



Instruction Manual

Visualizer™

Western Blot Detection Kit

Cat. #64-201 and #64-202

*For 1000 cm² of membrane,
20 mini-gel-sized blots*

For laboratory research use only.

Not intended for human or
animal diagnostic, therapeutic
or other clinical uses.

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I. Introduction and Principle

The Visualizer™ Western Blot Detection Kit was designed to make Western detection of proteins easier by providing the highest level of sensitivity with the least amount of background in a chemiluminescent reagent system. Western blotting has become one of the most common protein analysis techniques used in biomedical research today. It provides a non-radioactive, direct method for identifying proteins in a mixture as well as determining the relative amount of specific proteins in different samples.

Variations on standard Western blotting techniques are numerous. In general, a complex protein mixture (such as a cell lysate, cell extract or purified protein preparation) is fractionated on a gel (e.g. SDS-PAGE, native PAGE, IEF or 2-D PAGE) by electrophoresis. After separation, proteins are transferred to a membrane, which can be nitrocellulose or polyvinylidene fluoride (PVDF). For the best results with Visualizer™, we recommend using PVDF. Nitrocellulose can also be used, but more cell sample is needed compared to that with PVDF.

Enzyme-linked detection utilizing secondary antibodies (that can recognize virtually any primary antibody) covalently conjugated to enzymes such as horseradish peroxidase (HRP),

provided a significant advance in Western blotting. The Visualizer™ Western blot detection reagents, in the presence of HRP, will go through a catalyzed reaction that results in the steady and stable emission of luminescence. Imaging is performed on standard X-ray film or by using an image documenting station that is fitted with a chemiluminescent-sensitive camera. This provides the capability of making multiple exposures of a blot, securing valuable data, extending the dynamic range of detection and making quantitation easier and more accurate.

Upstate's Visualizer™ Western Blot Detection Kit utilizes a superior version of the chemiluminescent HRP substrate luminol that results in the fastest and most sensitive detection of an antigen while providing a long lasting signal and the highest signal to noise ratio of any competing product on the market. These kits include the chemiluminescent substrates paired with an HRP-Conjugated Secondary Antibody to either rabbit or mouse IgG. The kit has been carefully designed to provide users with the most convenient and highest quality Western blot detection reagents available today. Combined with Upstate's array of primary antibodies, the Visualizer™ Western Blot Detection Kit will provide you with the results your research has always needed.

II. System Components

A. Reagents Supplied

All components are stable for 18 months when stored as indicated.

Visualizer™ Western Detection Reagents

Component	Amt.	Conc.	Storage	Cat. #
Detection Reagent A	20 ml	–	4°C	20-275A
Detection Reagent B	2x20 ml	–	4°C	20-275B

Secondary Antibody HRP-Conjugates

Component	Amt.	Conc.	Storage	Cat. #
Goat anti-Mouse IgG	500 µg	1 µg/µl	COA*	12-349
Goat anti-Rabbit IgG	500 µg	1 µg/µl	COA*	12-348

B. Required Materials Not Provided

- SDS-PAGE Reagents and Apparatus
- Western Transfer Reagents and Apparatus
- PVDF, Nitrocellulose or Other Membrane
- Rocker or Shaker
- Membrane Incubation Containers
- Forceps

- Paper Towels or Wipes
- Primary Antibody
- Wash Buffer
- Blocking Buffer
- X-Ray Film
- Plastic Wrap (i.e. Saran Wrap)
- Dark Room or Digital Imaging Station
- X-Ray Film Development Reagents and Equipment
- Ponceau Stain (optional)
- Stripping Buffers (optional)

C. Preparation of Reagents

Wash Buffer may be water, TBS or PBS. Some users prefer to use water or PBS, but TBS may result in a reduced background. Many blotting procedures utilize PBS or TBS containing Tween-20, from 0.05% to 0.5%; Tween-20 may reduce non-specific background on the membrane, but too much may interfere with primary antibody binding resulting in loss of signal. The optimum Wash Buffer formulation for a particular antibody should be empirically determined.

PBS-Phosphate-Buffered Saline

PBS formulations may vary from user to user; one formulation is:

	To Prepare 1x Reagent:		To Prepare 10x Reagent:	
	Amt. (g)	Final Conc. (mM)	Amt. (g)	Final Conc. (mM)
KH_2PO_4	0.144	1.06	1.44	10.6
$\text{K}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$	0.795	2.96	7.95	29.6
NaCl	9.000	155.00	90.00	1550.0

Dissolve potassium phosphate and NaCl in 800 ml water, adjust pH to 7.4 and then dilute to 1 L with water. Autoclave if reagent will be stored for more than a few days.

TBS-Tris-Buffered Saline

TBS formulations may vary from user to user; one formulation is:

	To Prepare 1x Reagent:		To Prepare 10x Reagent:	
	Amt. (g)	Final Conc. (mM)	Amt. (g)	Final Conc. (mM)
Tris Base	3.03	25	30.3	250
NaCl	9.00	155	90.0	1550

Dissolve Tris and NaCl in 800 ml water, adjust pH to 7.4 and then dilute to 1 L with water. Autoclave if reagent will be stored for more than a few days.

Tween-20 Formulations in PBS or TBS

	To 1 L of 1x PBS or TBS, add:			
Amount of Tween-20 (ml)	0.5	1	2.5	5
For a Final Tween-20 % of	0.05%	0.1%	0.25%	0.5%

Blocking Buffer - 3% Blocking Reagent in Wash Buffer.
Prepare just prior to use. Dissolve 0.9 g of Blocking Reagent in 30 ml of Wash Buffer.

Stripping Buffer I

	Amount	Final Conc.
Tris	3.1 ml, (1 M stock)	62.5 mM
SDS	1.0 g	2%
β -mercaptoethanol	0.34 ml	100 mM

Dissolve SDS in 40 ml water, add Tris, adjust pH to 6.8, add β -mercaptoethanol and then dilute to 50 ml with water.

Stripping Buffer II

	Amount	Final Conc.
Glycine	1.5 g	0.4 M
SDS	0.1 g	0.2%
Tween-20	1.0 ml	2%

Dissolve Glycine and SDS in 40 ml water, adjust pH to 2.2, add Tween-20 and then dilute to 50 ml with water.

III. Immunoblotting, Detection & Miscellaneous Procedures

Please read all instructions thoroughly before proceeding. There are many variations on the general procedure outlined below. Although Upstate endeavors to provide specific instructions for each of our antibodies intended for use in Western blotting, the optimal conditions for use of primary antibodies from other suppliers may need to be empirically determined. If the specific instructions provided with a primary antibody deviate from those outlined below, always default to the specific instructions.

A. Immunoblotting Procedure

1. Prepare samples and perform SDS-PAGE following the instructions provided with your specific electrophoresis system.
2. Transfer the gel to a PVDF (recommended) or nitrocellulose membrane following all instructions provided with your specific blotting device and membrane.
3. Rinse the membrane twice with Wash Buffer to remove transfer buffer and gel particles. If desired, the membrane may be stained with Ponceau S to visualize protein bands. (i.e., to examine protein loading before proceeding with the detection, see section C on page 15).

4. Incubate the membrane in freshly prepared Blocking Buffer, with agitation, for 20 to 30 minutes at room temperature (or overnight at 4°C).
5. Incubate the membrane 1-2 hours at room temperature (or overnight at 4°C) in the appropriate primary antibody diluted in freshly prepared Blocking Buffer with agitation during the incubation. Dilute as recommended for the particular antibody.
6. Wash the membrane 3-5 times (3-5 minutes each) with Wash Buffer.
7. Incubate the membrane for 1 hour at room temperature (or overnight at 4°C) in the appropriate secondary antibody HRP conjugate diluted in freshly prepared Blocking Buffer with agitation during in the incubation. Dilute as recommended for the particular conjugate (*see the Certificate of Analysis*).
8. Wash the membrane 3-5 times (3-5 minutes each) with Wash Buffer.
9. (Optional) Rinse the membrane 4-5 times with water.

B. Chemiluminescent Detection

1. Prepare Visualizer™ Working Solution prior to use by combining in a 1:2 ratio Detection Reagent A and Detection Reagent B respectively. You will need approximately 3 ml of Working Solution for a single mini-gel sized blot (3 ml per 50 cm² of membrane). Mix the solution well and protect from excessive exposure to light. The Visualizer™ Working Solution is stable at room temperature for several hours. For best results, allow the Visualizer™ Working Solution to warm to room temperature before use.
2. Transfer the membrane(s) from the last wash to a fresh tray or dish. Weigh boats, pipette tip boxes or other glass/plastic dishes work well. It is best if the tray or dish is only slightly larger than the membrane and is not made of polystyrene plastic.
3. Pipette the Visualizer™ Working Solution onto the blotted membrane and incubate 5 minutes at room temperature. The membrane should be completely covered with Visualizer™ Working Solution.
4. Remove the membrane(s) from the Visualizer™ Working Solution with forceps (never touch the membrane with ungloved fingers), drain excess Visualizer™ Working Solution and then gently touch the corner of the

membrane to a paper towel or filter paper to remove the last drop. **DO NOT** allow the membrane to become dry, as the enzyme and substrate require moisture to function.

5. Place the membrane(s) on a firm surface such as a small glass plate and seal it with plastic wrap being careful not to have any air bubbles trapped between neither the plate and membrane nor the membrane and plastic wrap.
6. Develop an image for the Western blot using either a dark room and X-ray film or a digital imaging station.
7. Exposure time may vary from a few seconds to a few minutes (or longer), depending upon the amount of antigen being detected.
8. After all desired images have been obtained, membranes may be stripped and reprobbed or stained with Ponceau S and/or dried as desired. Dried membranes may be placed within a clean, safe bag which can then be sealed with most common impulse heat sealers for long term storage. The stored membranes can be rehydrated and then reprobbed at a later date.

C. Optional Procedure – Ponceau Staining

Membranes may be stained with Ponceau S either before Immunoblotting or after Detection. If staining is done before Immunoblotting, the membrane must be destained before proceeding. **DO NOT** allow the membrane to become dry before Immunoblotting and Detection. If staining is performed after Detection, the membrane may be destained to the point where protein bands are distinct, then dried and sealed within plastic as a permanent record.

1. Incubate the membrane in Ponceau S working solution for 5-10 minutes at room temperature with agitation. (10x stock is 2% Ponceau S in 30% trichloroacetic acid and 30% sulfosalicylic acid; dilute 1:10 with water for use; Sigma, cat. #P7767)
2. Remove the Ponceau S solution (the solution may be saved and reused several times).
3. Rinse in water until protein bands are distinct. Change water as necessary. Destaining will be rapid, requiring only 1-2 minutes. If the Immunodetection is complete, the membrane may be dried for storage at this point.

4. If Immunodetection has yet to be performed, the position of molecular weight markers or lanes may be marked with a pencil at this time.
5. The membrane may be rinsed a few more times with water to remove most of the residual stain from the bands. The last of the stain will be removed during blocking of the membrane (*Step A.4 on page 12*)

D. Optional Procedure – Stripping and Reprobing

Chemiluminescent detection is fully compatible with stripping blots and reprobing with different antibodies. Some antigen may be lost with each strip/reprobe cycle, so it is recommended that detection of antigens expected to be present in the least amounts be performed first before any membrane stripping. There are two commonly used stripping procedures. Neither has a strong advantage or disadvantage; the best method to use for a particular antigen/antibody must be empirically determined. If a membrane becomes dry, it will be difficult to strip. It is imperative that membranes to be stripped are kept moist. Generally, if stripping and reprobing is likely, it is a good idea to put membranes into PBS immediately after the final X-ray film exposure has been obtained.

Method #1: Hot SDS/ β -mercaptoethanol

1. Incubate the membrane in Stripping Buffer I (20 ml per blot or more) for 30 min at 70°C.
2. Wash the membrane with PBS until the odor of β -mercaptoethanol can no longer be detected.

Method #2: Acid Glycine

1. Incubate the membrane in Stripping Buffer II (20 ml per blot or more) 2 x 30 min at room temperature.
2. Wash the membrane with 3 x 5 minutes with PBS.
After either stripping procedure, block the membrane and proceed with detection using a new primary antibody.

E. Abbreviated Immunoblotting and Detection Procedure for Experienced Users

Immunoblotting

1. Prepare samples and perform SDS-PAGE.
2. Transfer the gel to a membrane (PVDF is recommended).
3. Rinse the membrane twice with Wash Buffer and stain with Ponceau S if desired (Section III, part C).
4. Block the membrane by incubating it for at least 20-30 minutes at room temperature.
5. Incubate the membrane 1-2 hours at room temperature (or overnight at 4°C) with the appropriate primary antibody.
6. Wash the membrane 3-5 times (3-5 minutes each) with Wash Buffer.
7. Incubate the membrane for 1 hour at room temperature (or overnight at 4°C) with the appropriate secondary antibody HRP conjugate and dilution.
8. Wash the membrane 3-5 times (3-5 minutes each) with Wash Buffer.

Detection

1. Prepare Visualizer™ Working Solution (a 1:2 ratio of Detection Reagent A to Detection Reagent B) and allow solution to come to room temperature. You will need 3 ml Visualizer™ Working Solution per 50 cm² of membrane.
2. Transfer the membrane(s) from the last wash to a fresh tray or dish.
3. Add Visualizer™ Working Solution and incubate 5 minutes at room temperature.
4. Remove the membrane(s) from the Working Solution and drain excess.
5. Place the membrane(s) on a glass plate and wrap with plastic (i.e. Saran Wrap).
6. In a dark room, expose X-Ray film to the membrane(s) and develop film or use digital imaging station in place of film.
7. Strip and reprobe the membrane, if desired.
8. Stain the membrane with Ponceau S, dry it and seal it within plastic, if desired, for storage.

IV. Troubleshooting

The following tips address most problems encountered during Western blotting:

Smear Pattern or Distorted Bands

- **Uneven contact between gel and membrane:** cassettes used should allow a tight fit, leading to even pressure over the entire surface of the gel and membrane.
- **For Tris-Glycine gels, gel not equilibrated in buffer prior to transfer:** the gel should be soaked in Towbin transfer buffer containing methanol for 15-30 minutes before assembling the transfer sandwich. For precast gels, make sure to follow the manufacturer's instructions for gel preparation for transfer.

"Bald Spots"

- **Bubbles between gel and membrane:** bubbles create areas of low transfer efficiency. Bubbles should be completely removed when putting together the transfer sandwich.

Incomplete Transfer

- **Incomplete protein transfer:** this often occurs with high molecular weight proteins, especially when using a methanol-based transfer buffer. One way to prevent

this is by using a nylon membrane, which does not require methanol in the transfer buffer. Adding SDS to the transfer buffer and using higher field strengths also improve protein transfer.

- **Proteins transferred through membrane:** this may occur when working with proteins of very low molecular weight. Optimizing/shortening transfer times and using a double layer of membrane usually enhances retention of small proteins. PVDF membranes are superior to nitrocellulose for preventing proteins from going through the membrane.
- **Inappropriate transfer buffer used:** the most stable and commonly used buffers are Tris-Glycine based (Towbin Transfer Buffer).
- **Impurities in the transfer buffer:** this will lead to a pattern on the membrane that mirrors the holes in the transfer cassette. Fresh buffer should be prepared for each transfer.
- **The wrong transfer buffer was used based on the isoelectric point (pI) of the protein being detected:** The pH of the transfer buffer used and the pI of the protein being detected will determine the direction of the protein transfer. If the pH of the buffer and the pI of the protein are near equal, the protein will remain

in the gel. If the pH of the buffer is lower than the pI of the protein, the protein will have a net negative charge and migrate towards the positive electrode. If the pH of the buffer is higher than the pI of the protein, the protein will have a net positive charge and migrate towards the negative electrode. Adding SDS to the buffer is one way to make sure all proteins in the gel have a net negative charge and therefore migrate towards the positive electrode. For pre-cast, neutral pH gels, consult the manufacturers instructions.

High Background

- **Cross-reactivity between blocking agent and primary antibody:** this will result in overall membrane background. Usually, the addition of detergent (Tween-20) to the Washing Buffer will eliminate the problem. If background persists, changing the blocking agent is recommended.
- **Concentration of either primary and/or secondary antibody too high or incubation time too long:** the higher the antibody concentration and the longer the incubation time, the greater the non-specific binding. Raising the incubation temperature (e.g. to 37°C) is recommended over lengthening the incubation time.

Also, many short washing steps are better than a few long ones.

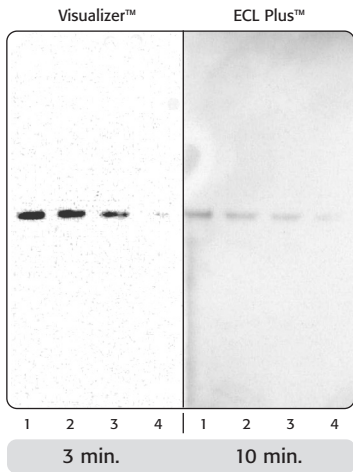
- **Membrane drying during incubation process:** care should be taken to keep membrane from drying out during incubation.

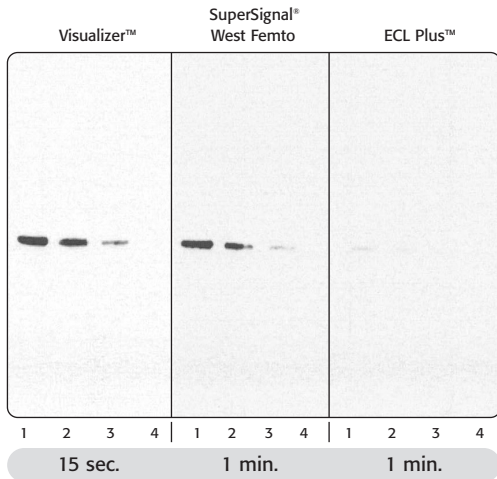
Little or No Signal

- **Antigen is not recognized by primary antibody:** this can occur especially with monoclonal antibodies that were raised against a native protein. In some cases, a non-reducing gel system may need to be used.
- **Inhibition of secondary antibody conjugate:** HRP-labeled antibodies should not be used in conjunction with sodium azide or hemoglobin.
- **Detergent is too harsh:** SDS, Nonidet P-40 and Triton X-100 disrupt binding between proteins. Tween-20 is the most commonly used and recommended detergent for washing and incubation solutions.
- **Try a different membrane:** For example, PVDF is a more sensitive membrane than nitrocellulose. In addition, there are many different manufacturers of each that have different protein binding capabilities.
- **Load more sample per lane.**

V. Sample Data (see page 26 for legend)

A. p38 (PVDF)



B. cdk2 (Nitrocellulose)

Western detection reagent product comparison. Four different quantities of HeLa cell lysate (cat. #12-501) were run on an Invitrogen 4-12% NuPAGE Bis/Tris gel in MES buffer and transferred to a PVDF (Panel A) or nitrocellulose (Panel B) membrane.

A. Lanes 1-4 respectively: 100 ng, 75 ng, 50 ng and 25 ng HeLa cell lysate.

B. Lanes 1-4 respectively: 1,000 ng, 700 ng, 400 ng and 100 ng HeLa cell lysate.

Blots were blocked for 1 hour at room temperature in 5% nonfat milk PBST.

Primary antibody concentrations used:

A. Anti-p38 α /SAPK2 α , clone 2F11 (cat. #05-454): 1 μ g/ml

B. Anti-cdk2 (cat. #06-505): 1 μ g/ml for Visualizer™ and ECL Plus™ and 200 ng/ml for SuperSignal® West Femto

Secondary antibody concentrations used:

A. 200 ng/ml goat, anti-mouse IgG HRP

B. Visualizer™ and ECL Plus™ at 20 ng/ml and SuperSignal® West Femto at 10 ng/ml goat, anti-rabbit IgG HRP.

Membranes were washed and each section was placed into its respective Western detection reagent for 5 minutes and brought back together for exposure to film. Exposure times are noted under each section.

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www.upstate.com

United States 706 Forest Street,
Charlottesville, Virginia 22903-5231

tel 434 975 4300 or toll free 800 233 3991

fax 434 220 0480 or toll free 866 831 3991

e-mail info@upstate.com

tech support 800 548 7853 or

techserv@upstate.com

Europe Unit 3 Mill Square,
Featherstone Road, Wolverton Mill South,
Milton Keynes MK12 5YU UK

tel +44 (0) 1908 552820

fax +44 (0) 1908 552821

e-mail euinfo@upstate.com

tech support +44 (0) 1908 552842,
freephone (UK only) 0800 0190 555 or

eurotechserv@upstate.com