | Incubate blot with diluted conjugate for one hour at room temperature. | |
| 6. | During the conjugate incubation step, prepare 0.05 mL/cm$^2$ of membrane to be detected. | *Prepare KPL PhosphaGLO in advance to allow it to come to room temperature prior to its use. Cover with foil to minimize light exposure.* |
| 7. | After the conjugate incubation, wash as described in step 3. | |
| 8. | Pour off the remaining wash buffer from the blot and place the membrane on a sheet protector or a dry tray. | |
| 9. | Rinse membrane 2 x 2 minutes with 1X Assay buffer or 0.2 M Tris, pH 9.5-9.7. | |

| STEPS | | CRITICAL POINTS |
|---|---|---|
| 10. | Gently pipette 0.05 mL/cm$^2$ of previously prepared PhosphaGLO over the entire membrane. Incubate without rocking for 1 minute. | *Example: for a 10 x 10 cm blot, use 5 mL of KPL PhosphaGLO. The surface tension of the substrate will keep it on the surface of the membrane.* |
| 11. | Lift the membrane with forceps and blot the excess substrate onto a piece of filter paper. Seal the membrane in clear plastic and expose to X-ray film for 10 seconds to 1 minute. Adjust exposure time as needed. | *Excessive substrate on the blot will contribute to background.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.* |
| 12. | *Optional:* Chemiluminescent Imager Detection. Incubate the blot for twice the time typically used for film. If the imager provides stacking capabilities, capture exposures at 5 minute intervals for 1 hour to maximize signal. The optimal exposure can be chosen. | *Follow the manufacturer's recommendations regarding the set up and operation of the imager.* |

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## TROUBLESHOOTING

### Problem 1: No Signal

| Possible Cause | Corrective Measure |
|---|---|
| • Inactive alkaline phosphatase | Verify enzyme activity by mixing 10 µL of diluted conjugate with 1 mL of substrate (the substrate should glow in the dark). |
| • No binding of conjugate to the primary antibody | Confirm correct specificity of the conjugate for the primary antibody; i.e. no AP-anti-rabbit 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 | Ensure correct orientation of the membrane during the assay and film exposure. |
| • Missed step in procedure | Review procedure to ensure all steps were followed. |

### 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. |
| • Insufficient protein loaded or transferred | Increase the amount of protein loaded onto the gel. |
| • Insufficient incubation of primary antibody | Increase the incubation times for weak primary antibodies. |
| • Insufficient exposure time | Increase the time of exposure to film. |
| • Excessive washing beyond recommended procedure | Follow the procedure as written. |

### Problem 3: Excessive signal, nonspecific bands or general background

| Possible Cause | Corrective Measure |
|---|---|
| • Overexposure of film to signal | Expose the membrane to film for a shorter period of time. |
| • Insufficient blocking or washing | Increase blocking and washing time or increase number of washes. Vary type of block used. |
| • 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. Or, decrease the amount of primary antibody. |
| • Excessive protein loaded on the gel | Decrease the amount of protein loaded onto the gel. |

### 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. Check with the membrane vendor for correct procedures. |

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**Stripping and Re-probing a Western Blot**
This protocol is adapted from Kaufmann, *et. al*.[11]. After performing protein transfer, detection with KPL PhosphaGLO and film exposure, membranes may be stripped and re-probed 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 Block Solution.
4. Repeat detection procedure.

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

| RELATED PRODUCTS | CAT. NO. |
|---|---|
| KPL PhosphaGLO Reserve™ AP Substrate | 5430-0053 (55-60-02) |
| KPL Detector™ Block | 5920-0004 (71-83-00) |
| KPL Milk Diluent/Blocking Solution | 5140-0011 (50-82-01) |
| KPL 10% BSA Diluent/Blocking Solution | 5140-0006 (50-61-00) |
| KPL Wash Solution | 5150-0008 (50-63-00) |
| KPL Coating Solution | 5150-0014 (50-84-00) |
| KPL Phosphatase Assay Buffer | 5960-0017 (50-63-14) |



The product listed herein is for research use only and is not intended for use in human or clinical diagnosis.

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