upstate®
cell signaling solutions

Western Blot Detection Kit

Instruction Manual

Cat. No. 17-318

For 1000 cm^2 of Membrane
10 10x10 cm Blots
20 Mini-Gel-Sized Blots

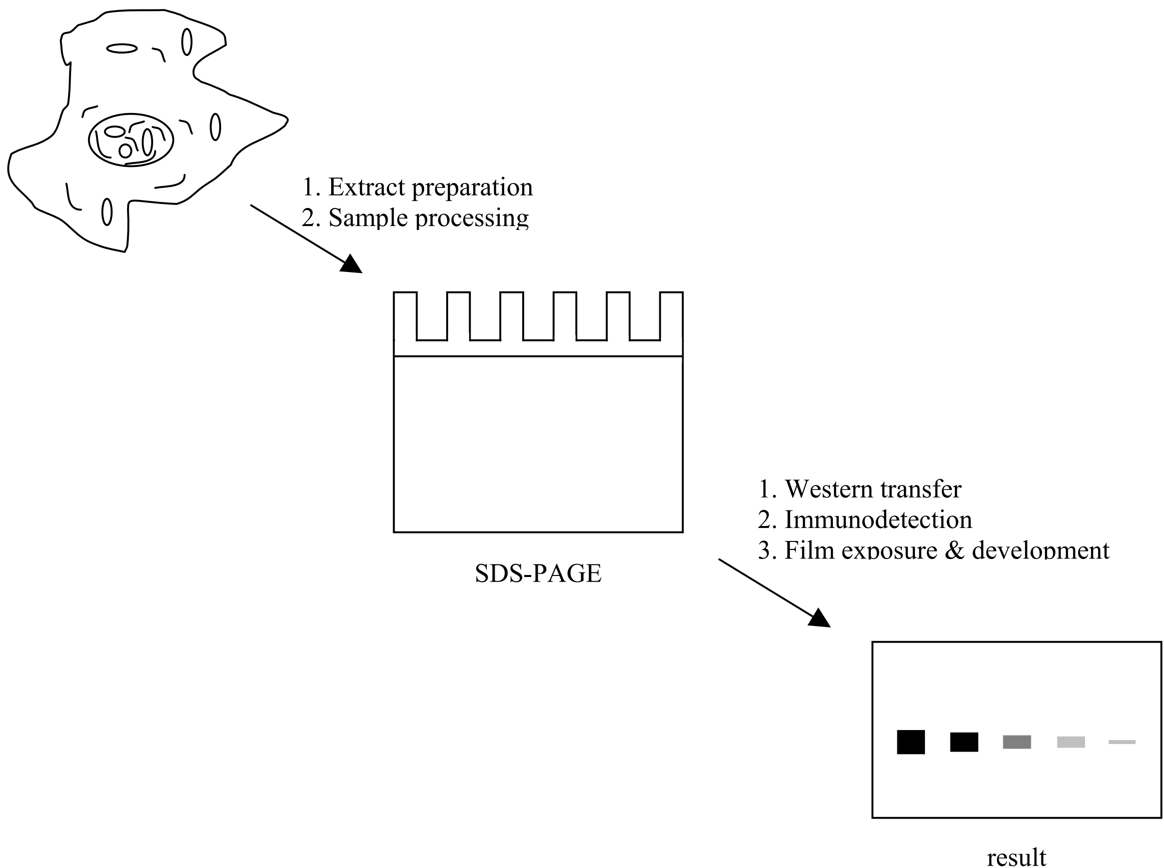

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Table of Contents

Section	Page
I. Introduction and Principle	2
II. System Components	
A. Reagents Supplied	3
B. Materials Required but not Supplied	3
C. Preparation of Reagents	3-4
III. Immunoblotting, Detection, & Miscellaneous Procedures	
A. Use of Pyronin Y	5
B. Immunoblotting Procedure	5
C. Chemiluminescent Detection	6
D. Use of Double-It Reagent	7
E. Ponceau Staining	8
F. Stripping and Reprobing	8
G. Abbreviated Procedure for Experienced Users	9
IV. Troubleshooting	10
V. Sample Data	11
VI. References	12

I. Introduction and Principle

Western blotting has become one of the most common protein analysis techniques used in biomedical research today. Western blotting provides a direct method for identifying, monitoring, and determining the relative amount of specific proteins in large numbers of different samples. The technique is fairly straightforward and can be performed quite rapidly. The general technique of western transfer and immunodetection can be readily used with SDS-PAGE, native PAGE, IEF or 2-D PAGE with only minor variations on the general method.

Variations on standard western blotting techniques are numerous. In general, a complex protein mixture (such as a cell lysate or extract, or a purified protein preparation) is fractionated on a gel by electrophoresis. After separation, proteins are transferred to a membrane, which can be nitrocellulose, polyvinylidene fluoride (PVDF) or nylon; nitrocellulose and PVDF membranes are most commonly used for protein work. Usually, specific antibodies, known as primary antibodies, are used to detect specific protein antigens on the membrane.

Previously, detection of antibody-antigen immune complexes on a membrane was accomplished using ^{125}I-labeled Protein A. Binding of this reagent to immune complexes immobilized on a membrane permitted convenient imaging of blots with standard X-ray film. Many problems exist with this method, however, including the lack of reactivity of Protein A with a broad range of antibodies, and handling and disposal of the copious amounts of radioactive waste produced during membrane processing. In addition, detection of low-abundance antigens frequently required very long film exposures, delaying further experimentation.

Enzyme-linked detection, utilizing secondary antibodies (that can recognize virtually any primary) covalently conjugated to enzymes such as horseradish peroxidase (HRP), provided a significant advance in western blotting. Originally, enzyme substrates that produced colored reaction products were used; specific detection resulted in production of a colored band directly on the membrane. This technique is imperfect, however; only one copy of a result is obtained, making loss of data a problem, and it is easy to overdevelop the reaction, resulting in high nonspecific backgrounds. Poor sensitivity is also an issue with this method.

Today, outstanding non-radioactive detection procedures now exist that provide the required sensitivity without many of the problems associated with earlier techniques. The development of the chemiluminescent HRP substrate luminol has enabled rapid and high-sensitivity detection. Imaging is performed on standard X-ray film, providing the capability of making multiple exposures of a blot, securing valuable data and extending the dynamic range of detection (a high-intensity band on a short-exposure film can easily be compared with a low-intensity band on a long-exposure film).

Upstate's Western Blot Detection Kit utilizes LumiGLO®, a superior version of the chemiluminescent HRP substrate luminol, and includes HRP-Conjugated Secondary Antibodies that recognize rabbit, mouse and sheep IgGs, providing very broad detection capability. The kit also includes a number of items designed to eliminate many common problems encountered in blotting. Pyronin Y Reagent precisely marks gel lane positions on the membrane; Blocking Reagent fills unoccupied sites on membranes; phosphorescent Autorad Orientation Markers facilitate alignment of X-ray films with the membrane; Film Exposure Folders provide a wrinkle- and static-free plastic holder for membranes during film exposure and for permanent storage of dry membranes; Double-It Reagent reduces nonspecific film background, improving visualization of low-intensity bands. The kit has been carefully designed to provide users with the most convenient and highest quality western blot detection kit available today. Combined with Upstate's array of primary antibodies, the 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 1 year when stored as indicated.

Component	Amount	Conc.	Storage	Cat. No.
LumiGLO®* Chemiluminescent Substrate	(100ml)	-	4ºC	20-212
Reagent A	50ml	-	-	-
Reagent B	50ml	-	-	-
Secondary Antibody HRP-Conjugates	-	-	-20ºC	-
Rabbit anti-Sheep IgG	125µl	1µg/µl	-	12-342**
Goat anti-Mouse IgG	125µl	2 µg/µl	-	12-349**
Goat anti-Rabbit IgG	75µl	1 µg/µl	-	12-348**
Blocking Reagent	20g	-	RT	20-200
Double-It Reagent	20ml	-	RT	20-213
Autorad Orientation Markers	10 markers	-	RT	20-135**
Film Exposure Folders	20 folders	7.5x5.5 in.	RT	20-211
Pyronin Y Solution	10µl	10mg/ml	RT	20-214

* **LumiGLO®** is a registered trademark of Kirkegaard & Perry Laboratories, Inc.
** This Cat. No. indicates the same material, but in a different pack size.

B. Materials Required but not Supplied

SDS-PAGE Reagents and Apparatus
Western Transfer Reagents and Apparatus
Nitrocellulose or other Membrane
Rocker or Shaker
Incubation Containers
Forceps
Paper Towels or Wipes
Specific Primary Antibody(ies)
Wash Buffer
Blocking Buffer
X-Ray Film
X-Ray Film Exposure Cassette
Dark Room
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 TBS, but PBS 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 nonspecific 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, but water is often a good starting point.

PBS - Phosphate-Buffered Saline
PBS formulations may vary from user to user; one formulation is:

	To Prepare **1X** Reagent:		To Prepare **10X** Reagent:	
	Amount (g)	Final Conc. (mM)	Amount (g)	Final Conc. (mM)
KH_2PO_4	0.144	1.06	1.44	10.6
$K_2HPO_4 \cdot 7H_2O$	0.795	2.96	7.95	29.6
NaCl	9.00	155	90.0	1550

Dissolve potassium phosphate and NaCl in 800ml water, adjust pH to 7.4, and then dilute to 1L 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:	
	Amount (g)	Final Conc. (mM)	Amount (g)	Final Conc. (mM)
Tris Base	3.03	25	30.3	250
NaCl	9.00	155	90.0	1550

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

Tween-20 Formulations in PBS or TBS

	To 1L of **1X PBS or TBS**, add:			
Amount of Tween-20 (ml)	0.5	1	2.5	5
For a final Tween-20 Conc. of:	0.05%	0.1%	0.25%	0.5%

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

Stripping Buffer I

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

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

Stripping Buffer II

	Amount	Final Conc.
Glycine	1.5g	0.4M
SDS	0.1g	0.2%
Tween-20	1ml	2%

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

Pyronin Y is a non-reactive dye that can be used to conveniently mark lane positions, facilitating procedures requiring a membrane to be cut for probing with multiple primary antibodies. Pyronin Y will produce a pink band on membranes that will be retained during processing, but will not interfere with antibody recognition or chemiluminescent detection.

To use Pyronin Y, simply add 2μl of the 10mg/ml stock to 1ml of 2X SDS-PAGE Sample Buffer. Use the sample buffer as you normally would. The Pyronin Y dye front (pink) will run slightly above the bromophenol blue in your sample buffer, and will also transfer to the membrane.

If desired, the top of membranes may be marked with Pyronin Y as well. Stop electrophoresis when the dye front is half-to-three-quarters of the way down the gel, apply a small amount of 1X SDS-PAGE sample buffer (containing Pyronin Y) into the lanes, and then resume electrophoresis. This will result in a pink spot at the top and bottom of the each lane of the gel.

B. Immunoblotting Procedure

1. Prepare samples (with or without Pyronin Y) and perform SDS-PAGE following all instructions provided with your specific electrophoresis system.
2. Transfer the gel to a nitrocellulose (or other) membrane following all instructions provided with your specific blotting device.
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 E below).
4. Block the membrane by incubating it for 20 - 30 minutes at room temperature (or overnight at 4°C) with agitation in freshly prepared Blocking Buffer.
5. Incubate the membrane 1 - 2 hours at room temperature (or overnight at 4°C) with agitation in the appropriate primary antibody diluted in freshly prepared Blocking Buffer (dilute as recommended for the particular antibody).
6. Wash the membrane 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 agitation in the appropriate secondary antibody HRP conjugate diluted in freshly prepared Blocking Buffer (dilute as recommended for the particular conjugate; see the Certificate of Analysis).
8. Wash the membrane 5 times (3 - 5 minutes each) with Wash Buffer.
9. Rinse the membrane 4 - 5 times with water.

C. Chemiluminescent Detection

1. Prepare LumiGLO® Working Reagent prior to use by combining equal volumes of the Substrate Solution A and Solution B. You will need approximately 5ml of Working Reagent for a single mini-gel sized blot (1 ml per 10 cm^2 of membrane). Allow LumiGLO® Working Reagent to warm to room temperature before use.
2. Place an open Film Exposure Folder on a flat level surface. Attach an Autorad Orientation Marker (Catalog # 20-135) to the Folder if desired. The marker should be positioned so as not to obscure the membrane(s) when the folder is closed.
3. Transfer the membrane(s) from the last water rinse to a fresh tray or dish. Weigh boats, pipette tip boxes, or other glass or plastic dishes work well. It is best if the tray or dish is only slightly larger than the membrane.
4. Add LumiGLO® Working Reagent and incubate 1 minute at room temperature. The membrane should be completely covered with Reagent.
5. Remove the membrane(s) from the Working Reagent with forceps, drain excess Reagent, 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.
6. Place the membrane(s) in the open Film Exposure Folder, and then close the folder, being sure that there are no air bubbles between the membrane and the upper layer of the Folder.
7. Place the Folder in an appropriate X-Ray film exposure cassette.
8. In a dark room, expose X-Ray film to the membrane(s). Exposure time may vary from a few seconds to a few minutes (or longer), depending upon the amount of antigen being detected.
9. Develop the X-Ray film(s).
10. After all desired films have been obtained, membranes may be stripped and reprobed, or stained with Ponceau S and/or dried as desired. Dried membranes may be placed within a Film Exposure Folder, which can then be sealed with most common impulse heat sealers. This permanent record of the blot may then be stored in a lab notebook. Dried films may be treated with Double-It Reagent to eliminate background.

D. Use of Double-It Reagent

Most X-Ray film (such as Kodak X-Omat AR5) is double-sided; the chemical emulsion, which produces a dark band in response to exposure to light, is coated on both sides of the film plastic. Chemiluminescent reagents typically only expose one side of the film, the side closest to the membrane (the "bottom" surface of the film). However, when the film is developed, the emulsion on both sides of the film is developed. The development of the unexposed film surface can often result in a hazy or foggy film (noise), which effectively reduces the intensity of a specific band (signal).

Double-It Reagent can be used to remove the noise from the side of the film that was not exposed to the signal (the "upper" surface). Care is needed however, as Double-It Reagent will also remove bands if it is applied to the wrong ("bottom") surface of the film.

Upstate is not responsible for loss of specific bands if Double-It Reagent is used on the wrong surface of the film. Use Double-It Reagent only on the top surface of your X-ray film.

It is strongly recommended that Double-It Reagent be tested in a small and unimportant area of the film to ensure that the correct side is being used. Simply apply a small amount of the Reagent to an area on the blot that exhibits background but is not important in interpretation of the result. A good choice to aid in the determination of film orientation is the Autorad Orientation Marker (Catalog # 20-135). If the marker signal can be properly read, and is not erased after Double-It Reagent is applied to the marker signal area, the film is right side up.

1. Dry the X-Ray film before using Double-It Reagent.
2. Determine which side of the film is upper (containing only background noise) and lower (exposed by the membrane, containing specific band signal).
3. Place the film upper side up on a piece of white paper (or a light box) in a fume hood. Double-It Reagent is a proprietary formulation containing bleach. Some individuals may be sensitive to bleach and bleach fumes, so use of Double-It Reagent in a fume hood is recommended.
4. Dilute the Double-It Reagent as appropriate to obtain the desired result. Undiluted Reagent may be spotted onto the film and wiped around with a paper towel or wipe; all areas of the film contacted by reagent will be cleared of background. Dilution with water will weaken the reagent; a 1:5 dilution will only be strong enough to clear background from the spot it first comes into contact with.
5. Double-It Reagent may be applied directly to the film (for more dilute preparations) or to a paper towel. Gently rubbing the Reagent on the film will cause the nonspecific background to disappear, resulting in a film with much higher clarity. This effectively increases the Signal-to-Noise ratio of a specific band; for faint bands that can only just be seen without this procedure, the Signal-to-Noise ratio may be doubled.

E. 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 a Film Exposure Folder (or wrapped in 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, Catalog # 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)

F. 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.

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.

Hot SDS/β-ME

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

Acid Glycine

1. Incubate the membrane in Stripping Buffer II (20ml 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.

G. Abbreviated Immunoblotting and Detection Procedure for Experienced Users

Note: This abbreviated procedure is provided as a convenience to outline and facilitate completion of the assay. It is recommended that a photocopy of the page be used. As a step is completed, it may be checked off in the appropriate box. After completion of the assay, the page can then be incorporated into your lab notebook if desired.

Immunoblotting

☐ 1. Prepare samples (with or without Pyronin Y) and perform SDS-PAGE.

☐ 2. Transfer the gel to a nitrocellulose (or other) membrane.

☐ 3. Rinse the membrane twice with Wash Buffer and stain with Ponceau S if desired.

☐ 4. Block the membrane by incubating it for 20 - 30 minutes at room temperature.

☐ 5. Incubate the membrane 1 - 2 hour at room temperature (or overnight at 4°C) with the appropriate primary antibody.

☐ 6. Wash the membrane 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.

☐ 8. Wash the membrane 5 times (3 - 5 minutes each) with Wash Buffer.

☐ 9. Rinse the membrane 4 - 5 times with water.

Detection

☐ 1. Prepare LumiGLO® Working Reagent (equal volumes of Substrate Solution A & B).

☐ 2. Place an open Film Exposure Folder (with an Autorad Orientation Marker) on a flat level surface.

☐ 3. Transfer the membrane(s) from the last water rinse to a fresh tray or dish.

☐ 4. Add LumiGLO® Working Reagent and incubate 1 minute at room temperature.

☐ 5. Remove the membrane(s) from the Working Reagent and drain excess Reagent.

☐ 6. Place the membrane(s) in a Film Exposure Folder.

☐ 7. Place the Folder in an appropriate X-Ray film exposure cassette.

☐ 8. In a dark room, expose X-Ray film to the membrane(s).

☐ 9. Develop the X-Ray film(s).

☐ 10. Strip and reprobe the membrane, if desired.

☐ 11. Stain the membrane with Ponceau S, dry it, and seal it within a Film Exposure Folder, as desired.

☐ 12. Clear film background with Double-It Reagent if desired.

IV. Troubleshooting

The following tips address most problems encountered during western blotting:

Smeared 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.
- Gel not equilibrated in buffer prior to transfer: the gel should be soaked in transfer buffer containing methanol for 15 to 30 minutes before assembling the transfer sandwich.

"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.
- Inappropriate transfer buffer used: the most stable and commonly used buffers are Tris-Glycine based.
- 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.

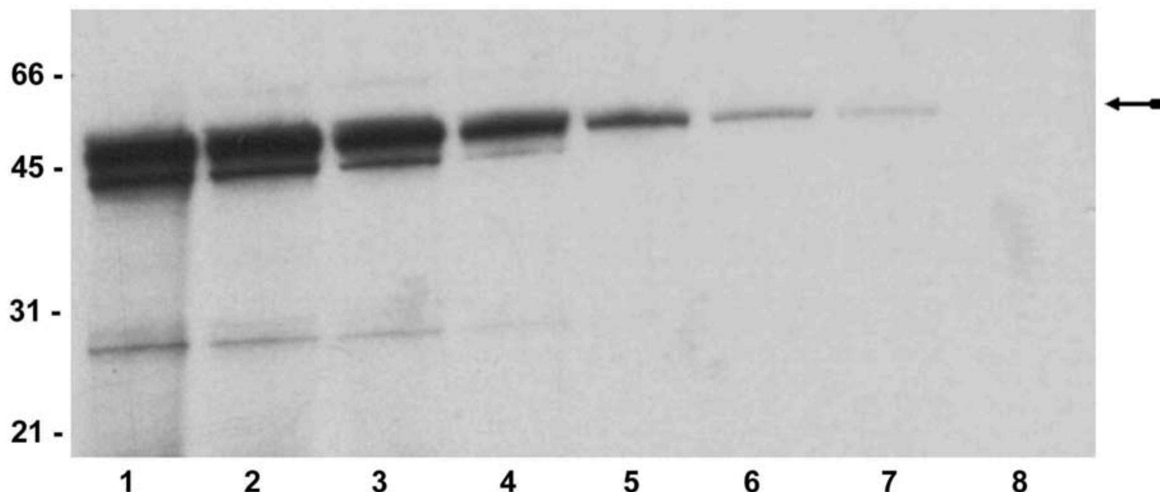
High Background

- 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 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 37ºC) 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

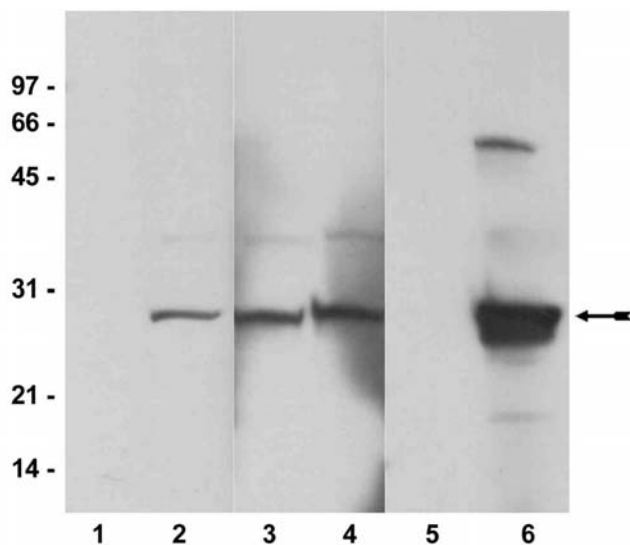
- Antigen is not recognized by primary antibody: this can occur especially with monoclonal antibodies that were raised against a native protein. In some cases, a non-reducing gel system may need to be used.
- Inhibition of secondary antibody conjugate: HRP-labeled antibodies should not be used in conjunction 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.

V. Sample Data



Immunoblot Analysis

20µg (lane 1), 10µg (lane 2), 5µg (lane 3), 2.5µg (lane 4), 1.0µg (lane 5), 0.5µg (lane 6), 0.25µg (lane 7) and 0.1µg (lane 8) samples of L6 cell lysate were resolved by electrophoresis, transferred to nitrocellulose and probed with Anti-MAP Kinase 1/2 (0.5µg/ml). Proteins were visualized using a goat anti-rabbit secondary antibody conjugated to HRP. LumiGLO® chemiluminescent substrate was used as the detection system. Film was exposed 1 second before developing, and was treated with Double-It Reagent after drying. Arrow indicates MAP Kinase 1/2 (42-44kDa).



HSP27 Phosphorylation Assay

HSP27 recombinant protein (Catalog # 14-381) was phosphorylated in a non-radiographic assay by MAPKAP Kinase 2 (Catalog # 14-337). To demonstrate phosphorylation, 0.1µg samples of non-phosphorylated (lanes 1, 3, 5) and phosphorylated (lanes 2, 4, 6) HSP27 were resolved by electrophoresis, transferred to nitrocellulose and probed using the antibodies described below. Visualization was achieved using HRP-conjugated secondary antibodies and the LumiGLO® chemiluminescent detection system. Film was exposed 6 seconds before developing, and was treated with Double-It Reagent after drying. Arrow indicates HSP27/phospho-HSP27 (~27kDa).

Lanes 1, 2

Primary antibody: Anti-phospho-HSP27 (Ser78) sheep immunoaffinity purified IgG (Catalog # 07-035), 0.5µg/ml.
Secondary antibody: Rabbit anti-sheep IgG, HRP-conjugate (Catalog # 12-342B), 1:2000 dilution.

Lanes 3, 4

Primary antibody: Anti-HSP27, rabbit polyclonal IgG (Catalog # 06-478), 0.5µg/ml.
Secondary antibody: Goat anti-rabbit IgG, HRP-conjugate (Catalog # 12-348B), 1:5000 dilution.

Lanes 5, 6

Primary antibody: Anti-phospho-HSP27 (Ser78) clone JBW502, mouse monoclonal IgG (Catalog #05-645), 0.5µg/ml.
Secondary antibody: Goat anti-mouse IgG, HRP-conjugate (Catalog # 12-349B), 1:2000 dilution.

VI. References

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