COLUMBIA BIOSCIENCES

WESTERN BLOT DETECTION KIT
Buffers and detection reagents for up to ten 10 x 10 cm^2 blots

Fluorescent detection via:
Goat anti-mouse SureLight® P3

Cat. #: WK-P112

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KIT CONTENTS

Item	**Qty**
1X Blocking Buffer	50 mL
10X Wash Buffer	50 mL
2° Antibody - Goat anti-Mouse SureLight® P3	1 vial

STORAGE CONDITIONS

- Kits are shipped with a cold pack for next day delivery, and are warranted for six months from date of shipment if stored at 4°C.
- Phycobiliprotein solutions, once reconstituted, should not be frozen and should be protected from light.

PRIOR TO USE

- Dilute wash buffer to 1X strength by adding 9X volume reagent grade water.
- To use entire amount, add bottle of 10X buffer to 450 mL water.
- Reconstitute SureLight® P3 detection reagent with 1.5 ml reagent grade water

Supplies Needed

***NOTE**: Collect the following supplies before starting the procedure.*

User supplies:

Reagent grade water
Western Blot Membranes
Membrane Tray
Primary Antibodies
Forceps
Fluorescent imager

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PROTOCOL

NOTE: *Please read the entire procedure as well as the TIPS section before beginning.*

Western blot should be prepared using standard blotting procedures for the membrane being used. Allow blot to dry for at least 1 hour before detection. Dry blots can be stored overnight at room temperature if stored between filter paper.

NOTE: *Membranes should be handled only by the edges using clean forceps. Take care to not touch the membrane with gloved or bare hands.*

NOTE: *Do not write on the membrane with an ink pen or marker because they will fluoresce. Use a pencil to write on the membrane to avoid this problem.*

After the gel has been transferred to the blotting membrane:

1. Soak membrane in 1xPBS for at least 2 minutes.
2. Place membrane in incubation tray and block using 1X blocking buffer for 1 hour with gentle shaking. Use enough blocking buffer to completely cover the membrane.
3. Prepare the primary antibody dilution in blocking buffer. Prepare enough volume to completely cover the membrane when incubating.
4. Incubate the blot in the diluted primary antibody for 1 – 4 hours at room temperature with gentle shaking or overnight at 4^oC.
5. Wash the membrane by pouring off the primary antibody solution, rinse with 1X wash buffer, cover blot with 1X wash buffer and shake on a platform shaker for 5 minutes at room temperature, pour off wash solution, repeat 3 more times.
6. Reconstitution of detection reagents:
    a. Add 1.5 ml of dH_2O to the SureLight® P3 secondary antibody reagent
    b. Allow 20-30 minutes for full solubility before using.
7. Dilute the secondary antibody conjugates in blocking buffer. Prepare enough volume to completely cover the membrane.
    Recommended reagent dilutions:
    SureLight® P3 Goat anti-mouse – 1:50
8. Add secondary antibody to membrane after washing is complete. Shield the membrane from light (cover with foil for example) while it is incubating.
9. Incubate for 60 minutes at room temperature with gentle shaking.
10. Wash membrane as in step 5.
11. Rinse membrane with 1X PBS with no Tween®-20 and allow it to dry before scanning.

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TIPS & RECOMMENDATIONS

- **Follow the protocol carefully.**
- **Recommended scanner excitation and emission parameters:**
-

		Excitation	Emission
SureLight® P3	**Laser**	594/614/635 nm (Red)	
	Filter Set	615 nm peak, 10-30 nm bandpass	660 nm peak, 10-30 nm bandpass
	Instrument Preset	Cy™5, AlexaFluor® 647	

- No single blocking reagent will be optimal for every antigen-antibody pair. Some primary antibodies may exhibit greatly reduced signal or different nonspecific binding in different blocking solutions. If you have difficulty detecting your target protein, changing the blocking solution may dramatically improve performance. If the primary antibody has worked well in the past using chemiluminescent detection, try that same blocking solution for detection.
- To avoid background speckles on blots, use high-quality ultra pure water for buffers. Rinsing previously-used incubation boxes with methanol can reduce background contamination of future blots.
- Never perform Western incubations or washes in dishes that have been used for Coomassie staining.
- Membranes should be handled only by their edges, with forceps.
- Always pour off antibody solution and washes from the same corner of the box to ensure complete removal of previous solutions.
- After handling membranes that have been incubating in antibody solutions, clean forceps thoroughly with distilled water and/or methanol, then rinse with distilled water.
- Always clean the scanning surface prior to scanning to remove dust, buffer residue, and smudges that may affect image quality or contaminate the membrane. The scanning surface can be wiped with methanol, rinsed with a small volume of water, followed by a final rinse with isopropanol to help prevent blot contamination from the scan bed. Pressurized “canned air” may be helpful in removing dust and lint.
- If using a silicone mat to cover your membranes, carefully clean the surface of the mat that will touch the membrane with isopropanol. Blot the mat dry and avoid rubbing or wiping the mat with tissue, as this creates more lint and leads to speckling.
- Do not wrap the membrane in plastic when scanning.
- Store the secondary antibody vials at 4°C in the dark. Do not thaw and refreeze antibodies, as this will affect performance. Minimize exposure to light and take care not to introduce contamination into the vial. Dilute immediately prior to use. If particulates are seen in the antibody solution, centrifuge before use.
- Protect membrane from light during secondary antibody incubations and washes.
- Use gels that contain the narrowest well size possible to minimize load volume and concentrate the target protein.
- The best transfer conditions, membrane, and blocking agent for each experiment will vary, depending on the antigen, sample type, and antibody.
- For proteins <100 kDa, the recommended transfer buffer is 1X Tris-glycine buffer with 20% methanol and no SDS. Addition of SDS to the transfer buffer can greatly reduce binding of transferred proteins to the membrane (for both PVDF and nitrocellulose).
- For proteins >100 kDa, decrease the methanol concentration in the transfer buffer to 10%.
- Soak the gel in transfer buffer for 10-20 minutes before setting up the transfer. Soaking equilibrates the gel and removes SDS so that it will not be carried over into the transfer tank.
- To maximize retention of transferred proteins on the membrane, allow the membrane to air-dry completely after transfer (approximately 1-2 hours).

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- Do not over-block. Extended blocking times can cause loss of target protein from the membrane.

TECHNICAL ASSISTANCE

Phycobiliproteins have been tested extensively in customer applications. If you have any questions or experience difficulties regarding any aspect of our products, please contact us:

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Our customers are an important source of information regarding advanced or specialized uses of our products. We encourage you to contact us if you have any suggestions about product performance or new applications and techniques.

TROUBLESHOOTING GUIDE

Problem	Possible Cause	Solution / Prevention
High background, uniformly distributed	BSA used for blocking	Blocking solutions containing BSA may cause high membrane background. Try switching to a different blocker.
	Not using optimal blocking reagent.	Compare different blocking buffers to find the most effective for your system; try blocking longer. Try diluting blocker 1:1 in 1X PBS.
	Background on nitrocellulose	Increase amount of Tween® 20 added to the diluted antibodies, staying in the range of 0.1-0.2%. Add SDS to diluted secondary antibody, staying in the range of 0.01-0.02%.
	Background on PVDF	Reduce Tween® 20 in diluted antibodies to 0.1%. Add 0.01-0.02% SDS to diluted secondary antibody.
	Membrane autofluorescence	Scan an unused dry membrane to check for autofluorescence using the same image acquisition parameters as the "high background Western blot.
	Antibody concentrations too high	Optimize primary and secondary antibody dilutions
	Insufficient washing.	Increase number of washes and buffer volume.
		Make sure that 0.1% Tween® 20 is present in wash buffer and increase if needed. Note that excess Tween® 20 (0.5-1%) may decrease signal.
	Cross-reactivity of antibody with contaminants in blocking buffer	Use Blocking Buffer instead of milk. Milk-based blockers may contain IgG that can cross-react with anti-goat antibodies. This can significantly increase background and reduce antibody titer. Milk-based blockers may also contain endogenous biotin or phospho-epitopes that can cause higher background.
	Inadequate antibody volume used	Increase antibody volume so entire membrane surface is sufficiently covered with liquid at all times (use heat-seal bags if volume is limiting). Do not allow any area of membrane to dry out.
		Use agitation for all antibody incubations
	Membrane contamination	Always handle membranes carefully and with forceps. Do not allow membrane to dry. Use clean dishes, bags, or trays for incubations.
Uneven, blotchy or speckled background	Blocking multiple membranes together in small volume	If multiple membranes are being blocked in the same dish, ensure that blocker volume is adequate for all membranes to move freely and make contact with liquid.
	Membrane not fully wetted or allowed to partially dry	Keep membrane completely wet at all times. This is particularly crucial if blot will be stripped and re-used.
		If using PVDF, remember to first pre-wet in 100% methanol.
	Contaminated forceps, dishes, or transfer equipment	Always carefully clean forceps after they are dipped into an antibody solution, particularly dye-labeled secondary antibody. Dirty forceps can deposit dye on the membrane that will not wash away.
		Use clean dishes, bags or trays for incubations
	Dirty scanning surface or silicone mat	Clean scanning surface and mat carefully before each use. Dust, lint, and residue will cause speckles.
	Incompatible marker or pen used to mark membrane	Use only pencil to mark membranes.

Problem	Possible Cause	Solution / Prevention
Weak or no signal	Not using optimal blocking reagent	Primary antibody may perform substantially better with a different blocker. Try diluting blocker 1:1 in 1X PBS.
	Insufficient amount of antibody used.	Primary antibody may be of low affinity. Increase amount of antibody or try a different source.
		Extend primary antibody incubation time (try 4-8 hrs at room temperature, or overnight at 4°C).
		Increase amount of primary or secondary antibody, optimizing for best performance.
	Primary or secondary antibody may have lost reactivity due to age or storage conditions.	Use fresh or unexpired antibodies.
	Too much detergent present; signal being washed away	Decrease Tween® 20 and/or SDS in diluted antibodies. Recommended SDS concentration is 0.01-0.02%, but some antibodies may require an even lower concentration.
	Insufficient antigen loaded	Load more protein on the gel. Use the narrowest possible well size to concentrate antigen.
	Protein did not transfer well	Check transfer buffer choice and blotting procedure.
		Use pre-stained molecular weight marker to monitor transfer, and stain gel after transfer to make sure proteins are not retained in gel.
	Protein lost from membrane during incubations	Reduce blocking times or decrease high concentrations of detergent in diluted antibodies
	Proteins not retained on membrane during transfer	Allow membrane to air dry completely (1 - 2 hr) after transfer. This helps make the binding irreversible.
		Addition of 20% methanol to transfer buffer may improve antigen binding. ***Note:*** *methanol decreases pore size of gel and can hamper transfer of large proteins.*
		SDS in transfer buffer may interfere with binding of transferred proteins, especially for low molecular weight proteins. Try reducing or eliminating SDS. ***Note:*** presence of up to 0.05% SDS does improve transfer efficiency of some proteins.
		Small proteins may pass through membrane during transfer (“blow-through”). Use membrane with smaller pore size or reduce transfer time.
Nonspecific or unexpected bands	Antibody concentrations too high.	Reduce the amount of antibody used.
		Reduce antibody incubation times.
		Increase Tween® 20 in diluted antibodies.
		Add or increase SDS in diluted secondary antibodies.
	Not using optimal blocking reagent.	Choice of blocker may affect background bands. Try a different blocker.
	Cross-reactivity between antibodies in a two-color experiment	Double-check the sources and specificities of the primary and secondary antibodies used
		Use only highly cross-adsorbed secondary antibodies.
		There is always potential for cross-reactivity in two-color experiments. Use less secondary antibody to minimize this.
		Always test primary and secondary antibody sets on separate blots first so you know what bands to expect and where.
		Avoid using mouse and rat antibodies together, if possible. Because the species are so closely related, anti-mouse will react with rat IgG to some extent, and anti-rat with mouse IgG. Sheep and goat antibodies may exhibit the same behavior.
	Bleedthrough of signal from one channel into other channel	Reduce signal in further experiments by reducing the amount of protein loaded or antibody used.

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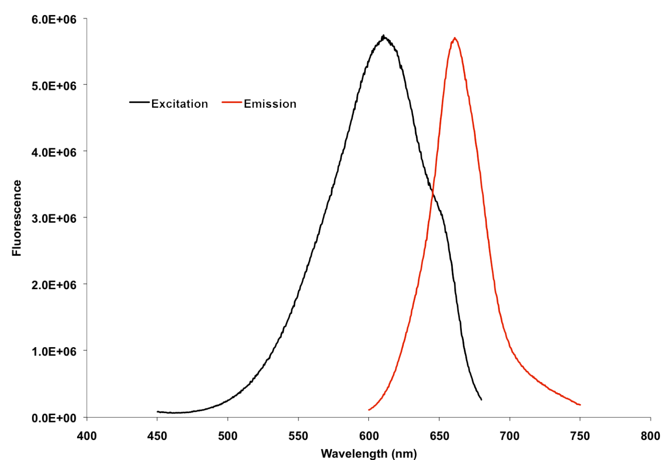
APPENDIX A: SPECTRAL PROPERTIES

SureLight® P3

Spectral Characteristics

- Visible absorption maximum   614 nm
- Emission maxima   662 nm

Fluorescence (normalized to absorbance, excitation 450–650 nm, emission 600–750 nm)



TRADEMARKS

SureLight® is a trademark of Columbia Biosciences Corporation
Tween®-20 is a registered trademark of ICI Americas, Inc.

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