KPL PhosphaGLO AP Substrate

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

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*
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.	

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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.	*D ŇNc d aN c 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

Possible Cause	Corrective Measure
Poor transfer of protein to membrane	Follow 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 -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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