PhosphaGLO Reserve™ AP Substrate

Catalog No.	Size
55-60-02	100 mL
55-60-01	30 mL

DESCRIPTION

PhosphaGLO Reserve AP Substrate contains a dioxetane-based chemiluminescent substrate designed for use with phosphatase-labeled (AP) reporter molecules. PhosphaGLO Reserve offers great improvements in the way of signal intensity. 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) are from samples that are precious.

PhosphaGLO Reserve AP Substrate is provided as a stable one-component solution. The product provides rapid and accurate identification of proteins that are of low abundance and potentially limited availability. Given the increased sensitivity, less target may be required on Western blots and ELISAs.

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 when performing ELISA.

NOTE: This product may arrive frozen.

CONTENTS

Catalog No. 55-60-01 contains:
1 x 30 mL PhosphaGLO Reserve AP Substrate

Catalog No. 55-60-02 contains:
1 x 100 mL PhosphaGLO Reserve AP Substrate

STORAGE/STABILITY

Store this at 2 – 8°C. If the product arrives frozen, allow it to thaw prior to use. PhosphaGLO Reserve 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.

PRODUCT SAFETY AND HANDLING

See MSDS (Material Safety Data Sheet) for this product.

RELATED PRODUCTS

Product	Catalog No.
PhosphaGLO AP Substrate, 30 mL	55-60-04
PhosphaGLO AP Substrate, 100 mL	55-60-03
Phosphatase Assay Buffer (10X), 200 mL	50-63-14
Detector™ Block, 240 mL	71-83-00
Milk Diluent/Blocking Solution Concentrate Kit, 200 mL	50-82-01
10% BSA Diluent/Blocking Solution Kit, 200 mL	50-61-00
10% BSA Diluent/Blocking Solution, 1 L	50-61-10
Wash Solution Concentrate (20X), 800 mL	50-63-00
Coating Solution Concentrate, 50 mL	50-84-00

PHOSPHAGLO RESERVE AP SUBSTRATE USER'S GUIDE

⇒ PhosphaGLO Reserve can be used with nitrocellulose and PVDF membranes. For best results, nitrocellulose is recommended.

⇒ PhosphaGLO Reserve should be protected from light and warmed to room temperature prior to use.

⇒ PhosphaGLO Reserve is an extremely sensitive substrate. Insufficient washing of membranes or contamination of substrate with AP will result in non-specific background.

⇒ Because of PhosphaGLO Reserve's super sensitivity, the AP conjugate should be titrated to give the best results.

⇒ Do not allow PhosphaGLO Reserve to contact the film. If this occurs, PhosphaGLO Reserve solution will cause dark spots to appear on the film.

⇒ PhosphaGLO Reserve 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

PhosphaGLO Reserve 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 antibody
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 for the assay. 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 will need optimization as slight differences in activity can result in major differences in background.

⇒ Recommended conjugate dilutions should be tested in a range from 1/10,000 to 1/100,000 of a 0.1 mg/mL stock.

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
1 hour
⇓
Wash Membrane
3 x 5 minutes
1 x 10 minutes
⇓
Rinse with Assay buffer
2x2 minutes
⇓
Incubate with PhosphaGLO Reserve Substrate
1 minute
⇓
Expose to Film
10 seconds - 10 minutes

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STEPS	CRITICAL POINTS
1. Block the membrane by immersing in block solution (1X Detector Block is recommended) using a minimum of 0.2 mL/cm^2 of membrane. Block at room temperature for 1 hour with gentle rocking, or stationary at 2 - 8°C overnight.	*Example: for a 10 x 10 cm blot, use 20 mL of block solution. 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 or 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 cm^2 membrane). Wash membrane 3 times for 5 minutes each, followed by one 10-minute wash.	*1X TBS-TWEEN™ may also be used.*
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/cm^2 of membrane.	*Example: 2 µL conjugate + 20 mL diluent. Suggested diluents include Detector Block and TBS/PBS-TWEEN. The optimal dilution may vary for different lots of conjugate. Ttitrate 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 PhosphaGLO Reserve. Prepare 0.05 mL/cm^2 of membrane to be detected.	*Allow PhosphaGLO Reserve to come to room temperature prior to use. Cover with foil to minimize light exposure.*

STEPS	CRITICAL POINTS
7. After the conjugate incubation, wash as described in step 3.	
8. Pour off 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.	
10. Gently pipette 0.05 mL/cm^2 of PhosphaGLO Reserve over the entire membrane. Incubate ***without*** rocking for 1 minute.	*Example: for a 10 x 10 cm blot, use 5 mL of PhosphaGLO Reserve.* *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 (in a dark room, the substrate should glow).
• No binding of conjugate to the primary antibody	Confirm correct specificity of the conjugate for the primary antibody; *i.e.* no anti-rabbit AP 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 to target	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.
• 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 Reprobing a Western Blot

This protocol is adapted from Kaufmann, *et. al.*[11]. After performing protein transfer, detection with PhosphaGLO Reserve 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 Block Solution.
4. Repeat detection procedure.

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