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Grasp the Proteome™

Western Blotting Handbook and Troubleshooting Guide

Featuring the SuperSignal® West Family of Products

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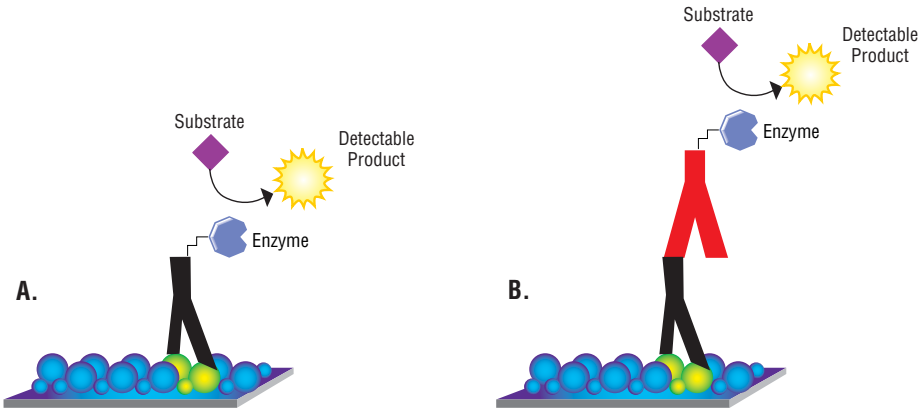
Introduction

The term "blotting" refers to the transfer of biological samples from a gel to a membrane and their subsequent detection on the surface of the membrane. Western blotting (also called immunoblotting because an antibody is used to specifically detect its antigen) was introduced by Towbin, *et al.* in 1979 and is now a routine technique for protein analysis. The specificity of the antibody-antigen interaction enables a single protein to be identified in the midst of a complex protein mixture. Western blotting is commonly used to positively identify a specific protein in a complex mixture and to obtain qualitative and semiquantitative data about that protein.

The first step in a Western blotting procedure is to separate the macromolecules using gel electrophoresis. Following electrophoresis, the separated molecules are transferred or blotted onto a second matrix, generally a nitrocellulose or polyvinylidene fluoride (PVDF) membrane. Next, the membrane is blocked to prevent any nonspecific binding of antibodies to the surface of the membrane. The transferred protein is complexed with an enzyme-labeled antibody as a probe. An appropriate substrate is then added to the enzyme and together they produce a detectable product such as a chromogenic or fluorogenic precipitate on the membrane for colorimetric or fluorometric detection, respectively. The most sensitive detection methods use a chemiluminescent substrate that, when combined with the enzyme, produces light as a byproduct. The light output can be captured using film, a CCD camera or a phosphorimager that is designed for chemiluminescent detection. Whatever substrate is used, the intensity of the signal should correlate with the abundance of the antigen on the blotting membrane.

Detailed procedures for detection of a Western blot vary widely. One common variation involves direct vs. indirect detection as shown in Figure 1. With the direct detection method, the primary antibody that is used to detect an antigen on the blot is also labeled with an enzyme or fluorescent dye. This detection method is not widely used as most researchers prefer the indirect detection method for a variety of reasons.

In the indirect detection method, a primary antibody is added first to bind to the antigen. This is followed by a labeled secondary antibody that is directed against the primary antibody. Labels include biotin, fluorescent probes such as fluorescein or rhodamine, and enzyme conjugates such as horseradish peroxidase or alkaline phosphatase. The indirect method offers many advantages over the direct method.



1A. Direct Detection **1B. Indirect Detection**

Figure 1A. In the direct detection method, **labeled primary antibody** binds to antigen on the membrane and reacts with substrate, creating a detectable signal. **1B.** In the indirect detection method, **unlabeled primary antibody** binds to the antigen. Then, a **labeled secondary antibody** binds to the primary antibody and reacts with the substrate.

Advantages of Direct Detection (Figure 1A)

- It is a quick methodology because only one antibody is used
- Cross-reactivity of secondary antibody is eliminated
- Double staining is easily achieved using different labels on primary antibodies from the same host

Disadvantages of Direct Detection (Figure 1A)

- Immunoreactivity of the primary antibody may be reduced as a result of labeling
- Labeling of every primary antibody is time-consuming and expensive
- There is no flexibility in choice of primary antibody label from one experiment to another
- Little signal amplification

Advantages of Indirect Detection (Figure 1B)

- Sensitivity is increased because each primary antibody contains several epitopes that can be bound by the labeled secondary antibody, allowing for signal amplification
- A wide variety of labeled secondary antibodies are available commercially
- Since many primary antibodies can be made in one species and the same labeled secondary antibody can be used for detection, it is versatile
- Immunoreactivity of the primary antibody is not affected by labeling
- Different visualization markers can be used with the same primary antibody

Disadvantages of Indirect Detection (Figure 1B)

- Cross-reactivity may occur with the secondary antibody, resulting in nonspecific staining
- An extra incubation step is required in the procedure

Protein Detection Made Easy*

For detection of proteins that cannot be efficiently transferred to a membrane, Pierce has developed UnBlot® Technology that allows positive identification of proteins directly in a gel. (Product #s 33500, 33505, 33510 and 33515)

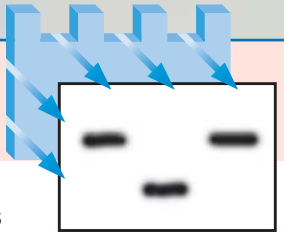
STEP 1 SDS-PAGE

Separate protein sample by electrophoresis.

- PAGEprep® Protein Clean-up and Enrichment Kit (Product # 26800)
- Precise™ Protein Gels (many available, see *www.piercenet.com*)
- Tris-HEPES-SDS Running Buffer (Product # 28398)
- ImmunoPure® Lane Marker Reducing Sample Buffer (5X) (Product # 39000)
- ImmunoPure® Lane Marker Non-Reducing Sample Buffer (5X) (Product # 39001)
- BlueRanger® Prestained Protein Molecular Weight Marker Mix (Product #s 26681 and 26685)
- Chemiluminescent BlueRanger® Prestained Peroxidase-labeled Protein Molecular Weight Marker Mix (Product # 26651)
- TriChromRanger™ Prestained Protein Molecular Weight Marker Mix (Product # 26691)
- ColorMeRanger™ Unstained Protein Molecular Weight Marker Mix (Product # 26671)

STEP 2 Electro-Transfer

Transfer proteins to membrane.

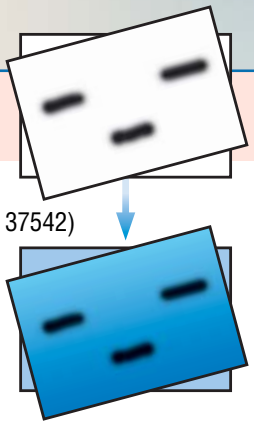


- MemCode™ Reversible Protein Stain Kit for Nitrocellulose Membranes (Product # 24580) and for PVDF Membranes (Product # 24585)
- Tris-Glycine Transfer Buffer (Product # 28380)
- Qentix™ Western Blot Signal Enhancer (Product # 21050)
- Nitrocellulose Membrane, 0.2 µm (Product #s 77012, 88013 and 88024)
- Nitrocellulose Membrane, 0.45 µm (Product #s 77010, 77011, 88014 and 88025)
- Capture™ PVDF Membrane, 0.45 µm (Product #s 88585 and 88518)

*For a detailed Western blotting protocol, see pages 50-51.

STEP 3 Blocking

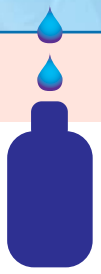
Block nonspecific sites.



- StartingBlock™ Blocking Buffer in PBS (Product # 37538) and in TBS (Product # 37542)
- StartingBlock™ T20 Blocking Buffer (Contains 0.05% Tween®-20) in PBS (Product # 37539) or TBS (Product # 37543)
- SuperBlock® Buffer in PBS (Product # 37515) and in TBS (Product # 37535)
- SuperBlock® T20 Blocking Buffer (Contains 0.05% Tween®-20) in PBS (Product # 37516) or TBS (Product # 37536)
- SuperBlock® Blocking Buffer – Blotting in PBS (Product # 37517) and in TBS (Product # 37537)
- Casein in PBS (Product # 37528) and in TBS (Product # 37532)
- BSA in PBS (Product # 37525) and in TBS (Product # 37520)
- SEA BLOCK Buffer (Product # 37527)
- BLOTTO in TBS (Product # 37530)

STEP 4A Formulate Wash Buffers

Choose a buffer.



- Phosphate Buffered Saline (PBS, Product # 28372)
- Tris Buffered Saline (TBS, Product #s 28376 and 28379)
- Modified Dulbecco's PBS (Product # 28374)
- Carbonate-Bicarbonate Buffer Packs (Product # 28382)
- MES Buffered Saline (Product # 28390)
- BupH™ Borate Buffer Packs (Product # 28384)
- BupH™ Citrate-Carbonate Buffer Pack (Product # 28388)

Western Blotting the Pierce Way

STEP 4B
Formulate Wash Buffers

Add detergent to blocking/wash buffers to reduce nonspecific binding.

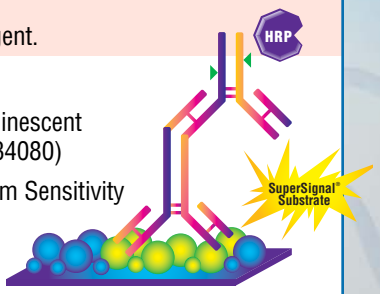
[Skip this step if you use StartingBlock™ T20 Blocking Buffer in PBS (Product # 37539) or TBS (Product # 37543) or SuperBlock® T20 Blocking Buffer in PBS (Product # 37516) or TBS (Product # 37536). These buffers already contain Tween®-20 Detergent at optimized concentrations.]

Surfact-Amps® Brand Detergents containing:

- Tween®-20 (Product # 28320) and Tween®-80 (Product # 28328)
- Triton® X-100 (Product # 28314) and Triton® X-114 (Product # 28332)
- Nonidet P-40 (Product # 28324)
- Brij®-35 (Product # 28316) and Brij®-58 (Product # 28336)

STEP 6
Enzyme Substrates

Add the detection reagent.



Chemiluminescent Substrates:

- SuperSignal® West Pico Chemiluminescent Substrate (Product #s 34077 and 34080)
- SuperSignal® West Femto Maximum Sensitivity Substrate (Product #s 34096 and 34095)
- SuperSignal® West Dura Extended Duration Substrate (Product #s 34076 and 34075)
- Lumi-Phos™ WB Substrate (Product # 34150)

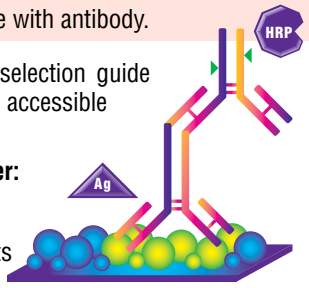
Colorimetric Substrates:

- 1-Step™ Chloronaphthol (Product # 34012)
- TMB-Blotting (Product # 34018)
- NBT/BCIP (Product # 34042)
- Metal Enhanced DAB (Product # 34065)

For convenience and economy, Pierce also offers complete Western blotting Kits that include chemiluminescent substrates, enzyme-conjugated antibodies, blocking buffers and standard buffers.

STEP 5
Primary and Secondary Detection Reagents

Incubate the membrane with antibody.



For a complete list, visit the antibody selection guide on our web site (www.piercenet.com) accessible under the Products tab.

For direct detection methods we offer:

- Monoclonal Antibodies
- Fluorescent Probes and Labeling Kits
- Enzyme Labeling Kits

For indirect detection methods we offer:

- Biotinylation Kits
- Protein A, Protein G and Protein L labeled with fluorescein, rhodamine, HRP, AP or biotin
- Avidin, Streptavidin and NeutrAvidin™ Biotin-Binding Protein labeled with fluorescein, rhodamine, HRP or AP
- Secondary antibodies labeled with fluorescein, rhodamine, HRP, AP or biotin

STEP 7
Film

Expose the membrane to X-ray film.

- CL-XPosure™ Film 5" x 7" sheets, (Product #s 34090 and 34092); 8" x 10" sheets, (Product #s 34091 and 34093)
- Erase-It® Background Eliminator Kit (Product # 21065)

STEP 8
Stripping Buffer

Reprobe the blot if necessary.

- Restore™ Western Blot Stripping Buffer (Product # 21059)
- IgG Elution Buffer (Product #s 21004 and 21009)

Transfer Protein to a Membrane

Following electrophoresis, the protein must be transferred from the electrophoresis gel to a membrane. There are a variety of methods that have been used for this process including diffusion transfer, capillary transfer, heat-accelerated convectional transfer, vacuum blotting transfer and electroelution. The transfer method that is used most commonly for proteins is electroelution or electrophoretic transfer because of its speed and transfer efficiency. This method uses the electrophoretic mobility of proteins to transfer them from the gel to the matrix. Electrophoretic transfer of proteins involves placing a protein-containing polyacrylamide gel in direct contact with a piece of nitrocellulose or other suitable protein-binding support and "sandwiching" this between two electrodes submerged in a conducting solution (Figure 2). When an electric field is applied, the proteins move out of the polyacrylamide gel and onto the surface of the membrane where the proteins become tightly attached. The resulting membrane is a copy of the protein pattern that was found in the polyacrylamide gel.

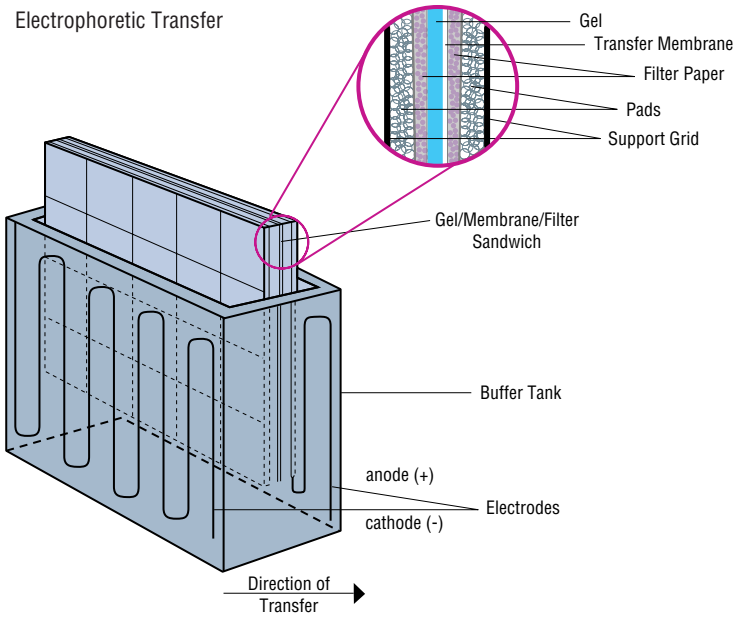


Figure 2. Electrophoretic transfer.

Transfer efficiency can vary dramatically among proteins, based upon the ability of a protein to migrate out of the gel and its propensity to bind to the membrane under a particular set of conditions. The efficiency of transfer depends on factors such as the composition of the gel, whether there is complete contact of the gel with the membrane, the position of the electrodes, the transfer time, size and composition of proteins, field strength, and the presence of detergents. Optimal transfer of proteins is generally obtained in low-ionic strength buffers and with low electrical current.

Pierce offers a wide selection of the most commonly used membranes for Western blotting including nitrocellulose and polyvinylidene difluoride (PVDF). Please refer to page 6 for a complete offering of transfer membranes.

At this stage, before proceeding with the Western blot, it is often desirable to stain all proteins on the membrane with a reversible stain to check the transfer efficiency. Although the gel may be stained to determine that protein left the gel, this does not ensure efficient binding of protein on the membrane. Ponceau S stain is the most widely used reagent for staining proteins on a membrane. However, it has limited sensitivity, does not photograph well and fades with time. Pierce MemCode™ Reversible Stain is a superior alternative for staining protein on nitrocellulose (Product # 24580) or PVDF (Product # 24585) membranes. MemCode™ Stain detects low nanogram levels of protein, is easily photographed, does not fade with time and takes less than 30 minutes to stain, photograph and erase.

Featured Product

MemCode™ Reversible Protein Stain for Nitrocellulose and PVDF Membranes

A great NEW alternative to Ponceau S stain.

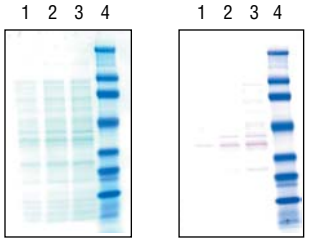
For years the red Ponceau S has been the best option for staining before Western blotting, despite its major shortcomings. MemCode™ Reversible Protein Stains decrease staining time, increase staining sensitivity and enhance the immunoreactivity of antigens in subsequent Western blotting. Try these new reversible protein stains for nitrocellulose and PVDF membranes and you will never use Ponceau S again.

Highlights:

- Sensitive, general protein stain that binds tightly to proteins
- Stain is protein-specific, avoiding interference from other biomolecules
- From stain to destain to band erasure in minutes
- Turquoise bands are easily photographed
- Stained bands do not fade with time
- Enhances Western blot detection
- All components are room temperature-stable

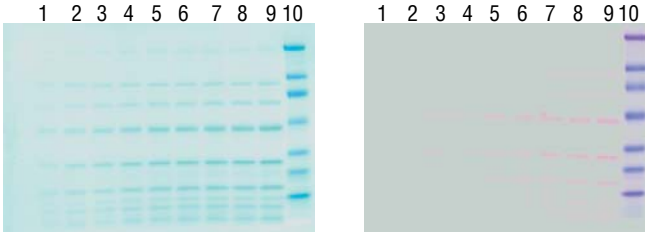
Table 1. Comparison of MemCode™ Reversible Protein Stain with Ponceau S

Ponceau S Reversible Stain	MemCode™ Reversible Protein Stain
• Weak-binding, low-sensitivity general protein stain	• Tight-binding, higher sensitivity general protein stain
• Detection limit: 250 ng	• Detection limit: 25-50 ng
• Red bands are difficult to photograph	• Turquoise blue bands are photographed easily
• Stained protein bands fade within hours	• Turquoise bands do not fade over time, but they can be reversed
• Typical staining time: 5 minutes	• Typical staining time: 60 seconds
	• Background eliminated quickly with low pH wash



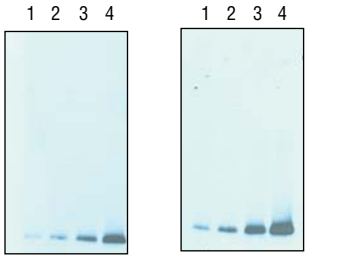
A. MemCode™ Stain B. Ponceau S Stain

Figure 3. MemCode™ Reversible Protein Stain and Ponceau S Stain: A comparison of GST lysate staining on nitrocellulose. Increasing amounts of GST Lysate protein were applied onto two 4-20% Tris-glycine SDS polyacrylamide gels. Both gels were electroblotted to nitrocellulose membrane. Blot **A** was treated with MemCode™ Stain for 30 seconds and destained according to the protocol. Blot **B** was stained with 0.1% Ponceau S stain for 5 minutes and destained. The blot stained with MemCode™ Stain demonstrates superior visual detection of bands. GST Lysate loading volumes (Lane 1-3). Lane 1: 5 µl, Lane 2: 10 µl, Lane 3: 15 µl, Lane 4: BlueRanger® Marker Mix (Product # 26681), 10 µl.



A. MemCode™ Stain B. Ponceau S Stain

Figure 4. Comparison of MemCode™ Reversible Protein Stain with Ponceau S stain on PVDF membrane. ColorMeRanger™ Unstained Protein M.W. Markers (Product # 26671) were serially diluted and applied to two 4-20% Tris-glycine-SDS polyacrylamide gels **Lanes 1-9**. Both gels were electroblotted to PVDF membrane. **Blot A** was stained with MemCode™ Stain for 1 minute and destained according to the protocol. **Blot B** was stained with 0.1% Ponceau S in 5% acetic acid for 5 minutes and destained according to the published protocol. **Lane 10.** BlueRanger® Prestained M.W. Marker Mix (Product # 26681).



A. Control B. MemCode™ Stain

Figure 5. Immunoblot analysis of GST by chemiluminescent detection after MemCode™ Staining, destaining and stain reversal. Different amounts of purified GST protein were applied to two 10% Tris-glycine SDS polyacrylamide gels. Both gels were electroblotted to nitrocellulose membranes. The control membrane (Panel **A**) was not treated with MemCode™ Reversible Protein Stain. Panel **B** was subjected to the staining, detaining and stain erasing protocol of the MemCode™ Kit. Both membranes were then probed with anti-GST incubated with goat anti-rabbit IgG-HRP conjugate and detected using Pierce SuperSignal® West Dura Substrate (Product # 34075). Lane 1: 125 pg, Lane 2: 250 pg, Lane 3: 500 pg and Lane 4: 1 ng.

MemCode™ Reversible Protein Stain Protocols

A. Nitrocellulose Membrane Staining Protocol

1. Wash membrane with ultrapure H_2O.
2. Add MemCode™ Stain. Shake 30 seconds. Protein bands appear turquoise in color.

B. Destaining Protocol

1. Rinse three times with MemCode™ Destain Solution.
2. Add MemCode™ Destain. Shake 5 minutes.
3. Rinse four times with ultrapure H_2O.
4. Wash on a shaker with ultrapure H_2O for 5 minutes.

C. Stain Erasing Protocol

1. Wash with MemCode™ Stain Eraser on a shaker for 2 minutes.
2. Rinse four times with ultrapure H_2O.
3. Wash with ultrapure H_2O on a shaker for 5 minutes.

A. PVDF Membrane Staining Protocol

1. Wash membrane with ultrapure H_2O.
2. Add MemCode™ Sensitizer. Shake for 2 minutes.
3. Add MemCode™ Stain. Shake for 1 minute. Protein bands appear turquoise in color.

B. Destaining Protocol

1. Rinse three times with MemCode™ Destain Solution.
2. Wash with Memcode™ Destain mixed 1:1 with MeOH on a shaker for 5 minutes.
3. Rinse five times with ultrapure H_2O.

C. Stain Erasing Protocol

1. Wash with MemCode™ Stain Eraser mixed 1:1 with MeOH on a shaker for 10-20 minutes.
2. Rinse five times with ultrapure H_2O.

PRODUCT #	DESCRIPTION	PKG. SIZE
24580	**MemCode™ Reversible Protein Stain Kit for Nitrocellulose Membranes** *Sufficient material to stain protein and reverse the stain from 10 (8 cm x 8 cm) nitrocellulose membranes.* Includes: MemCode™ Reversible Stain *A broad-spectrum stain for proteins transferred to nitrocellulose membranes.* MemCode™ Destain* *Enhances protein band detection by eliminating background stain.* MemCode™ Stain Eraser* *Reverses protein band staining on demand.*	Kit 250 ml 1,000 ml 500 ml
24585	**MemCode™ Reversible Protein Stain Kit for Polyvinylidene Difluoride Membrane** *Sufficient material to stain protein and reverse the stain from 10 (8 cm x 8 cm) PVDF membranes.* Includes: MemCode™ Sensitizer *PVDF membrane pre-treatment agent.* MemCode™ Reversible Stain *A broad-spectrum stain for proteins transferred to PVDF membrane.* MemCode™ Destain* *Enhances protein band detection by eliminating background stain.* MemCode™ Stain Eraser* *Reverses protein band staining on demand.*	Kit 250 ml 250 ml 1,000 ml 500 ml

**Reagent-grade methanol (required, but not supplied) supplements the Destain and Stain Eraser formulations.*

Blocking Nonspecific Binding Sites

In a Western blot, it is important to block the unreacted sites on the membrane to reduce the amount of nonspecific binding of proteins during subsequent steps in the assay. A variety of blocking buffers ranging from milk or normal serum to highly purified proteins have been used to block unreacted sites on a membrane. The blocking buffer should improve the sensitivity of the assay by reducing background interference. Individual blocking buffers are not compatible with every system. For this reason, a variety of blockers in both Tris buffered saline (TBS) and phosphate buffered saline (PBS) are available. The proper choice of blocker for a given blot depends on the antigen itself and on the type of enzyme conjugate to be used. For example, with applications using an alkaline phosphatase conjugate, a blocking buffer in TBS should be selected because PBS interferes with alkaline phosphatase. The ideal blocking buffer will bind to all potential sites of nonspecific interaction, eliminating background altogether without altering or obscuring the epitope for antibody binding.

For true optimization of the blocking step for a particular immunoassay, empirical testing is essential. Many factors can influence nonspecific binding, including various protein:protein interactions unique to a given set of immunoassay reagents. The most important parameter when selecting a blocker is the signal-to-noise ratio, which is measured as the signal obtained with a sample containing the target analyte as compared to that obtained with a sample without the target analyte. Using inadequate amounts of blocker will result in excessive background staining and a reduced signal-to-noise ratio. Using excessive concentrations of blocker may mask antibody:antigen interactions or inhibit the marker enzyme, again causing a reduction of the signal-to-noise ratio. When developing any new immunoassay, it is important to test several different blockers for the highest signal-to-noise ratio in the assay. No single blocking agent is ideal for every occasion because each antibody-antigen pair has unique characteristics. If a blocking buffer that does not cross-react with your system cannot be found, UnBlot® In-Gel Protein Detection is an alternative choice. The UnBlot® System specifically detects proteins within the gel and requires no blocking (see page 39 for more information).

Pierce offers a complete line of blocking buffers for Western blotting including BLOTTO, Casein, BSA, SEA BLOCK and the exclusive SuperBlock® and Starting Block™ Blocking Buffers.

Featured Products

Transfer Buffers

BupH™ Tris-Glycine and Tris Buffered Saline

Great for Western blots!

BupH™ Tris-Glycine Buffer Packs

Each pack yields 500 ml of 25 mM Tris and 192 mM glycine at a pH of approximately 8 when dissolved in 400 ml deionized water and 100 ml of methanol (20 liters total)

BupH™ Tris Buffered Saline Packs

Each pack yields 500 ml of 25 mM Tris, 0.15 M NaCl, pH 7.2 when dissolved in 500 ml deionized water (10 pack makes 5 liters total; 40 pack makes 20 liters total).

PRODUCT #	DESCRIPTION	PKG. SIZE
28380	BupH™ Tris-Glycine Buffer Packs	40 pack
28376	BupH™ Tris Buffered Saline Packs	40 pack
28379	BupH™ Tris Buffered Saline Packs	10 pack

Transfer Membranes

Nitrocellulose Membranes

PRODUCT #	DESCRIPTION	PKG. SIZE
88013	Nitrocellulose Membrane, 0.2 µm 7.9 cm x 10.5 cm	15/pkg.
88018	Nitrocellulose Membrane, 0.45 µm 33 cm x 3 m	1 roll
88014	Nitrocellulose Membrane, 0.45 µm 7.9 cm x 10.5 cm *Minimum 87 sheets when cut to 7.9 cm x 10.5 cm; minimum 52 sheets when cut to 11.5 cm x 12.5 cm.*	15/pkg.
88024	Nitrocellulose Membrane, 0.2 µm 8 cm x 8 cm	15/pkg.
77012	Nitrocellulose Membrane, 0.2 µm 8 cm x 12 cm	25/pkg.
88025	Nitrocellulose Membrane, 0.45 µm 8 cm x 8 cm	15/pkg.
77011	Nitrocellulose Membrane, 0.45 µm 8 cm x 12 cm	10/pkg.
77010	Nitrocellulose Membrane, 0.45 µm 8 cm x 12 cm	25/pkg.

Polyvinylidene Difluoride (PVDF) Membranes

PRODUCT #	DESCRIPTION	PKG. SIZE
88585	Capture™ PVDF Transfer Membrane, 0.45 µm 10 cm x 10 cm	10 sheets
88518	Capture™ PVDF Transfer Membrane, 0.45 µm 26.5 cm x 3.75 m	1 roll

Blocking Buffer Optimization

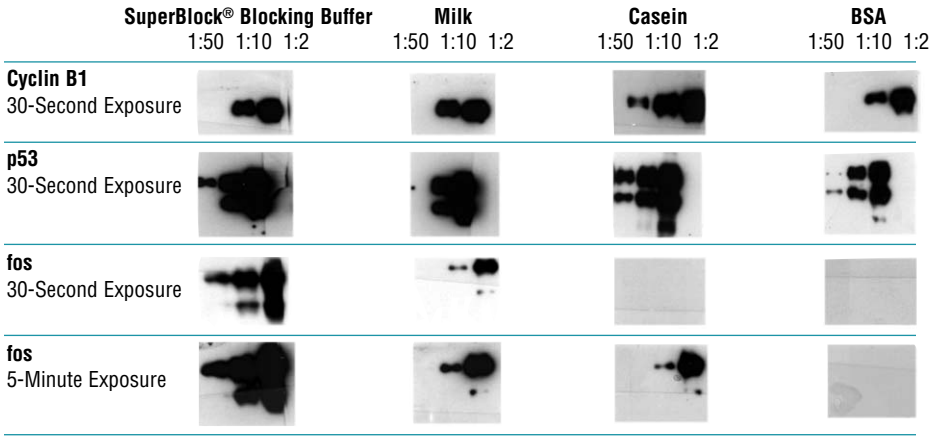


Figure 6: Blocking buffer optimization.

Blocking Buffers Application Chart

Product #	Blocking Buffer	ELISA	Western blot	Dot Blot	Immunohisto-chemistry	DNA/RNA Hybridizations
37538	StartingBlock™ (PBS) Blocking Buffer	✔	✔	✔	✔	
37542	StartingBlock™ (TBS) Blocking Buffer	✔	✔	✔	✔	
37539	StartingBlock™ T20 (PBS) Blocking Buffer	✔	✔	✔	✔	
37543	StartingBlock™ T20 (TBS) Blocking Buffer	✔	✔	✔	✔	
37515	SuperBlock® Blocking Buffer in PBS*	✔	✔	✔	✔	✔
37535	SuperBlock® Blocking Buffer in TBS*	✔	✔	✔	✔	✔
37517	SuperBlock® Blocking Buffer – Blotting in PBS		✔	✔	✔	
37537	SuperBlock® Blocking Buffer – Blotting in TBS		✔	✔	✔	
37516	SuperBlock® T-20 PBS Blocking Buffer	✔	✔	✔	✔	✔
37536	SuperBlock® T-20 TBS Blocking Buffer	✔	✔	✔	✔	✔
37527	SEA BLOCK Blocking Buffer	✔	✔	✔		
37520	Blocker™ BSA in TBS	✔	✔	✔	✔	✔
37525	Blocker™ BSA in PBS	✔	✔	✔	✔	✔
37532	Blocker™ Casein in TBS	✔	✔	✔	✔	✔
37528	Blocker™ Casein in PBS	✔	✔	✔	✔	✔
37530	Blocker™ BLOTTO in TBS	✔	✔	✔	✔	✔

*These blocking buffers are recommended for use when performing Western blots with SuperSignal® Chemiluminescent Substrates.

Blocking Buffer Optimization

The most appropriate blocking buffer for Western blotting use is often system-dependent. Determining the proper blocking buffer can help to increase the system's signal-to-noise ratio. Occasionally, when switching from one substrate to another, the blocking buffer that you are using will lead to diminished signal or increased background. Empirically testing various blocking buffers with your system can help achieve the best possible results. Avoid using milk as a blocking reagent for blots that rely on the avidin/biotin system because milk contains variable amounts of biotin. Although SuperBlock® Blocking Buffer (Product # 37515) often gives excellent results, we recommend testing several blocking reagents for their suitability in a particular system. There is no blocking reagent that will be the optimal reagent for all systems.

As shown in Figure 6, various proteins were analyzed by Western blotting to determine the optimal blocking condition for nonspecific sites. Recombinant Human Cyclin B1, Wild-Type p53 and Mouse fos Baculovirus lysates were diluted in Lane Marker Reducing Sample Buffer (1:50, 1:10 or 1:2) and separated electrophoretically on a 12% SDS-polyacrylamide gel. The proteins were transferred to nitrocellulose membrane and cut into strips. The membrane strips were blocked for 1 hour at RT with shaking in Blocker™ Casein in TBS, 1% BSA in TBS, SuperBlock® Blocking Buffer in TBS or 5% nonfat milk in TBS. Tween®-20 (0.05%) was added to all blocking buffers. The membranes were then incubated with the appropriate primary antibody at 0.5 µg/ml prepared in the different blocking solutions for one hour at RT with shaking. Each membrane strip was washed with TBS followed by a one-hour incubation in HRP-conjugated Goat anti-Mouse antibody prepared in the different blocking buffers at a 25 ng/ml concentration. The membranes were washed with TBS. A working solution of SuperSignal® West Pico Chemiluminescent Substrate was prepared and added to each membrane for 5 minutes. The membranes were removed and placed in sheet protectors prior to exposure to film for 30 seconds and 5 minutes as indicated. The film was developed per the manufacturer's instructions. The resulting blots were analyzed for signal:noise and compared. The results indicate that there is no blocking reagent that is optimal for all systems.

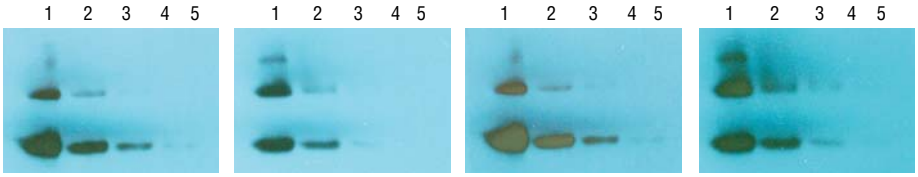
StartingBlock™ Blocking Buffer

Confused about all the blocking options available for Western blot and ELISA applications? StartingBlock™ Blocking Buffer simplifies the selection of a blocker.

Although no blocking buffer is ideal for every system, you can improve the odds dramatically with StartingBlock™ Blocking Buffer because it is compatible with the widest variety of antibodies.

For example: StartingBlock™ Blocking Buffers are compatible with biotin-containing systems, while milk-based protein blockers interfere. StartingBlock™ Buffers do not cross-react with rabbit antibodies, while many other blockers do. StartingBlock™ Blocking Buffers are also free of potentially interfering serum proteins.

StartingBlock™ Blocking Buffers offer a high level of performance – regardless of the system you choose for your Western blotting or ELISA application. In fact, they may be the only blockers you ever use.



7A 7B 7C 7D

Figure	7A	7B	7C	7D
Membrane Type	Nitrocellulose	PVDF	Nitrocellulose	PVDF
Film Exposure Time	30 minutes	30 minutes	24 hours*	24 hours*

**Full duration of SuperSignal® West Dura Chemiluminescent Substrate light emission.*

Figure 7A-D. Comparison of StartingBlock™ Blocking Buffer Performance after stripping and reprobing. Nitrocellulose vs. PVDF when probed for the transferrin receptor (CD71).

Highlights:

Compatible with a wide range of detection systems

- Works in both Western and ELISA applications
- Does not cross-react with rabbit antibodies
- Serum protein-free
- Biotin-free

Shorter blocking times

- Western blotting – 1-15 minutes
- ELISA – "no-wait" blocking capability

Strip and reprobe – no reblocking necessary

- Blots stay blocked with StartingBlock™ Blocker when our Restore™ Stripping Buffer (Product # 21059) is used, allowing reprobing of the same blot without re-blocking

Superior signal-to-noise ratios in ELISA applications

- Signal:noise ratios in the range of 10:1-20:1 have been realized with StartingBlock™ Blocking Buffer

PRODUCT #	DESCRIPTION	PKG. SIZE
37538	**StartingBlock™ (PBS) Blocking Buffer** *A protein-based blocker formulation in phosphate buffered saline (pH 7.5) for use in Western blotting and ELISA applications.*	1 liter
37542	**StartingBlock™ (TBS) Blocking Buffer** *A protein-based blocker formulation in Tris buffered saline (pH 7.5) for use in Western blotting and ELISA applications.*	1 liter

Starting Block™ Blocking Buffers are also available with an optimized amount of Tween®-20 to provide the lowest background.

PRODUCT #	DESCRIPTION	PKG. SIZE
37539	**StartingBlock™ T20 (PBS) Blocking Buffer** *A protein-based blocker formulation in phosphate buffered saline at pH 7.5 with 0.05% Tween®-20 and Kathon® Antimicrobial Agent.*	1 liter
37543	**StartingBlock™ T20 (TBS) Blocking Buffer** *A protein-based blocker formulation in Tris buffered saline at pH 7.5 with 0.05% Tween®-20 and Kathon® Antimicrobial Agent.*	1 liter

SuperBlock® Blocking Buffers

Guaranteed to be biotin-free.

Our most popular blocking buffer, SuperBlock® Blocking Buffer, now comes in both dry and liquid formats! Many researchers have discovered that SuperBlock® Blocking Buffer is the only blocking buffer needed for all of their applications.

Highlights:

- Fast blocking – blocks ELISA plates in two minutes or membranes in five to 10 minutes
- **Non-serum protein** solution yields a very high signal-to-noise ratio
- Plates blocked with SuperBlock® Blocking Buffer can be stored dry for up to 12 months
- Liquid formulations available in PBS or TBS

SuperBlock® Dry Blend (TBS) Blocking Buffer

Delivers the ultimate in space-saving convenience.

Highlights:

- Delivers even more economy and stability
- Each pouch reconstitutes to form 200 ml of SuperBlock® Blocking Buffer in TBS
- Room-temperature storage; small packaging takes up minimal shelf space

References

Ikeda, K., *et al.* (2003) *J. Biol. Chem.* **278**, 7725-7734.

Leclerc, G.J. and Barredo, J.C. (2001) *Clin. Cancer Res.* **7**, 942-951.

Subbarayan, V., *et al.* (2001) *Cancer Res.* **61**, 2720-276.

Walters, R.W., *et al.* (2002) *Cell* **100**, 789-799.

PRODUCT #	DESCRIPTION	PKG. SIZE
37515	SuperBlock® (PBS) Blocking Buffer	1 liter
37516	SuperBlock® T20 (PBS) Blocking Buffer (Contains 0.05% Tween®-20)	1 liter
37535	SuperBlock® (TBS) Blocking Buffer	1 liter
37536	SuperBlock® T20 (TBS) Blocking Buffer (Contains 0.05% Tween®-20)	1 liter
37517	SuperBlock® (PBS) Blocking Buffer – Blotting*	1 liter
37537	SuperBlock® (TBS) Blocking Buffer – Blotting*	1 liter
37545	SuperBlock® (TBS) Blocking Buffer Dry Blend Blocking Buffer Each pouch yields 200 ml when reconstituted.	5 pouches

** Formulated for precipitating enzyme substrates. Added ingredient to keep precipitate from flaking. Not recommended for chemiluminescent substrates.*

SEA BLOCK Blocking Buffer

No mammalian proteins, reducing the risk of nonspecific interaction.

Highlights:

- Made from steelhead salmon serum
- Functions as a universal blocker
- Offers reduced background
- Can be diluted up to 1:10 with buffer

References

Hypolite, J.A., *et al.* (2001). *Am. J. Physiol. Cell Physiol.* **280**, C254-264.

Wang, L., *et al.* (2002*). J. Clin. Invest.* **110**, 1175-1184.

PRODUCT #	DESCRIPTION	PKG. SIZE
37527	SEA BLOCK Blocking Buffer	500 ml

Blocker™ Casein

Ready-to-use solution (1% w/v) of Hammersten Grade casein for blocking nonspecific sites.

Highlights:

- Preformulated for ease of use
- Use when skim milk demonstrates high background problems
- Thimerosal-free formulation

PRODUCT #	DESCRIPTION	PKG. SIZE
37532	Blocker™ Casein in TBS 1% (w/v) Casein Hammersten Grade in TBS, Contains Kathon® Antimicrobial Reagent as preservative, pH 7.4.	1 liter
37528	Blocker™ Casein in PBS 1% (w/v) Casein Hammersten Grade in PBS, Contains Kathon® Antimicrobial Reagent as preservative, pH 7.4.	1 liter

Blocker™ BLOTTO

Ready-to-use blocking buffers made of nonfat dry milk.

Highlights:

- Preformulated for ease of use
- Anti-foaming agent added
- Available in TBS Buffer
- Merthiolate-free formulation

PRODUCT #	DESCRIPTION	PKG. SIZE
37530	Blocker™ BLOTTO in TBS 5% (w/v) nonfat powdered milk in TBS, 0.01% Anti-foam A, contains Kathon® Antimicrobial Reagent as preservative, pH 7.4.	1 liter

Blocker™ BSA

For all blocking applications.

Highlights:

- 10% solutions of high-quality Bovine Serum Albumin
- Concentrated formulation saves storage space
- No waiting for powder to dissolve with this ready-to-dilute liquid concentrate

PRODUCT #	DESCRIPTION	PKG. SIZE
37525	Blocker™ BSA in PBS (10X)	200 ml
37520	Blocker™ BSA in TBS (10X)	125 ml

Surfact-Amps® 20 Purified Detergent Solution

Specially purified form of Tween®-20.

Highlights:

- Guaranteed <1 milliequivalent of peroxides and carbonyl in a 10% solution
- Enhances signal-to-background ratio

PRODUCT #	DESCRIPTION	PKG. SIZE
28320	Surfact-Amps® 20 Purified Detergent Solution	6 x 10 ml

Washing the Membrane

Like other immunoassay procedures, Western blotting consists of a series of incubations with different immunochemical reagents separated by wash steps. Washing steps are necessary to remove unbound reagents and reduce background, thereby increasing the signal-to-noise ratio. Insufficient washing will allow high background, while excessive washing may result in decreased sensitivity caused by elution of the antibody and/or antigen from the blot. As with other steps in performing a Western blot, a variety of buffers may be used. Occasionally, washing is performed in a physiologic buffer such as Tris buffered saline (TBS) or phosphate buffered saline (PBS) without any additives. More commonly, a detergent such as 0.05% Tween®-20 (Product # 28320) is added to the buffer to help remove nonspecifically bound material. Another common technique is to use a dilute solution of the blocking buffer along with some added detergent. Including the blocking agent and adding a detergent in wash buffers helps to minimize background in the assay. For best results, use high-purity detergents, such as Surfact-Amps® Detergents for Western blotting.

BupH™ Dry Buffers

The most advanced, versatile, time-saving buffer product line available.

The ultimate in convenience …

1. Reach for the sealed foil pack sitting conveniently on your bench top.
2. Open, pour into beaker and add water.
3. The fresh buffer is ready to use in practical aliquots so there's no waste.

The ultimate in versatility …

1. Routine buffers are designed for use in Western blotting, dialysis, cross-linking, ELISAs, immunohistochemistry, protein plate-coating, biotinylation and other applications.
2. Using one buffer source maintains consistency and eliminates variables within the lab.

The ultimate in integrity …

1. BupH™ Buffers are protected from contamination and are fresh every time.
2. Carry out applications with confidence in buffer quality.
3. "Test-assured" with the Pierce commitment to quality management standards.

The ultimate in time savings …

1. Making routine buffers is no longer time-consuming.
2. No component measurement, pH adjustment, quality validation, preparation tracking or refrigeration hassles.
3. Move forward with your work by eliminating re-tests due to buffer problems.

BupH™ Phosphate Buffered Saline Packs

Great wash buffer for Western blots!

Each pack yields 500 ml of 0.1 M phosphate, 0.15 M NaCl, pH 7.0 when dissolved in 500 ml deionized water (20 liters total).

PRODUCT #	DESCRIPTION	PKG. SIZE
28372	BupH™ Phosphate Buffered Saline Packs	40 pack

BupH™ Tris Buffered Saline

Great wash buffer for Western blots!

Each pack yields 500 ml of 25 mM Tris, 0.15 M NaCl, pH 7.2 when dissolved in 500 ml deionized water (10 pack makes 5 liters total; 40 pack makes 20 liters total).

PRODUCT #	DESCRIPTION	PKG. SIZE
28380	BupH™ Tris-Glycine Buffer Packs	40 pack
28376	BupH™ Tris Buffered Saline Packs	40 pack
28379	BupH™ Tris Buffered Saline Packs	10 pack

Surfact-Amps® 20 Purified Detergent Solution

Specially purified form of Tween®-20.

Highlights:

- Can be added to PBS or TBS wash buffers to improve performance
- Guaranteed < 1 milliequivalent of peroxides and carbonyl in a 10% solution
- Enhances signal-to-background ratio

PRODUCT #	DESCRIPTION	PKG. SIZE
28320	Surfact-Amps® 20	6 x 10 ml

Primary and Secondary Antibodies

The choice of a primary antibody for a Western blot will depend on the antigen to be detected and what antibodies are available to that antigen. A huge number of primary antibodies are available commercially and can be identified quickly by searching sites such as *www.antibodyresource.com* or *www.sciquest.com* on the Internet. Alternatively, a primary antibody may be made to recognize the antigen of interest. For more information on producing a custom antibody, see the Antibody Production and Purification technical section of the Pierce Technical Handbook and Catalog. Both polyclonal and monoclonal antibodies work well for Western blotting. Polyclonal antibodies are less expensive and less time-consuming to produce and they often have a high affinity for the antigen. Monoclonal antibodies are valued for their specificity, purity and consistency that result in lower background. Crude antibody preparations such as serum or ascites fluid are sometimes used for Western blotting, but the impurities present may increase background. To obtain antibodies with the greatest specificity, they can be affinity-purified using the immobilized antigen. For more information on affinity purification, order your **FREE** Affinity Purification Handbook from a Pierce Customer Service representative at 800-874-3723 or 815-968-0747, or from your local Perbio Science Branch office or distributor.

A wide variety of labeled, secondary antibodies can be used for Western blot detection. The choice of secondary antibody depends upon the species of animal in which the primary antibody was raised (the host species). For example, if the primary antibody is a mouse monoclonal antibody, the secondary antibody must be an anti-mouse antibody obtained from a host other than the mouse. The host species of the secondary antibody often will not affect the experiment. However, secondary antibodies are available from several different host species and, if a secondary antibody causes high background in a particular assay, another host species may be chosen. Another option to reduce background is to use a secondary antibody that has been pre-adsorbed to serum proteins from other species. This pre-adsorption process removes antibodies that have the potential to cross-react with serum proteins, including antibodies, from those species. To expedite the process of choosing the appropriate secondary antibody, visit the Secondary Antibody Selection Guide on the Pierce web site, located under the Products tab, or the home page.

Antibodies for Western blotting are typically used as dilute solutions, ranging from 1/100-1/500,000 dilutions beginning from a 1 mg/ml stock solution. The optimal dilution of a given antibody with a particular detection system must be determined experimentally. More sensitive detection systems require that less antibody be used, which can result in substantial savings on antibody costs and allow a limited supply of antibody to be stretched out over more experiments. It also produces a side benefit of reduced background because the limited amount of antibody shows increased specificity for the target with the highest affinity. Antibody dilutions are typically made in the wash buffer containing a blocking agent. The presence of a small amount of blocking agent and detergent in the antibody diluent often helps to minimize background.

Pierce offers a wide variety of ImmunoPure® Labeled Secondary Antibodies for use in Western blotting. The labels include biotin, fluorescein, rhodamine, horseradish peroxidase and alkaline phosphatase. For the complete list of labeled secondary antibodies please refer to pages 13-15.

Antibody Labels

The choice of secondary antibody also depends upon the type of label that is desired. Many different labels can be conjugated to antibodies. Radioisotopes were used extensively in the past, but they are expensive, have a short shelf-life, offer no improvement in signal-to-noise ratio and require special handling. Alternative labels are biotin, fluorophores and enzymes. The use of fluorophores requires fewer steps; however, special equipment is needed to view the fluorescence. Also, a photograph must be taken if a permanent record of the results is desired. Enzymatic labels are used most commonly and, although they require extra steps, they can also be extremely sensitive.

Alkaline phosphatase (AP) and horseradish peroxidase (HRP) are the two enzymes that are used extensively as labels for protein detection. An array of chromogenic, fluorogenic and chemiluminescent substrates is available for use with either enzyme. For a detailed comparison of these two enzymes, see Table 2.

Alkaline phosphatase, a 140,000 dalton protein that is generally isolated from calf intestine, catalyzes the hydrolysis of phosphate groups from a substrate molecule, resulting in a colored or fluorescent product or the release of light as a byproduct. AP has optimal enzymatic activity at a basic pH (pH 8-10) and can be inhibited by cyanides, arsenate, inorganic phosphate and divalent cation chelators, such as EDTA. As a label for Western blotting, AP offers a distinct advantage over other enzymes. Because its reaction rate remains linear, detection sensitivity can be improved by simply allowing a reaction to proceed for a longer time period.

Horseradish peroxidase is a 40,000 dalton protein that catalyzes the oxidation of substrates by hydrogen peroxide, resulting in a colored or fluorescent product or the release of light as a byproduct. HRP functions optimally at a near-neutral pH and can be inhibited by cyanides, sulfides and azides. Antibody-HRP conjugates are superior to antibody-AP conjugates with respect to the specific activities of both the enzyme and antibody. In addition, its high turnover rate, good stability, low cost and wide availability of substrates make HRP the enzyme of choice for most applications.

Table 2. Comparison of horseradish peroxidase and alkaline phosphatase enzymes

	Horseradish Peroxidase	Alkaline Phosphatase
Size	40 kDa	140 kDa
Price	Relatively Inexpensive	Relatively Expensive
Stability (Storage)	Stable at < 0˚C	Unstable at < 0˚C
Number of Substrates	Many	Few
Kinetics	Rapid	Slower
pH optimum	5-7	8-10

Affinity-Purified Secondary Antibodies

ImmunoPure® Affinity-Purified Antibodies are available unconjugated or conjugated with biotin, alkaline phosphatase, horseradish peroxidase, fluorescein or rhodamine. $F(ab')_2$ fragments of antibodies to immunoglobulins are also available in unconjugated or conjugated forms. These $F(ab')_2$ fragments of antibodies are especially useful in assays in which binding between the Fc portions of antibodies and Fc receptor-bearing cells must be eliminated.

ImmunoPure® Polyclonal Antibodies are purified by immunoaffinity chromatography using antigens coupled to agarose gels. Affinity purification helps to eliminate nonspecific antibodies, resulting in an increase in sensitivity and specificity, with a decrease in background. The purification process involves an elution procedure, yielding antibodies with high avidity. These antibodies exhibit both maximal binding to antigens and minimal cross-reactivity to other molecules. Conjugated antibodies are affinity-purified prior to the conjugation process.

Selected ImmunoPure® Antibodies have been further purified by passing them through immobilized serum proteins from other species. This additional processing minimizes cross-reactivities to other species' serum proteins and is indicated by "min x Species Sr Prot." The key to abbreviations for the individual species is shown in Table 3.

Table 3: Key to abbreviations for individual species

Bv = Bovine	Gu = Guinea Pig	Hs = Horse	Rt = Rat
Ch = Chicken	Ha = Hamster	Ms = Mouse	Sh = Sheep
Gt = Goat	Hn = Human	Rb = Rabbit	Sw = Swine

ImmunoPure® Polyclonal Conjugated Antibodies contain bovine serum albumin as a stabilizer. Table 4 lists the typical working dilutions for the conjugated antibodies when doing ELISAs, immunoblotting or immunohistochemical techniques.

Table 4: Typical dilution ranges recommended for Pierce ImmunoPure® Polyclonal Conjugated Antibodies

Conjugate	ELISA	Immunoblotting	Immunohistochemistry
Alkaline Phosphatase	1:5,000-1:50,000	1:2,500-1:25,000	1:500-1:5,000
Peroxidase	1:5,000-1:200,000 (for SuperSignal® ELISA Products)	1:25,000-1:500,000 (for SuperSignal® West Products)	1:500-1:5,000
Fluorescein	—	—	1:50-1:200
Rhodamine	—	—	1:50-1:200

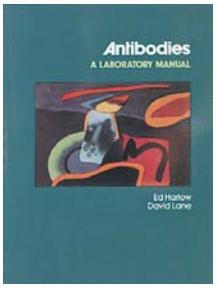
Storing Enzyme Conjugates

Pierce provides a variety of reagents to help preserve enzyme conjugate activity. Typically, conjugates are aliquoted in 50-100 µl increments using purified ethylene glycol (Product # 29810) as a preservative for -20°C storage. Conjugates can maintain activity for up to two years. An alternative to aliquoting is to use SuperFreeze™ Peroxidase Conjugate Stabilizer (Product # 31503), diluting the conjugate 1:1 in the stabilizer and storing it at -20°C for up to one year as a stock solution. Guardian™ Peroxidase Stabilizer/Diluents (Product #'s 37548 and 37552) allow peroxidase conjugates to be reconstituted and stored at 4°C as a 1:1,000 dilution or a 1:100,000 dilution stock solution.

Conjugate Stabilizers

PRODUCT #	DESCRIPTION	PKG. SIZE
37548	Guardian™ Peroxidase Conjugate Stabilizer/Diluent (SD)	200 ml
37552	Guardian™ Peroxidase Conjugate Stabilizer/Diluent (SD)	1 liter
31503	SuperFreeze™ Peroxidase Conjugate Stabilizer	25 ml
29810	Ethylene Glycol (50% aqueous solution)	200 ml

Using Antibodies: A Laboratory Manual



Few technical manuals have become standards in bioresearch like *Antibodies: A Laboratory Manual* by Ed Harlow and David Lane, which has enjoyed that status for more than a decade.

The authors, however, have raised the standard with the publication of their book *Using Antibodies: A Laboratory Manual*. Harlow and Lane have completely revised their guide for using antibody reagents in the laboratory. Chapters have been entirely rewritten, reorganized and updated to provide background, context and step-by-step instructions for techniques ranging from choosing the right antibody and handling it correctly, to the proper methods for characterizing antigens in cells and solutions. They've also added new chapters on tagging proteins and epitope mapping.

Rather than presenting an array of solutions for working with antibodies and antigens, Using Antibodies identifies the best approach to specific problems. These recommendations include more detail in the protocols, extensive advice on avoiding and solving problems, information regarding proper controls, and thorough illustration of theory, methods and results. The book also includes a bonus – a set of portable protocols that include step-by-step instructions for the most frequently used and essential techniques. The protocols are printed on durable cards, enabling them to be used easily at the bench.

This helpful guide, along with high-quality products from Pierce, will help you purify, immobilize, label and store antibodies and perform common procedures such as immunoprecipitation, Western blotting and ELISA.

PRODUCT #	DESCRIPTION	PKG. SIZE
15051	Using Antibodies: A Laboratory Manual Ed Harlow and David Lane Published by Cold Spring Harbor Laboratory Press, 1999. 495 pages; wire spiral-bound hardcover with nine separate portable protocols	1 book

** Sorry, books are nonreturnable.*

Affinity-Purified Secondary Antibodies

Specificity	Description	Host	Product # Pkg. Size Unconj.	Biotin-LC	Fluorescein	Rhodamine	Peroxidase	Alk. Phos.
Anti-BOVINE	Bovine IgG (H+L)	Goat	31100 2 mg	31710 2 ml				
	Bovine IgG (H+L)	Rabbit	31103 2 mg	31712 1.5 ml				
Anti-CHICKEN	Chicken IgY (H+L)	Rabbit	31104 2 mg	31720 1.5 ml	31501 1.5 mg		31401 1.5 ml	
Anti-GOAT	Goat IgG (H+L)	Donkey	31108 1.5 mg					
	Goat IgG (H+L) (min x HnMsRb Sr Prot)*	Mouse	31107 1.5 mg	31730 1 ml	31512 1 mg		31400 1 ml	
	Goat IgG (H+L)	Rabbit	31105 2 mg	31732 1.5 mg	31509 1.5 mg	31650 1.5 mg	31402 1.5 ml	31300 1 ml
	Goat IgG [F(ab')$_2$]	Rabbit	31153 2 mg	31753 1.5 ml	31553 1.5 mg		31403 1.5 ml	31405 1 ml
	Goat IgG (Fc)	Rabbit	31133 2 mg	31733 1.5 ml	31533 1.5 mg		31433 1.5 ml	31337 1 ml
Anti-GOAT F(ab')$_2$ Fragment of Host Antibody	Goat IgG (H+L) (min x Hn Sr Prot)*	Rabbit	31109 0.5 mg					31302 0.5 ml
Anti-GUINEA PIG	Guinea Pig IgG (H+L)	Goat	31114 2 mg					
Anti-HAMSTER	Hamster IgG (H+L)	Goat	31115 1.5 mg	31750 1.5 mg				
	Hamster IgG (H+L)	Rabbit	31120 2 mg		31587 1.5 mg	31652 1.5 mg		
Anti-HORSE	Horse IgG (H+L)	Goat	31116 2 mg	31760 1.5 mg				
Anti-HUMAN	Human IgG (H+L)	Goat	31130 2 mg	31770 1.5 mg	31529 2 mg	31656 2 mg	31410 2 ml	31310 1 ml
	Human IgG Gamma Chain Specific	Goat	31118 0.5 mg					
	Human IgG (H+L) (min x BvHsMs Sr Prot)*	Goat	31119 1.5 mg	31774 1.5 ml	31531 1.5 mg		31412 1.5 ml	
	Human IgG [F(ab')$_2$]	Goat	31122 2 mg					31312 1 ml
	Human IgG [F(ab')$_2$] (min x BvHsMs Sr Prot)*	Goat	31132 1.5 mg				31414 1.5 ml	
	Human IgG (Fc) (min x BvHsMs Sr Prot)*	Goat	31123 1.5 mg				31416 1.5 ml	
	Human IgM (Fc5μ)	Goat	31136 2 mg		31575 2 mg		31415 2 ml	
	Human IgM (μ)	Goat	31124 0.5 mg	31778 0.5 mg				
	Human IgM (Fc5μ) (min x Bv Sr Prot)*	Goat	31138 1.5 mg					
	Human IgA (α)	Goat	31140 2 mg		31577 2 mg		31417 2 ml	31314 1 ml
	Human IgG + IgM (H+L)	Goat	31134 2 mg	31776 2 ml				
	Human IgA + IgG + IgM (H+L)	Goat	31128 2 mg	31782 2 ml			31418 2 ml	31316 1 ml
	Human Kappa Chain	Goat	31129 0.5 mg	31780 0.5 mg				
	Human Lambda Chain	Goat	31131 0.5 mg					
	Human IgG (H+L) (min x Ms Sr Prot)*	Mouse	31135 2 mg				31420 1.5 ml	

*See Table 3 on page 12 for the Key to Abbreviations.

Affinity-Purified Secondary Antibodies

Specificity	Description	Host	Product # Pkg. Size Unconj.	Biotin-LC	Fluorescein	Rhodamine	Peroxidase	Alk. Phos.
Anti-HUMAN continued	Human IgG (H+L) (min x BvHsMs Sr Prot)*	Mouse	31137 1.5 mg	31784 1 ml				
	Human IgG (H+L)	Rabbit	31143 2 mg	31786 1.5 ml				
	Human IgG (H+L) (min x Ms Sr Prot)*	Rabbit	31147 1.5 mg					
	Human IgG (Fc)	Rabbit	31142 2 mg	31789 1.5 ml	31535 1.5 mg		31423 1.5 ml	31318 1 ml
	Human IgM (Fc5µ)	Rabbit	31149 2 mg					
Anti-HUMAN $F(ab')_2$ Fragment of Host Antibody	Human IgG (Fc)	Goat	31163 1 mg					
	Human IgG (H+L)	Goat	31165 1 mg					
	Human IgA + IgG + IgM (H+L)	Goat			31539 1 mg			
	Human IgG (min x MsBvHs Sr Prot)*	Mouse	31155 1.5 mg					
Anti-MOUSE	Mouse IgA (α) (min x Hn Sr Prot)*	Goat	31169 1 mg					
	Mouse IgA + IgG + IgM (H+L)	Goat	31171 2 mg					
	Mouse IgG (H+L)	Goat	31160 2 mg	31800 2 ml	31569 2 mg	31660 2 mg	31430 2 ml	31320 1 ml
	Mouse IgG (H+L) (min x BvHnHs Sr Prot)*	Goat	31164 1.5 mg	31802 1.5 mg	31541 1.5 mg	31661 1.5 mg	31432 1.5 ml	31322 1 ml
	Mouse IgG $[F(ab')_2]$	Goat	31166 2 mg	31803 2 ml	31543 2 mg		31436 2 ml	31324 1 ml
	Mouse IgG (Fc)	Goat	31168 2 mg	31805 2 ml	31547 2 mg	31663 2 mg	31437 2 ml	31325 1 ml
	Mouse IgG (Fc) (min x BvHnHs Sr Prot)*	Goat	31170 1.5 mg				31439 1.5 ml	31327 1 ml
	Mouse IgM (µ)	Goat	31172 2 mg	31804 0.5 mg	31992 2 mg	31662 2 mg	31440 2 ml	31326 1 ml
	Mouse IgM (µ) (min x BvHnHs Sr Prot)*	Goat	31176 1.5 mg		31585 1.5 mg	31664 1.5 mg		
	Mouse IgG + IgM (H+L)	Goat	31182 2 mg	31807 2 ml	31586 1.5 mg		31444 2 ml	31328 1 ml
	Mouse IgG + IgM (H+L) (min x BvHnHs Sr Prot)*	Goat	31184 1.5 mg				31446 1.5 ml	31330 1 ml
	Mouse IgG (H+L)	Horse	31181 1.5 mg	31806 1.5 mg				
	Mouse IgG (H+L)	Rabbit	31188 2 mg	31810 1.5 ml	31561 1.5 mg	31665 1.5 mg	31450 1.5 ml	31329 1 ml
	Mouse IgG (H+L) (min x Hn Sr Prot)*	Rabbit	31190 1.5 mg	31812 1 ml			31452 1 ml	31334 0.5 ml
	Mouse IgG $[F(ab')_2]$	Rabbit	31192 2 mg	31811 1.5 ml	31559 1.5 mg	31666 1.5 mg/	31451 1.5 ml	31331 1 ml
	Mouse IgG (Fc)	Rabbit	31194 2 mg	31813 1.5 ml	31555 1.5 mg		31455 1.5 ml	31332 1 ml
	Mouse IgM (µ)	Rabbit	31196 2 mg	31814 1.5 ml	31557 1.5 mg		31456 1.5 ml	31333 1 ml
	Mouse IgG + IgM (H+L)	Rabbit	31198 2 mg	31815 1.5 ml	31558 1.5 mg		31457 1.5 ml	31335 1 ml
	Mouse IgG (H+L) (min x BvHnHs Sr Prot)*	Goat	31185 1 mg		31565 1 mg		31438 0.5 ml	

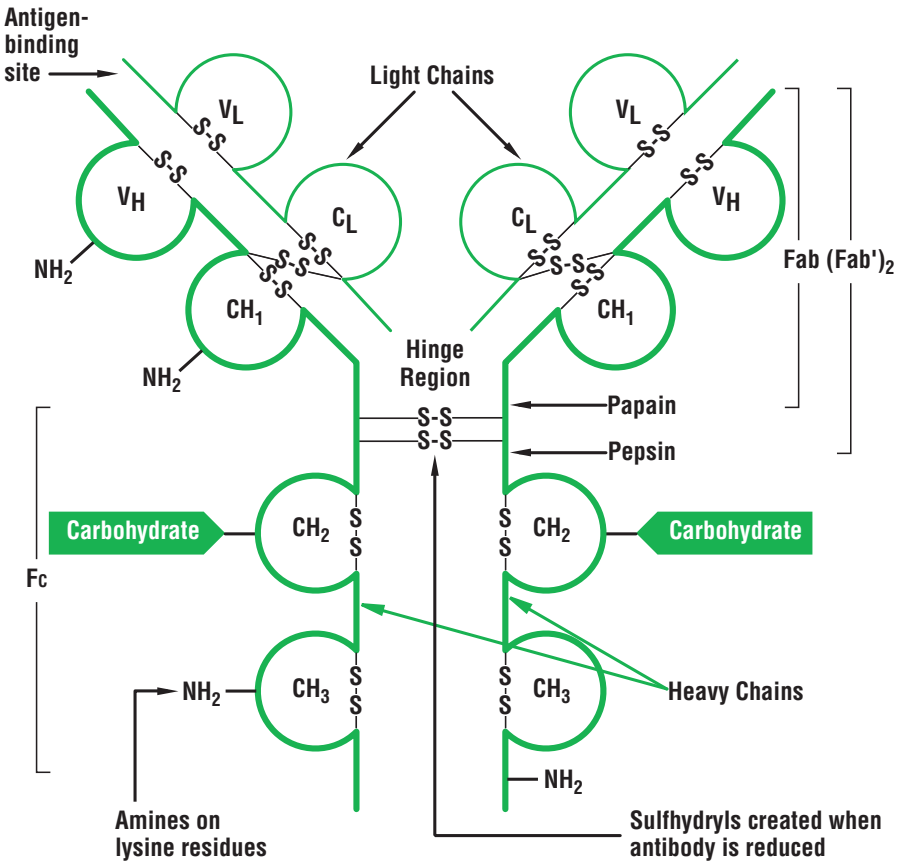
*See Table 3 on page 12 for the Key to Abbreviations.

Affinity-Purified Secondary Antibodies

Specificity	Description	Host	Product # Pkg. Size Unconj.	Biotin-LC	Fluorescein	Rhodamine	Peroxidase	Alk. Phos.
Anti-MOUSE $F(ab')_2$ Fragment of Host Antibody continued	Mouse IgM (µ)	Goat	31178 1 mg					
	Mouse IgM (µ) (min x BvHnHs Sr Prot)	Goat	31186 1 mg				31442 0.5 ml	
	Mouse IgG + IgM (H+L) (min x BvHnHs Sr Prot)	Goat					31448 0.5 ml	
	Mouse IgG (H+L)	Rabbit	31189 1 mg					
Anti-RABBIT	Rabbit IgG (H+L) (min x BvGtHnHsMsRtSh Sr Prot)	Donkey		31821 0.5 ml	31568 0.5 mg		31458 0.5 ml	31345 0.5 ml
	Rabbit IgG (H+L)	Goat	31210 2 mg	31820 1.5 mg		31670 2 mg	31460 2 ml	31340 1 ml
	Rabbit IgG (H+L) (min x Hn Sr Prot)	Goat	31212 1.5 mg	31822 1.5 ml	31583 1.5 mg		31462 1.5 ml	31342 1 ml
	Rabbit IgG $[F(ab')_2]$	Goat	31234 2 mg	31823 2 ml	31573 2 mg		31461 2 ml	31343 1 ml
	Rabbit IgG (Fc)	Goat	31216 2 mg				31463 2 ml	31341 1 ml
	Rabbit IgG (H+L) (min x GtHnMsSh Sr Prot)	Mouse	31213 1.5 mg	31824 1 ml	31584 1 mg	31674 1 mg	31464 1 ml	
Anti-RABBIT $F(ab')_2$ Fragment of Host Antibody	Rabbit IgG (H+L)	Goat	31214 1 mg		31579 1 mg			
	Rabbit IgG (H+L) (min x Hn Sr Prot)	Goat	31215 1 mg					
	Rabbit IgG (Fc)	Goat	31217 1 mg		31581 1 mg			
Anti-RAT	Rat IgG (H+L)	Goat	31220 2 mg	31830 2 ml	31629 2 mg	31680 2 mg	31470 2 ml	31350 1 ml
	Rat IgG $[F(ab')_2]$	Goat					31474 2 ml	
	Rat IgG (Fc)	Goat	31226 2 mg	31833 2 ml	31621 2 mg		31475 2 ml	31353 1 ml
	Rat IgM (µ)	Goat	31228 2 mg	31832 2 ml	31631 2 mg		31476 2 ml	31354 1 ml
	Rat IgG (H+L)	Rabbit	31218 2 mg	31834 1.5 mg				
	Rat IgG (H+L) (min x Ms Sr Prot)	Rabbit	31219 0.5 mg	31836 0.5 mg				
Anti-RAT $F(ab')_2$ Fragment of Host Antibody	Rat IgG (H+L)	Rabbit	31227 1 mg					
	Rat IgG + IgM (H+L)	Goat			31625 1 mg			
	Rat IgG (H+L) (min x Ms Sr Prot)	Mouse	31225 1 mg		31633 0.5 mg	31682 0.5 mg		
Anti-SHEEP	Sheep IgG (H+L)	Rabbit	31240 2 mg	31840 1.5 mg	31627 1.5 mg		31480 1.5 ml	31360 1 ml
	Sheep IgG (Fc)	Rabbit	31241 2 mg	31841 1.5 ml			31441 1.5 ml	31356 1 ml
	Sheep IgG $[F(ab')_2]$	Rabbit	31244 2 mg	31844 1.5 ml			31481 1.5 ml	31344 1 ml
Anti-SHEEP $F(ab')_2$ Fragment of Host Antibody	Sheep IgG (H+L)	Rabbit	31229 1 mg					
Anti-SWINE	Swine IgG (H+L)	Goat	31231 2 mg					

*See Table 3 on page 12 for the Key to Abbreviations.

Chemical cross-linking reagents have become an invaluable tool in the scientific community. These reagents are used in preparing antibody-enzyme conjugates and other labeled protein reagents. After the protein is conjugated to an appropriate enzyme, it may then be used as a detection reagent in a variety of assays and applications. A number of cross-linking methods have been used to prepare enzyme conjugates. For example, an *N*-hydroxysuccinimide ester can be prepared from a ligand of interest, then reacted with a primary amine on the surface of the enzyme. While this method is necessary in some applications, such as those in which the ligand does not contain a primary amine, it is not useful as a general-purpose method.



Antibody Modification Sites

Antibodies can be easily modified to contain labels such as biotin, fluorescent tags or enzymes to create reagents for Western blotting, ELISA, immunohistochemical staining and *in vivo* targeting. Pierce offers tools for a variety of antibody modification strategies.

Understanding the functional groups available on an antibody is the key to choosing the proper method for modification.

For example:

Primary amines (–NH_2) are found on lysine residues and the N-terminus. These are abundant and distributed over the entire antibody.

Sulfhydryl groups (–SH) are found on cysteine residues and are formed by selectively reducing disulfide bonds in the hinge region of the antibody.

Carbohydrate residues containing cis-diols can be oxidized (–CHO) to create active aldehydes. These are localized to the Fc region on antibodies and are more abundant on polyclonal antibodies.

Horseradish Peroxidase

Its high specific enzyme activity makes it the enzyme of choice.

Highlights:

- Superior to alkaline phosphatase and β-galactosidase conjugates due to the higher specific enzyme activity
- Small size (40 kDa) allows excellent cellular penetration
- Variety of substrates available
- Ideal in blotting and cytochemistry applications
- Used as the reporter enzyme for SuperSignal® Chemiluminescent Substrates

References

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Hosoda, H., *et al.* (1987). *Chem. Pharm. Bull.* **35**, 3336-3342.

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Wordinger, R.J., *et al.* (1987). Manual of Immunoperoxidase Techniques, 2nd Edition. Chicago: American Society of Clinical Pathologists Press, pp. 23-24.

Yolken, R.H. (1982). *Rev. Infect. Dis.* **4(1)**, 35-68.

Alkaline Phosphatase

A highly sensitive enzyme for ELISA and immunohistochemical applications.

Highlights:

- Purified form – ready to conjugate without prior dialysis
- Activity is not affected by exposure to antibacterial agents, such as sodium azide or thimerosal
- Specific activity >2,000 units/mg
- One unit is defined as the amount that will hydrolyze 1.0 µmole of *p*-nitrophenyl phosphate per minute at 37°C in 1.0 M diethanolamine, 0.5 mM $MgCl_2$, pH 7.8

Specific Activity per mg Protein

Buffer	25°C	37°C
0.1 M Glycine, 1.0 mM $ZnCl_2$, 1.0 mM $MgCl_2$, 6.0 mM *p*-Nitrophenyl phosphate, pH 10.4	>500	>1,000
1.0 M Diethanolamine, 0.5 mM $MgCl_2$, 15 mM *p*-Nitrophenyl phosphate, pH 9.8	>1,000	>2,000

References

Bulman, A.S. and Heyderman, E. (1981). *J. Clin. Pathol.* **34**, 1349-1351.

Cordell, J.L., *et al.* (1984). *J. Histochem. Cytochem.* **32**, 219-229.

Yolken, R.H. (1982). *Rev. Infect. Dis.* **4**, 35-68.

PRODUCT #	DESCRIPTION	PKG. SIZE
31490	ImmunoPure® Horseradish Peroxidase	10 mg
31491	ImmunoPure® Horseradish Peroxidase	100 mg
31391	ImmunoPure® Alkaline Phosphatase Calf intestinal. Supplied in Tris Buffer, pH ~7 Triethanolamine, 1 mM $MgCl_2$, 3 M NaCl, pH 7.6	20 mg
31392	ImmunoPure® Alkaline Phosphatase	100 mg

Maleimide activation – The heterobifunctional cross-linker SMCC (Product # 22360) and its water-soluble analog Sulfo-SMCC (Product # 22322) have more general utility in preparing immunologically active horseradish peroxidase or alkaline phosphatase conjugates. They are most useful when preparing conjugates of reduced IgG and $F(ab')_2$, because these methods involve the initial step of preparing a maleimide-activated (sulfhydryl-reactive) enzyme derivative. Studies have shown that the two-step maleimide method is superior to glutaraldehyde or metaperiodate methods for enzyme conjugation (Figure 8). The maleimide method gives higher yields with less polymerization, producing a conjugate preparation with superior immunoassay characteristics.

Maleimide-activated enzymes can be prepared using the heterobifunctional cross-linker Sulfo-SMCC. This reagent contains an *N*-hydroxysulfosuccinimide (Sulfo-NHS) functional group and a maleimide functional group and it is water-soluble due to the presence of the sulfonate ($-SO_3^-$) group on the *N*-hydroxysuccinimide ring. The sulfonate group also contributes to the stability of the molecule in aqueous solution. A study of the hydrolysis rate of the maleimide functional group from Sulfo-SMCC showed that it is less prone to hydrolysis to the maleamic acid than the non-sulfonated SMCC. The maleimide groups of Sulfo-SMCC exhibit no decomposition at pH 7 at 30°C within 6 hours. The Sulfo-NHS ester group reacts with primary amines on the enzyme surface to form a stable amide bond. After this first step of conjugation, the enzyme will have maleimide groups on its surface that react optimally toward sulfhydryl groups between pH 6.5 and 7.5 to form stable thioether bonds. Maleimide-mediated conjugation strategies are summarized in Figure 8.

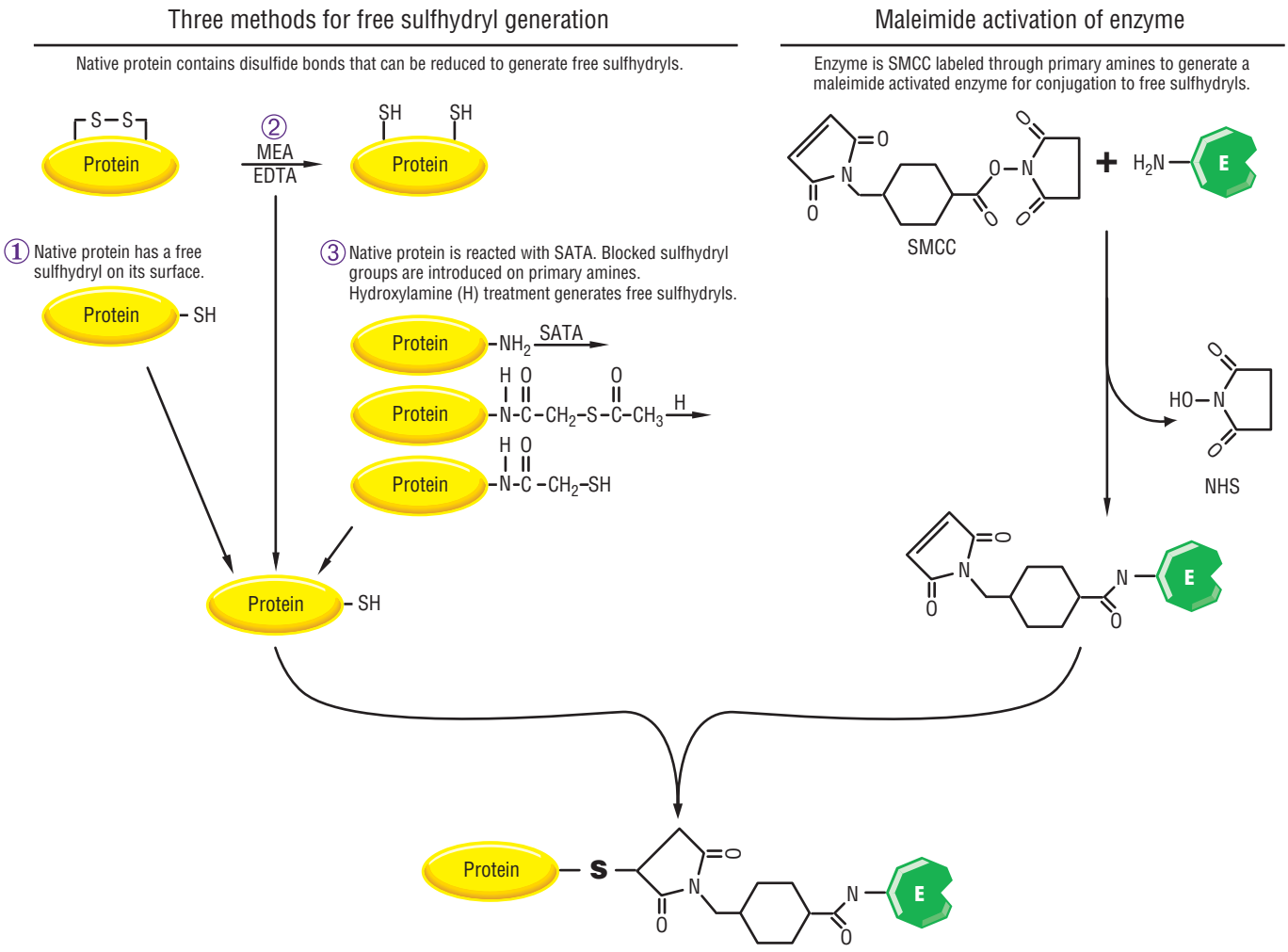

Figure 8. Three strategies for maleimide-mediated conjugation of enzymes.

Two reagents, Mercaptoethylamine•HCl (Product # 20408) and SATA (Product # 26102), are available to produce free sulfhydryls on macromolecules for conjugation to the maleimide-activated enzymes. For labeling antibody molecules, mild reduction with Mercaptoethylamine•HCl (MEA) results in two half-antibody fragments containing free sulfhydryl groups in the hinge region. Labeling in this area is advantageous because it directs the modification away from the antigen-binding region. Native proteins lacking a free sulfhydryl on their surface can be reacted with SATA to generate blocked sulfhydryl groups. The SATA molecule reacts with primary amines via its NHS ester end to form stable amide linkages. The acetylated sulfhydryl group (blocked) is stable until treated with hydroxylamine to generate the free sulfhydryls.

Pierce offers stable, preactivated enzyme derivatives that are reactive toward sulfhydryl (-SH) groups, EZ-Link™ Maleimide Activated Alkaline Phosphatase (Product # 31486) and Horseradish Peroxidase (Product # 31485). These products eliminate the first step of the two-step maleimide method, simplifying and facilitating the conjugation protocol, while saving several hours. They can be used to prepare enzyme conjugates directly from proteins, peptides or other ligands that contain a free –SH group. Two reagents, SATA and mercaptoethylamine•HCl, are also included in the kit formats to produce free sulfhydryls on macromolecules for conjugation.

PRODUCT #	DESCRIPTION	PKG. SIZE
31486	EZ-Link® Maleimide Activated Alkaline Phosphatase	2 mg
31493	EZ-Link® Maleimide Activated Alkaline Phosphatase Kit	Kit
	Includes: EZ-Link® Maleimide Activated Alkaline Phosphatase	2 mg
	Activation/Conjugation Buffer	20 ml
	BupH™ Tris Buffered Saline Pack	2 packs
	BupH™ Phosphate Buffered Saline Pack	1 pack
	Polyacrylamide Desalting Column	1 x 10 ml
	Mercaptoethylamine•HCl	6 mg
	SATA	2 mg
	Hydroxylamine	5 mg
	DMF	1 ml
	Column Extender	
31485	EZ-Link® Maleimide Activated Horseradish Peroxidase	5 mg
31494	EZ-Link® Maleimide Activated Horseradish Peroxidase Kit	Kit
	Includes: EZ-Link® Maleimide Activated Horseradish Peroxidase	5 mg
	Activated Horseradish Peroxidase Conjugation Buffer	20 ml
	2-Mercaptoethylamine•HCl	6 mg
	SATA	2 mg
	Dimethylformamide	1 ml
	Hydroxylamine•HCl	5 mg
	Polyacrylamide Desalting Column	1 x 10 ml

EZ-Link® Maleimide Activated Peroxidase References

Choi, J.Y., *et al.* (2002). *J. Biol. Chem.* **277**, 21630-21638.

Seo, Y.R., *et al.* (2002). *PNAS* **99**, 14548-14553.

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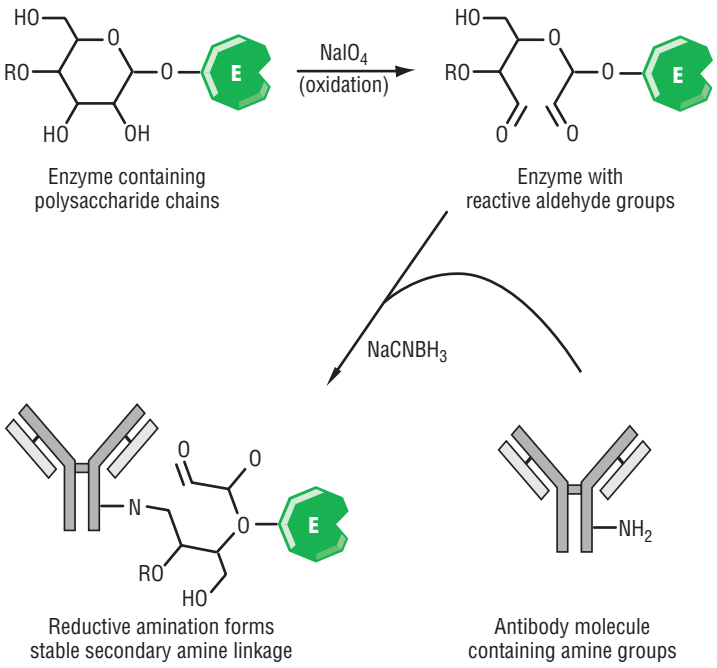

Figure 9. Conjugation scheme for periodate oxidation and subsequent reductive amination.

Periodate – Glycoproteins such as horseradish peroxidase, glucose oxidase and most antibody molecules can be activated for conjugation by treatment with periodate. Oxidizing polysaccharide residues in a glycoprotein with sodium periodate provides a mild and efficient way of generating reactive aldehyde groups for subsequent conjugation with amine- or hydrazide-containing molecules via reductive amination (Figure 9). Some selectivity of monosaccharide oxidation may be accomplished by regulating the concentration of periodate in the reaction medium. In the presence of 1 mM sodium periodate, sialic acid groups are specifically oxidized at adjacent hydroxyl residues, cleaving off two molecules of formaldehyde and leaving one aldehyde group. At higher concentrations of sodium periodate (10 mM or greater), other sugar residues will be oxidized at points where adjacent carbon atoms contain hydroxyl groups. This reaction should be performed in the dark to prevent periodate breakdown and for a limited period of time (15-30 minutes) to avoid loss of enzymatic activity.

Cross-linking with an amine-containing protein takes place under alkaline pH conditions through the formation of Schiff base intermediates. These relatively labile intermediates can be stabilized by reduction to a secondary amine linkage with sodium cyanoborohydride. Reductive amination has been done using sodium borohydride or sodium cyanoborohydride; however, cyanoborohydride is the better choice because it is more specific for reducing Schiff bases and will not reduce aldehydes. Small blocking agents such as lysine, glycine, ethanolamine or Tris can be added after conjugation to quench any unreacted aldehyde sites. Ethanolamine and Tris are the best choices for blocking agents because they contain hydrophilic hydroxyl groups with no charged functional groups.

The pH of the reductive amination reaction can be controlled to affect the efficiency of the cross-linking process and the size of the resultant antibody-enzyme complexes formed. At physiological pH, the initial Schiff base formation is less efficient and conjugates of lower molecular weight result. At more alkaline pH (i.e., pH 9-10), Schiff base formation occurs rapidly and with high efficiency, resulting in conjugates of higher molecular weight and greater incorporation of enzyme when oxidized enzyme is reacted in excess. Low molecular weight conjugates may be more optimal for immunohistochemical staining or blotting techniques in which penetration of the complex through membrane barriers is an important consideration. Washing steps also more effectively remove excess reagent if the conjugate is of low molecular weight, thus maintaining low background in an assay. By contrast, conjugates of high molecular weight are more appropriate for ELISA procedures in a microplate format, where high sensitivity is important and washing off excess conjugate is not a problem.

Glutaraldehyde – Another method for conjugation uses glutaraldehyde, one of the oldest homo-bifunctional cross-linking reagents used for protein conjugation. It reacts with amine groups to create cross-links by one of several routes. Under reducing conditions, the aldehydes on both ends of glutaraldehyde will couple with amines to form secondary amine linkages. The reagent is highly efficient at protein conjugation but has a tendency to form various high-molecular weight polymers, making results difficult to reproduce.

EZ-Link® Activated Peroxidase References

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EZ-Link® Plus Activated Peroxidase References

Glover, L., (2002). *Eur. J. Biochem.* **269**, 4607-4616.

Nawa, M., *et al.* (2000). *Clin. Diagn. Lab. Immunol.* **7**, 774-777.

Völkel, T., *et al.* (2001). *Protein Eng.* **14**, 815-823.

PRODUCT #	DESCRIPTION	PKG. SIZE
31487	EZ-Link® Plus Activated Peroxidase (Periodate Activated)	1 mg
31488	EZ-Link® Plus Activated Peroxidase (Periodate Activated)	5 x 1 mg
31489	EZ-Link® Plus Activated Peroxidase Kit (Periodate Activated)	Kit
	Includes: EZ-Link® Plus Activated Peroxidase	5 x 1 mg
	Sodium Cyanoborohydride Solution	1 x 0.5 ml
	Quenching Buffer	25 ml
	BupH™ Phosphate Buffered Saline Pack	500 ml
	BupH™ Carbonate Buffer Pack	500 ml
31496	EZ-Link® Activated Peroxidase (Glutaraldehyde Activated)	1 mg
31495	EZ-Link® Activated Peroxidase (Glutaraldehyde Activated)	5 mg
31497	EZ-Link® Activated Peroxidase Antibody Labeling Kit (Glutaraldehyde Activated)	Kit
	Includes: EZ-Link® Activated Peroxidase	5 mg
	Conjugation Buffer	50 ml
	Lysine	250 mg
	Immobilized Protein A/G Column	0.5 ml
	Gentle Ag/Ab Binding Buffer	200 ml
	Gentle Ag/Ab Elution Buffer	200 ml

EZ-Label™ Fluorescent Labeling Kits

Make your own fluorescent-labeled antibody in less than two hours!

EZ-Label™ Kits are designed for labeling any size protein – small or large – even if you have only a small amount of your protein. Protein sample volumes ranging from 50 µl-1 ml can be used, with protein concentration up to 10 mg/ml for each reaction. EZ-Label™ Kits were specially developed and optimized for the most efficient labeling.

EZ-Label™ Kits contain everything you need to successfully label your antibody or protein:

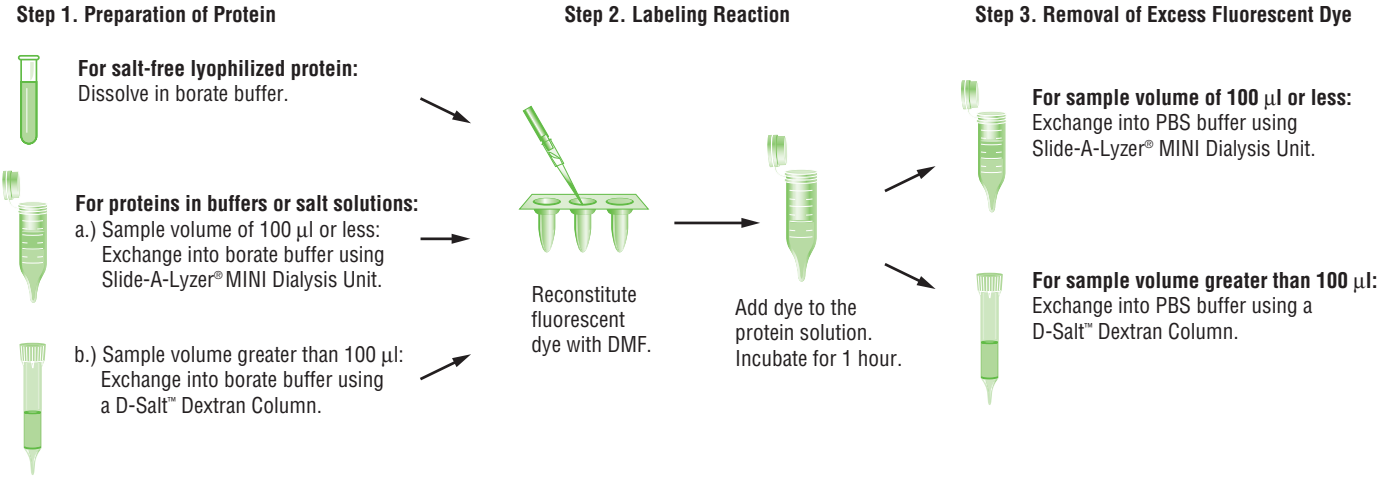
- Fluorescent dye provided in individual microtubes, eliminating the need to weigh dye
- Conveniently packaged dimethylformamide (DMF) to prepare the fluorescent dye solution
- Pre-made borate and phosphate buffers – just add water to the powder and they are ready-to-use
- Pre-packed, ready-to-use desalting columns for fast buffer exchange when your protein sample volume is greater than 100 µl
- Slide-A-Lyzer® MINI Dialysis Units for easy buffer exchange when your protein sample volume is less than or equal to 100 µl
- Amber reaction tubes – no handling in the dark required

EZ-Label™ Kit	Excitation Wavelength (nm)	Emission Wavelength (nm)
Fluorescein Protein Labeling Kit	491	518
Rhodamine Protein Labeling Kit	544	576
Fluorescein Isothiocyanate (FITC) Protein Labeling Kit	494	520

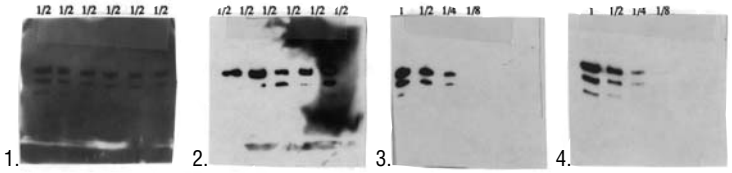
These kits contain sufficient reagents to perform five fluorescent labeling reactions, which use up to 10 mg/ml of protein for each reaction (50 µl-1 ml volume of protein).

PRODUCT #	DESCRIPTION	PKG. SIZE
53000	**EZ-Label™ Fluorescein Protein Labeling Kit**	Kit
	Sufficient for five coupling reactions.	
	Includes: No-Weigh™ Pre-Measured Fluorescein Microtubes	6 x 1 mg microtubes
	Dimethylformamide (DMF)	1 ml
	BupH™ Borate Buffer Packs	5 packs
	BupH™ Phosphate Buffered Saline Packs	5 packs
	D-Salt™ Dextran Desalting Columns	5 columns
	Slide-A-Lyzer® MINI Dialysis Unit Pack	5 units
	Reaction Tubes	5 tubes
53002	**EZ-Label™ Rhodamine Protein Labeling Kit**	Kit
	Sufficient for five coupling reactions.	
	Includes: No-Weigh™ Pre-Measured Rhodamine Microtubes	6 x 0.5 mg microtubes
	Dimethylformamide (DMF)	1 ml
	BupH™ Borate Buffer Packs	5 packs
	BupH™ Phosphate Buffered Saline Packs	5 packs
	D-Salt™ Dextran Desalting Columns	5 columns
	Slide-A-Lyzer® MINI Dialysis Unit Pack	5 units
	Reaction Tubes	5 tubes
53004	**EZ-Label™ Fluorescein Isothiocyanate (FITC) Protein Labeling Kit**	Kit
	Sufficient for five coupling reactions.	
	Includes: No-Weigh™ Pre-Measured FITC Microtubes	6 x 1 mg microtubes
	Dimethylformamide (DMF)	1 ml
	BupH™ Borate Buffer Packs	5 packs
	BupH™ Phosphate Buffered Saline Packs	5 packs
	D-Salt™ Dextran Desalting Columns	5 columns
	Slide-A-Lyzer® MINI Dialysis Unit Pack	5 units
	Reaction Tubes	5 tubes

See how easy the fluorescent labeling procedure is when you use EZ-Label™ Kits.



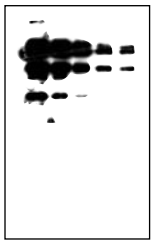
Optimizing Antibody Concentration



Mouse anti-Human p53	1/500 (1 µg/ml)	1/500 (1 µg/ml)	1/1,000 (0.5 µg/ml)	1/1,000 (0.5 µg/ml)
Goat anti-Mouse HRP	1/1,000 (1 µg/ml)	1/5,000 (0.2 µg/ml)	1/10,000 (0.1 µg/ml)	1/20,000 (0.05 µg/ml)
Exposure Time	30 seconds	30 seconds	1 minute	1 minute

Figure 10. Example of signal intensity on a Western blot when using SuperSignal® West Pico Substrate and antibodies at various concentrations. Recombinant Human Wild-Type p53 Baculovirus lysate at various concentrations was electrophoretically separated and transferred to nitrocellulose membrane. The membrane was blocked with BSA and then incubated with various dilutions of mouse anti-human p53 starting at the manufacturer's recommended dilution. Horseradish peroxidase-labeled goat anti-mouse was added at different concentrations and the signal was developed with SuperSignal® West Pico Substrate. The exposure times were also varied as indicated above. In blot 1, the blot was totally black due to both the primary and secondary antibody concentrations being too high. In blot 2, the background is inconsistent but very dark, again a result of too much primary and secondary antibody. In blots 3 and 4, the signal-to-noise was much better because both the primary and secondary antibody concentrations were reduced. Neither blots 3 nor 4 had background signal.

Primary Antibody 1:500
Secondary Antibody 1:5,000



Primary Antibody 1:5,000
Secondary Antibody 1:50,000

Figure 11. Example of signal intensity on a Western blot using SuperSignal® West Dura Substrate and antibodies at various concentrations.

In Figure 11, blots were optimized with SuperSignal® West Dura Chemiluminescent Substrate. The blot with a primary antibody concentration of 1:500 and a secondary antibody concentration of 1:5,000 shows what happens when the antibody levels are too high. The background is not excessively high, but the bands are too intense and blur together, resulting in poor resolution. A large number of nonspecific bands are also visible. An optimal blot was achieved by using a 1:5,000 primary antibody dilution and a 1:50,000 secondary antibody dilution.

Because every new Western blot is unique, there is no "perfect" antibody concentration for every blot. Therefore, every new Western blot needs to be optimized to find out which antibody concentration is most appropriate to that particular combination of membranes, proteins and antibodies. Optimization is even more crucial when key components of a system are changed, such as switching from a colorimetric substrate like chlororonaphthol (CN) to more sensitive chemiluminescent substrates such as SuperSignal® West Products. The first step of optimizing the blotting conditions usually involves optimizing the antibody concentrations (or dilutions) through the use of a dot blot protocol. The next step is usually the optimization of the blocking buffer by testing cross-reactivity of several different buffers with the blotting system's key components (see page 7).

Dot Blot Protocol for Optimization of Antigen and Antibody Concentrations

The optimal antibody concentrations to use with a given antigen are dependent on the antigen and antibody themselves. The affinity/avidity of the antibody for the antigen and the specific activity of both the primary and secondary antibody will vary. The optimal antigen and antibody concentrations can be determined by performing complete Western blots with varying concentrations of antigen and antibody. Alternatively, a faster and easier method is to perform a dot blot procedure. The following is an example of a dot blot protocol using SuperSignal® West Pico Substrate. When using other substrates from Pierce, refer to the instruction booklet for recommended antigen/antibody concentrations.

Optimizing Antibody Concentration

Note: All antibody dilutions assume a starting concentration of ~1 mg/ml.

1. Prepare dilutions of the protein sample in either TBS or PBS. The proper dilution will depend on the antigen concentration present in the sample because the concentration of the antigen of interest often is not known. It is necessary to test a wide range of dilutions. SuperSignal® West Pico Substrate has picogram-level detection sensitivity so sample dilutions can range from the low microgram to low picogram levels. If too much antigen is applied, the immunoassay results may show any or all of the following: detection of nonspecific bands, blurred banding patterns and rapid signal deterioration.

2. Prepare nitrocellulose membranes. The number of membrane pieces needed depends on how many different dilutions of primary and/or secondary antibody will be screened. Typically, one or two dilutions of the primary antibody are tested with two or three different dilutions of the secondary antibody. For example: 1/1,000 primary with 1/50,000 secondary, 1/1,000 primary with 1/100,000 secondary, 1/5,000 primary with 1/50,000 secondary, and 1/5,000 primary with 1/100,000 secondary.

3. Place **dry** nitrocellulose membranes on a paper towel. Dot antigen dilutions onto the membranes. Apply the smallest possible volume to the membranes (2-5 µl works well) because the greater the volume that is applied, the more diffuse the signal will be. Allow the antigen dilutions to dry on the membranes for 10-30 minutes or until no visible moisture remains.

4. Block the nonspecific sites on the nitrocellulose membranes by incubating them in blocking buffer that contains 0.05% Tween®-20 (blocker/Tween®-20) for 1 hour at RT with shaking.

5. Prepare the primary antibody dilutions (1/1,000-1/5,000) in blocker/Tween®-20 and apply to the membranes. Incubate for 1 hour at RT with shaking.

	SuperSignal® West Pico Substrate	SuperSignal® West Femto Substrate	SuperSignal® West Dura Substrate	Lumi-Phos™ WB Substrate
Recommended Primary Antibody Dilutions (from 1 mg/ml stock)	1/1,000-1/5,000 or 0.2-1.0 µg/ml	1/5,000-1/100,000 or 0.01-0.2 µg/ml	1/1,000-1/50,000 or 0.02-1.0 µg/ml	1/200-1/2,000 or 0.5-5.0 µg/ml

6. Wash the membrane 4-6 times in TBS or PBS, using as large a volume of wash buffer as possible. Add 0.05% Tween®-20 to the wash buffer to help reduce nonspecific background. For each wash, suspend the membrane in wash buffer and agitate for approximately 5 minutes. Pour off the wash buffer and repeat. Brief rinses of the membranes before incubation in the wash buffer may increase the wash step efficiency.

7. Prepare dilutions of the secondary antibody/HRP conjugate (1/20,000-1/100,000) in blocker/Tween®-20. Add the secondary antibody dilutions to the membranes and incubate for 1 hour with shaking.

	SuperSignal® West Pico Substrate	SuperSignal® West Femto Substrate	SuperSignal® West Dura Substrate	Lumi-Phos™ WB Substrate
Recommended Secondary Antibody Dilutions (from 1 mg/ml stock)	1/20,000-1/100,000 or 10-50 ng/ml	1/100,000-1/500,000 or 2.0-10 ng/ml	1/50,000-1/250,000 or 4.0-20 ng/ml	1/5,000-1/25,000 or 40-200 ng/ml

8. Wash the membrane again as described in Step 6.

9. Prepare the substrate working solution by mixing equal volumes of the Luminol/Enhancer Solution and the Stable Peroxide Solution. Prepare a sufficient volume to ensure that the blot is completely wetted with substrate and the blot does not dry out during incubation. **Recommended volume: 0.125 ml/cm^2 of blot surface.**

10. Incubate the membrane in the SuperSignal® West Pico Substrate Working Solution for 5 minutes.

11. Remove the membrane from the substrate and place in a plastic sheet protector or other protective wrap.

12. Place the blot against the film – protein side up – and expose. Any standard or enhanced autoradiographic film can be used. A recommended first exposure is 30-60 seconds. Exposure time can be varied to obtain optimum results. Alternatively, use a CCD camera or other imaging device; however, these devices may require longer exposure times.

13. On an optimized blot, the SuperSignal® West Pico Substrate-generated signal should last for up to eight hours. The blot can be re-exposed to film or an imaging device as needed to obtain the optimal results. Longer exposure times may be necessary as the blot ages. If optimal results are not achieved, repeat this procedure using different antigen and/or antibody dilutions.

As with the other components in a Western blotting system, there are many substrate choices available. The appropriate substrate choice depends on the enzyme label (AP or HRP), desired sensitivity, and desired form of signal or method of detection. Chromogenic substrates have been employed most widely and offer perhaps the simplest and most cost-effective method of detection. When these substrates come in contact with the appropriate enzyme, they are converted to insoluble, colored products that precipitate onto the membrane and require no special equipment for processing or visualizing. Substrates such as TMB (3,3´,5,5´-tetramethyl benzidine), 4-CN (4-chloro-1-naphthol) and DAB (3,3´-diaminobenzidine tetrahydrochloride) are available for use with HRP (Figure 12). For use with AP, NBT (nitro-blue tetrazolium chloride), BCIP (5-bromo-4-chloro-3´-indolylphosphate *p*-toluidine salt) and Fast Red (naphthol AS-MX phosphate + Fast Red TR Salt) are available (Figure 13). The performance of a particular substrate may vary dramatically when obtained from different suppliers because performance can be affected by the concentration and purity of the substrate and by other additives and buffer components that are a part of the formulation.

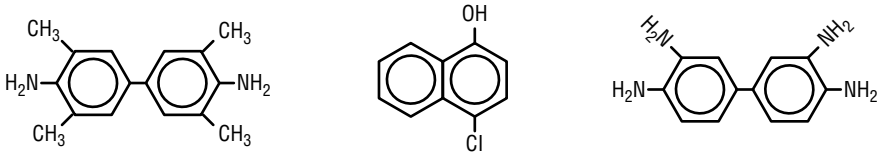


Figure 12. Chromogenic substrates for Western blotting with HRP.

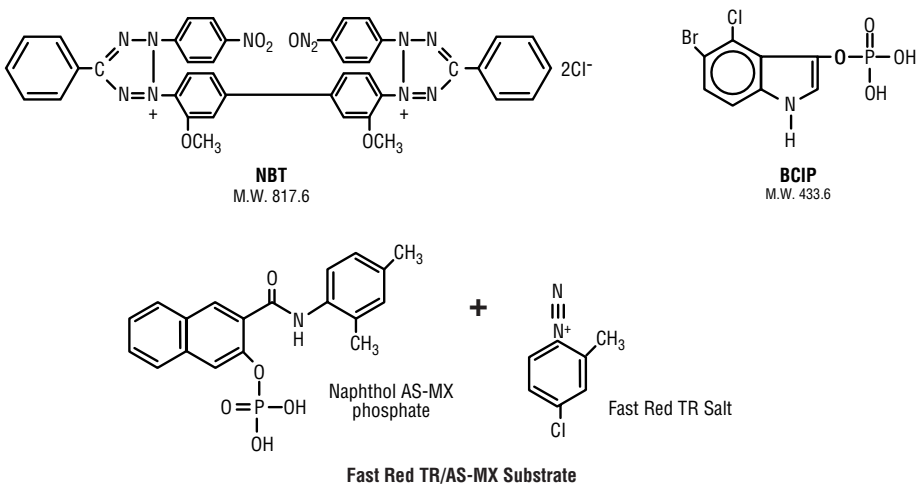


Figure 13. Chromogenic substrates for Western blotting with AP.

Peroxide must be added to a substrate for colorimetric detection with HRP. Because of its extremely short shelf life at the desired concentration, hydrogen peroxide traditionally was added to a buffer, along with the substrate, immediately prior to use. As a result, these substrates typically have a useful shelf life of only a few hours. Many precipitating HRP substrates from Pierce are supplied with, or come prepared in, Stable Peroxide Substrate Buffer (Product # 34062). The Stable Peroxide Substrate Buffer is a 10X concentrate that offers several advantages. It is less corrosive than the traditional 30% stock solution of hydrogen peroxide and, because fewer preparation steps are involved, it provides more consistent results. Although the Stable Peroxide Substrate Buffer is provided as a 10X concentrate, it is also stable at a 1X concentration.

PRODUCT #	DESCRIPTION	PKG. SIZE
34062	Stable Peroxide Buffer (10X)	100 ml

Substrates for HRP

TMB, with a molecular weight of 240.4, is most often used as a substrate for HRP in ELISAs. However, in the presence of HRP and peroxide, a water-soluble blue product is generated that can be precipitated onto a membrane. 1-Step™ TMB – Blotting (Product # 34018) is a single-component peroxidase substrate for Western blotting and immunohistochemistry. Precipitating the product results in dark blue bands where the enzyme is located. 1-Step™ TMB – Blotting is well suited to applications that require a large signal-to-noise ratio.

PRODUCT #	DESCRIPTION	PKG. SIZE
34018	1-Step™ TMB Blotting	250 ml

4-CN has a molecular weight of 178.6 and can be used for chromogenic detection of HRP in blotting and histochemistry. This precipitate is not as sensitive or as stable as TMB and DAB, but the alcohol-soluble precipitate photographs well and has a distinct blue-purple color that can be useful in double-staining applications.

PRODUCT #	DESCRIPTION	PKG. SIZE
34012	1-Step™ CN	250 ml
34010	4-Chloro-1-Napthol Powder	25 g powder
34011	4-Chloro-1-Napthol Tablets	50 tablets

DAB has a molecular weight of 214.1 and yields a brown precipitate in the presence of HRP and peroxide. The brown, insoluble product can be readily chelated with osmium tetroxide. This property makes DAB ideal for electron microscopy. The color produced by DAB can be intensified with the addition of metals such as nickel, copper, silver and cobalt that form complexes. The color produced by the metal complexes is darker than the color produced by DAB alone, enhancing the sensitivity in staining applications.

PRODUCT #	DESCRIPTION	PKG. SIZE
34002	DAB Substrate Kit	275 ml
	Includes: DAB (10X) Stable	25 ml
	Peroxide Buffer	250 ml
34065	Metal Enhanced DAB Substrate Kit	275 ml
	Includes: 10X Metal Enhanced DAB	25 ml
	Stable Peroxide Buffer	250 ml

The individual benefits of 4-CN and DAB are often combined into a single substrate mixture, **CN/DAB** Substrate. The CN/DAB Substrate has excellent sensitivity, yielding a dark black precipitate that photographs well. The CN/DAB Substrate works well in Western blotting and dot blotting applications.

PRODUCT #	DESCRIPTION	PKG. SIZE
34000	CN/DAB Substrate Kit	275 ml
	Includes: CN/DAB (10X)	25 ml
	Stable Peroxide Buffer	250 ml

Substrates for Alkaline Phosphatase

NBT, with a molecular weight of 817.6, is a member of a class of heterocyclic organic compounds known as tetrazolium salts. Upon reduction, the compound yields NBT-formazan, a highly colored, water-insoluble product. The substrate is widely used for immunochemical assays and techniques because the color produced by the formazan is linear and stable over a wide dynamic range.

PRODUCT #	DESCRIPTION	PKG. SIZE
34035	Nitro-Blue Tetrazolium Chloride	1 g powder

BCIP has a molecular weight of 433.6, and hydrolysis by alkaline phosphatase results in a blue-purple precipitate that can be deposited on nitrocellulose or nylon membranes. BCIP can be used as a chromogenic substrate for both immunoblotting and immunohistochemical studies.

PRODUCT #	DESCRIPTION	PKG. SIZE
34040	5-Bromo-4-Chloro-3'-indolyphosphate *p*-toluidine Salt	1 g powder

An ideal system for blotting or staining applications with AP is the combination of NBT and BCIP. Together, they yield an intense, black-purple precipitate that provides much greater sensitivity than either substrate alone. This reaction proceeds at a steady rate, allowing accurate control of its relative sensitivity. **NBT/BCIP** characteristically produces sharp band resolution with little background staining of the membrane.

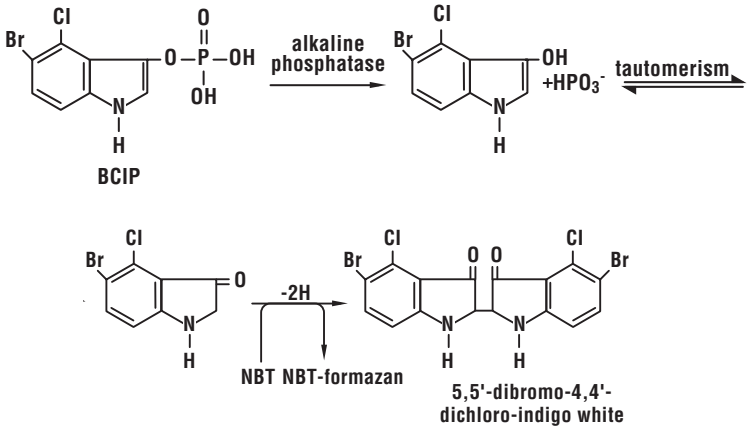


Figure 14. Reaction of alkaline phosphatase with BCIP and NBT.

PRODUCT #	DESCRIPTION	PKG. SIZE
34042	1-Step™ NBT/BCIP	250 ml
34070	1-Step™ NBT/BCIP Plus Suppressor	100 ml

Fast Red TR/AS-MX Substrate is a sensitive substrate that results in a bright red precipitate on transfer membranes. Alkaline phosphatase hydrolyzes the naphthol phosphate ester substrate. The naphthol then couples to the colorless chromogen, which is a diazonium salt, resulting in a precipitating azo dye. This substrate is ideal for double staining in both immunoblotting and immunohistochemical studies. The bright red precipitate contrasts well with horseradish peroxidase insoluble substrates, including CN/DAB and TMB. The red precipitate fades upon drying but will reappear when rehydrated with water. When double-staining immunoblots, the Fast Red TR/AS-MX solution must be added before a horseradish peroxidase substrate solution to prevent excessive background color development.

PRODUCT #	DESCRIPTION	PKG. SIZE
34034	Fast Red TR/AS-MX	Kit
	Includes: Fast Red TR Salt	250 mg
	Napthol AS-MX	35 ml
	Phosphate Concentrate	250 ml
	Substrate Buffer	

When energy in the form of light is released from a substance because of a chemical reaction the process is called chemiluminescence. Luminol is one of the most widely used chemiluminescent reagents and its oxidation by peroxide results in creation of an excited state product called 3-aminophthalate. This product decays to a lower energy state by releasing photons of light (Figure 15).

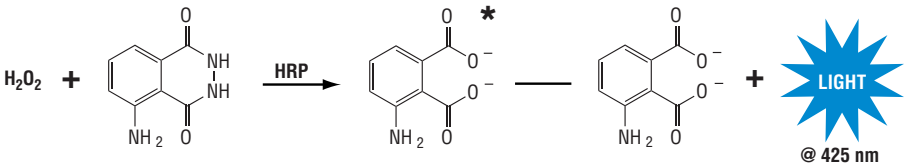


Figure 15. Luminol is oxidized in the presence of horseradish peroxidase and hydrogen peroxide to form an excited state product (3-aminophthalate). The 3-aminophthalate emits light at 425 nm as it decays to the ground state.

Chemiluminescent substrates have steadily gained in popularity throughout the past decade because they offer several advantages over other detection methods. These advantages have allowed chemiluminescence to become the detection method of choice in most protein laboratories. Using chemiluminescence allows multiple exposures to be performed to obtain the best image. The detection reagents can be stripped away and the entire blot reprobed to visualize another protein or to optimize detection of the first protein. A large linear response range allows detection and quantitation over a large range of protein concentrations. Most importantly, chemiluminescence yields the greatest sensitivity of any available detection method. Using HRP as the enzyme label and SuperSignal® West Femto Chemiluminescent Substrate (Product # 34095), lower detection limits in the low femtogram range are possible because the enhancers in this substrate greatly intensify the emitted light and extend the signal duration.

Chemiluminescent substrates differ from other substrates in that the light detected is a transient product of the reaction that is only present while the enzyme-substrate reaction is occurring. This is in contrast to substrates that produce a stable, colored product; these colored precipitates remain on the membrane after the enzyme-substrate reaction has terminated. On a chemiluminescent Western blot, the substrate is the limiting reagent in the reaction; as it is exhausted, light production decreases and eventually ceases. A well-optimized procedure using the proper antibody dilutions will produce a stable output of light over a period of several hours, allowing consistent and sensitive detection of proteins. When the antibody is not diluted sufficiently, too much enzyme is present and the substrate is used up quickly. A stable output of light will never be achieved. This is the single greatest cause of symptoms such as variability, dark background with clear bands and decreased sensitivity in Western blotting experiments with chemiluminescence. To avoid this problem, it is crucial to optimize the amount of antibody used for detection. Antibody suppliers typically suggest a dilution range for using their antibody on a Western blot. This dilution range is often appropriate for blots detected with a relatively insensitive chromogenic substrate, but a much greater dilution is generally required for optimum performance with a sensitive chemiluminescent substrate.

Table 5. Advantages of enhanced chemiluminescence

Sensitive
- Intense signal with low background
- Requires less antigen and antibody

Fast
- Rapid substrate processing of blot
- Signal generated within seconds

Nonhazardous
- No health hazards
- No waste disposal problems

Stable
- Unlike radioisotopes, the shelf life is long
- Store at 4°C

Hard-copy results
- Results are captured on X-ray film
- No fading or tearing of brittle membrane over time
- Permanent record

Film results
- Signal remains glowing for an extended period of time
- Ability to place blot against film at various times
- Ability to optimize the developing method

Ability to reprobe the blot
- Nonisotopic probes can be stripped off the membrane
- Immunodetection can be repeated

Large linear response
- Can detect a large range of protein concentrations

Quantitative
- The X-ray film can be scanned using a reflectance densitometer or using an imaging device, such as a CCD camera

Table 6. Blotting cost comparison between SuperSignal®, ECL™ and Western Lightning™ Products

Substrate Cost Comparison	SuperSignal® West Pico Substrate	ECL™ Substrate	Western Lightning™ Substrate
Membrane (8 x 10 or 8 x 12)	$ 5.66	$ 8.30	$12.40
TBS Wash Buffer	$ 1.24	$ 1.24	$ 1.30
SuperBlock® Blocking Buffer	$ 4.48	$ 4.48	$ 4.69
Primary Antibody*	**$ 3.15**	**$31.50**	**$ 5.78**
Secondary Antibody	$ 0.04	$ 0.47	$ 0.47
Substrate	$ 3.72	$ 5.46	$ 7.88
Film	$ 1.20	$ 2.28	$ 2.12
Total Blotting Cost	**$19.49**	**$53.73**	**$34.64**

**Endogen's Anti-CD54 (Product # MA5407, 500 µg) was used at the substrate manufacturer's recommended starting dilution.*

Costs are based on January 2004 U.S. list prices for an 8 x 10 cm mini gel following manufacturer's instructions.

Table 7. A comparison of SuperSignal® West Substrates

	SuperSignal® West Pico Chemiluminescent Substrate	SuperSignal® West Dura Extended Duration Substrate	SuperSignal® West Femto Maximum Sensitivity Substrate
Primary Benefit	• Twice the signal for about half the price of competing products	• Extended signal duration is ideal for use with imaging equipment	• The most sensitive chemiluminescent substrate for HRP detection available
Lower Detection Limit*	• Low-picogram (10^{-12}) • Mid-attomole (10^{-17})	• Mid-femtogram (10^{-14}) • High-zeptomole (10^{-19})	• Low-femtogram (10^{-15}) • Mid-zeptomole (10^{-20})
Signal Duration	• 6-8 Hours	• 24 Hours	• 8 Hours
Suggested Antibody Dilutions**	• Primary: 1/1,000-1/5,000 • Secondary: 1/20,000-1/100,000	• Primary: 1/1,000-1/50,000 • Secondary: 1/50,000-1/250,000	• Primary: 1/5,000-1/100,000 • Secondary: 1/100,000-1/500,000
Room Temperature (RT) Working Solution Stability	• 24 Hours	• 24 Hours	• 8 Hours
Stock Solution Shelf Life	• 1 year at RT	• 1 year at RT	• 1 year at 4°C or 6 months at RT

*Lower detection limits were determined using Streptavidin-HRP or Biotinylated-HRP as the ligand.
**Please follow recommended antibody dilutions. SuperSignal® Substrates are much more sensitive than other substrates, so it is critical that you follow these guidelines. Failure to do so could result in unsatisfactory results.

SuperSignal® West Pico Chemiluminescent Substrate

Twice as much signal for about 40% less than the price of the ECL™ System.

In side-by-side comparisons using identical conditions, blots incubated in SuperSignal® West Pico Chemiluminescent Substrate exhibit at least twice the intensity of blots treated with the ECL™ System.

SuperSignal® West Pico Chemiluminescent Substrate costs much less than the ECL™ System.

	SuperSignal® West Pico Substrate (Product # 34080)	**ECL™ Western Blotting Detection Substrate** (Product # RPN2106)
2004 U.S. List Price per cm^2 of membrane	**$0.05**	**$0.07**

More stable

SuperSignal® West Pico Substrate is room temperature (RT)-stable for months, with no discernable loss in activity. RT stability frees up valuable cold-room space and saves time because there is no need to wait for the reagents to warm up.

Long signal

With signal duration of more than six hours, there is adequate time to optimize the exposure conditions. In most cases, there is no need to rerun samples and repeat the blotting procedure.

Highlights:

- Economy – costs less per ml than other chemiluminescent substrates
- Longer light emission – strong light emission over a working day allows you to make several exposures
- High intensity – signal is twice as intense as other compatibly priced luminol-based systems
- Picogram sensitivity – highly sensitive for the rapid development of a wide range of protein levels
- Excellent stability – 24-hour-plus working solution stability; kit is stable for at least one year at ambient temperature
- Saves antibody – primary and secondary antibodies are diluted further so they can be used for more blots

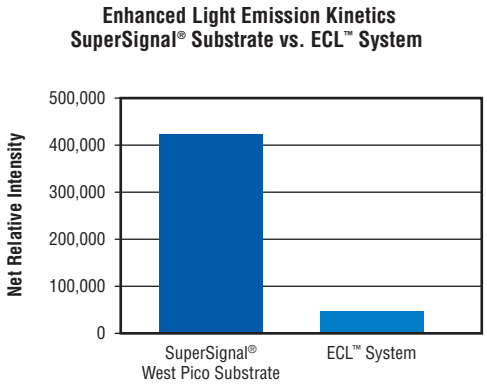


Figure 17. Net relative intensity six hours after incubation.

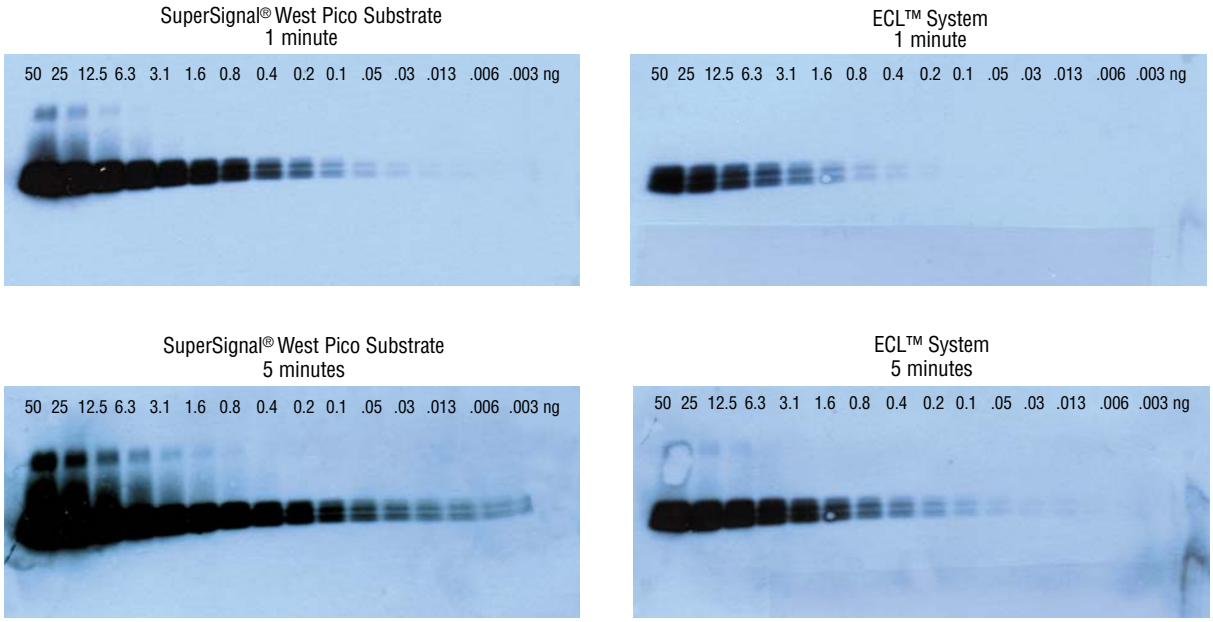


Figure 16. 50 ng of Recombinant Mouse IL-2 was serially diluted down to 0.003 ng and electrophoresis was performed. The gels were transferred to nitrocellulose membranes, blocked and incubated with a 1 µg/ml dilution of rat anti-mouse IL-2. After washing, the membranes were incubated with 20 ng/ml dilutions of HRP-conjugated goat anti-rat antibody. The membranes were washed again and then incubated with substrate that was prepared according to the manufacturer's instructions. Blots were exposed to film for one- and five-minute exposures.

Table 8. A conversion protocol for using SuperSignal® Substrates

Step-by-step Conversion Protocol	ECL™ Substrate	SuperSignal® West Pico Substrate
1. Perform standard electrophoresis and blotting.	Use their Hybond™ Nitrocellulose Membrane.	Use any nitrocellulose membrane.
2. Block the nonspecific sites.	Add blocking reagent, incubate and wash.	Add blocking reagent, incubate and skip the wash!
3. Add diluted primary antibody; incubate for 1 hour, then wash.	Optimization Range: **1/100-1/1,500 dilution**	Optimization Range: **1/1,000-1/5,000 dilution**
4. Add diluted secondary antibody (HRP-labeled); incubate for 1 hour, then wash.	Optimization Range: **1/1,500-1/50,000 dilution**	Optimization Range: **1/20,000 - 1/100,000 dilution**
5. Prepare chemiluminescent substrate.	Mix equal volumes of both solutions.	Mix equal volumes of both solutions.
6. Incubate the substrate on the blot.	Incubate blot with Working Solution without agitation for precisely 1 minute.	Incubate blot with Working Solution with agitation for ~5 minutes.
	It's recommended that you work quickly once ECL™ Working Solution has been added to the membrane.	The signal lasts for hours, so take your time!
7. Expose to film.	Immediately expose to film for 1 minute.	Expose to film for 1 minute.

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Messenger, M.M., *et al.* (2002). *J. Biol. Chem.* **277**, 23054-23064.

PRODUCT #	DESCRIPTION	PKG. SIZE
34080	SuperSignal® West Pico Chemiluminescent Substrate	500 ml
	Includes: Luminol/Enhancer	250 ml
	Stable Peroxide Buffer	250 ml
34077	SuperSignal® West Pico Chemiluminescent Substrate	100 ml
	Includes: Luminol/Enhancer	2 x 25 ml
	Stable Peroxide Buffer	2 x 25 ml
34079	SuperSignal® West Pico Chemiluminescent Substrate Trial Kit	50 ml
	Includes: Luminol/Enhancer	25 ml
	Stable Peroxide Buffer	25 ml

SuperSignal® Western Blotting Kits

For convenience and ease-of-use, nothing beats a complete Western blotting kit!

The Standard Detection Kits provide:

- HRP-conjugated Anti-Rabbit IgG, Anti-Mouse IgG or NeutrAvidin™ Biotin-Binding Protein
- SuperSignal® West Pico Substrate

The Complete Detection Kits provide:

- HRP-conjugated Anti-Rabbit IgG, Anti-Mouse IgG or NeutrAvidin™ Biotin-Binding Protein
- SuperBlock® Blocking Buffer
- TBS Wash Buffer
- SuperSignal® West Pico Substrate

PRODUCT #	DESCRIPTION	PKG. SIZE
34081	SuperSignal® West Pico Complete Mouse IgG Detection Kit	Kit
34082	SuperSignal® West Pico Mouse IgG Detection Kit	Kit
34084	SuperSignal® West Pico Complete Rabbit IgG Detection Kit	Kit
34083	SuperSignal® West Pico Rabbit IgG Detection Kit	Kit
34086	SuperSignal® West Pico Complete Biotinylated Protein Detection Kit	Kit
34085	SuperSignal® West Pico Biotinylated Protein Detection Kit	Kit

SuperSignal® West Dura Extended Duration Substrate

Specially formulated for use with CCD cameras.

Along with advances in cooled CCD technology has come an overall reduction in the cost of imaging instruments. It is increasingly common to see these instruments in today's research labs. Pierce developed SuperSignal® West Dura Extended Duration Substrate to meet the needs of researchers using this efficient new technology. Cooled CCD cameras, which offer the advantages of instant image manipulation, higher sensitivity, greater resolution and a larger dynamic range than film, also eliminate the need for film processing equipment and a darkroom. However, this technology has one drawback – it requires a substrate that produces an intense signal that is strong enough, and of long enough duration, to be captured by the cameras. Pierce developed SuperSignal® West Dura Substrate to meet this need. By combining 24-hour light emission with ultraintensity, SuperSignal® West Dura Substrate allows researchers to take full advantage of all the features offered by imaging instruments.

Because SuperSignal® West Dura Substrate provides the maximum light duration, which allows for multiple extended exposures, an IL-2 blot was repeated in comparison to the acridan-based HRP chemiluminescent substrate (ECL Plus™ Substrate) using the manufacturer's recommended dilutions and membrane. ECL Plus™ Substrate strongly recommends the use of PVDF. The proteins were transferred to PVDF for the ECL Plus™ Substrate and to nitrocellulose for the SuperSignal® System. The primary antibody for both substrates was used at a 1 µg/ml dilution.

A 10 ng/ml dilution was used for the secondary antibody SuperSignal® West Dura Substrate and a 20 ng/ml dilution was used for ECL Plus™ Substrate. A five-minute film exposure showed a high signal-to-noise ratio for the SuperSignal® West Dura System with detection down to 3 pg of IL-2 (Figure 17A). It showed high background for the acridan system and detection down to only 800 pg of IL-2 (Figure 17B). A 30-minute exposure at F1.6 on the CCD camera demonstrated detection down to 12.5 pg of IL-2 with the SuperSignal® Product (Figure 17C). When the ECL Plus™ Blot was exposed to the CCD camera at F1.6, the exposure was stopped at 15 minutes because of the intense background. Signal was difficult to distinguish above background (Figure 17D).

Reference

Tokumaru, H., *et al.* (2001). *Cell* **104**, 421-432.

Highlights:

- 24-hour light emission – 10 times longer than that offered by other enhanced chemiluminescent substrates for HRP; make multiple exposures for publication-quality blots
- Great sensitivity – see bands you've never been able to see before with femtogram-level sensitivity
- Save your antibody – antibodies can be diluted much further when using SuperSignal® West Dura Extended Duration Substrate than with other chemiluminescent substrates; perform 25-50 times more blots than possible with other chemiluminescent substrates
- Comes with HRP-labeled secondary antibodies
- Intense signal that is generated immediately and easily detected on film or chemiluminescent imager systems
- Working solution is stable for at least 24 hours; the kit itself is stable for at least one year with ambient shipping conditions

PRODUCT #	DESCRIPTION	PKG. SIZE
34075	**SuperSignal® West Dura Extended Duration Substrate**	100 ml
	Includes: Luminol/Enhancer	50 ml
	Stable Peroxide Buffer	50 ml
	HRP-Conjugated Goat Anti-Rabbit	1 ml
	HRP-Conjugated Goat Anti-Mouse	1 ml
34076	**SuperSignal® West Dura Extended Duration Substrate**	200 ml
	Includes: Luminol/Enhancer	100 ml
	Stable Peroxide Buffer	100 ml
	HRP-Conjugated Goat Anti-Rabbit	1 ml
	HRP-Conjugated Goat Anti-Mouse	1 ml
37071	**SuperSignal® West Dura Extended Duration Substrate Trial Kit**	20 ml
	Includes: Luminol/Enhancer	10 ml
	Stable Peroxide Buffer	10 ml

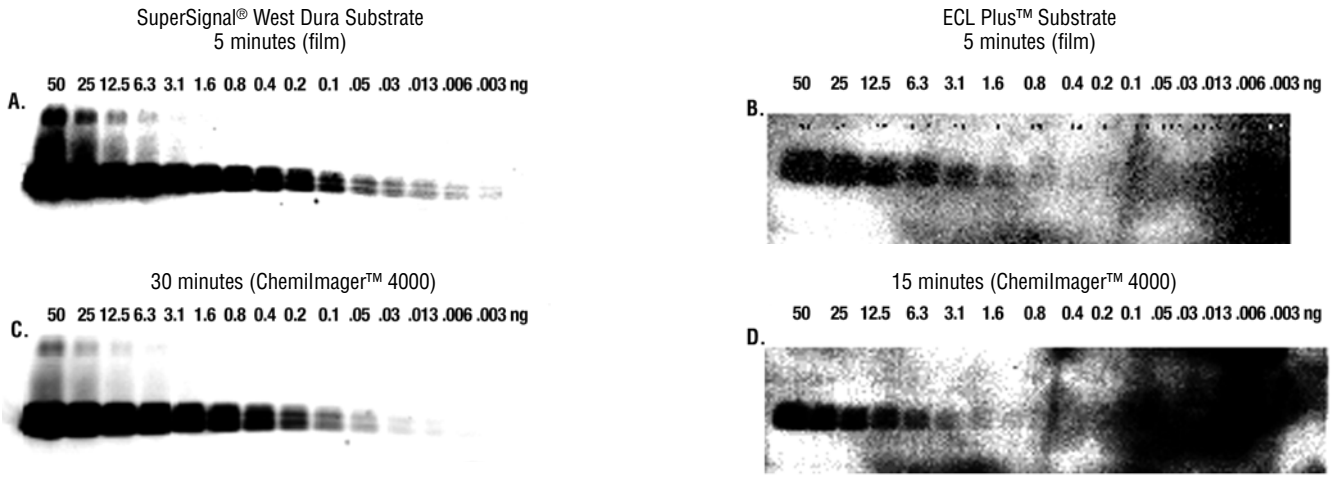

Figure 17. 50 ng of recombinant mouse IL-2 was serially diluted to 0.003 ng and electrophoresis was performed. The gel to be used for SuperSignal® West Dura Substrate was transferred to nitrocellulose membrane and the gel to be used for ECL Plus™ Substrate was transferred to PVDF membrane. The membranes were blocked and then incubated with a 1 µg/ml dilution of the primary antibody, rat anti-mouse IL-2. After washing, the membranes were incubated with the secondary antibody, HRP-conjugated goat anti-rat IgG. The membranes were washed again and then incubated with substrates that were prepared according to the manufacturer's instructions. Each membrane was exposed to X-ray film for 5 minutes. The SuperSignal® West Dura Substrate membrane was exposed to the ChemiImager™ 4000 for 30 minutes and the ECL Plus™ Blot was exposed for 15 minutes. (The exposure of the ECL Plus™ Blot was not extended to 30 minutes due to the high background that had already accumulated at 15 minutes.)

SuperSignal® West Femto Maximum Sensitivity Substrate

SuperSignal® West Femto Maximum Sensitivity Substrate provides the ultimate sensitivity for Western blotting, allowing users to see protein bands that were never visualized before.

Highlights:

- Sensitive – reach low-femtogram detection limits, that's zeptomole-level detection
- Economical – conserve precious primary antibodies with up to 1/100,000 dilutions and secondary antibodies to 1/500,000
- Comes with HRP-labeled secondary antibodies
- Intense – releases the most intense signal generated by chemiluminescent systems, making it easy to capture an image on film or via an imager system

Lower detection limit

- Low-femtogram (10^{-15})
- Mid-zeptomole (10^{-20})

Signal duration

- 8 hours

Suggested antibody dilutions (from 1 mg/ml stock)

- Primary: 1/5,000-1/100,000
- Secondary: 1/100,000-1,500,000

Reagent stability

- 1 year at 4˚C or 6 months at RT

PRODUCT #	DESCRIPTION	PKG. SIZE
34095	SuperSignal® West Femto Maximum Sensitivity Substrate *Sufficient substrate for 800 cm² of blotting membrane.* Includes: Luminol/Enhancer Solution Stable Peroxide Solution HRP-Conjugated Goat Anti-Rabbit HRP-Conjugated Goat Anti-Mouse	100 ml 50 ml 50 ml 1 ml 1 ml
34096	SuperSignal® West Femto Maximum Sensitivity Substrate *Sufficient substrate for 800 cm² of blotting membrane.* Includes: Luminol/Enhancer Solution Stable Peroxide Solution HRP-Conjugated Goat Anti-Rabbit HRP-Conjugated Goat Anti-Mouse	200 ml 100 ml 100 ml 1 ml 1 ml
34094	SuperSignal® West Femto Maximum Sensitivity Substrate Trial Kit Includes: Luminol/Enhancer Solution Stable Peroxide Solution	20 ml 10 ml 10 ml

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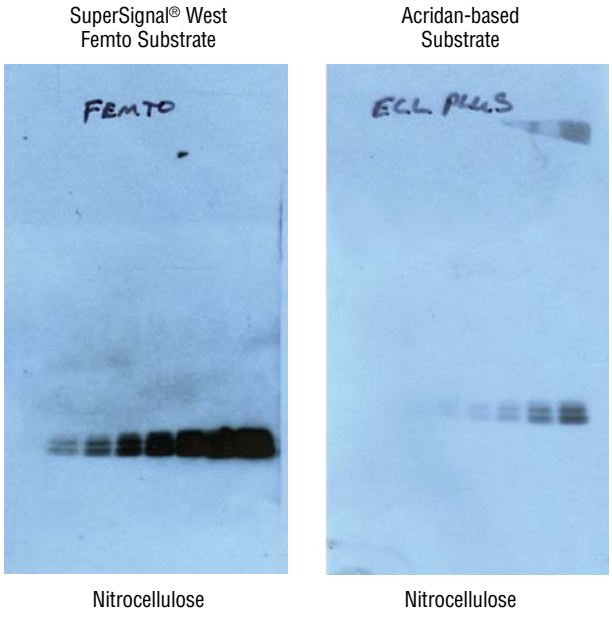

Figure 18. A comparison between SuperSignal® West Femto and ECL Plus™ Substrates. Two-fold serial dilutions of mouse IL-2 from 1,000 to 15.6 pg were detected with both substrates. The primary antibody dilution was 1/2,000 and the secondary antibody dilution was 1/300,000. Both antibodies had a 1 mg/ml starting concentration.

Lumi-Phos™ WB Chemiluminescent Substrate

A chemiluminescent substrate for alkaline phosphatase detection that provides the best of both worlds – high sensitivity and low background.

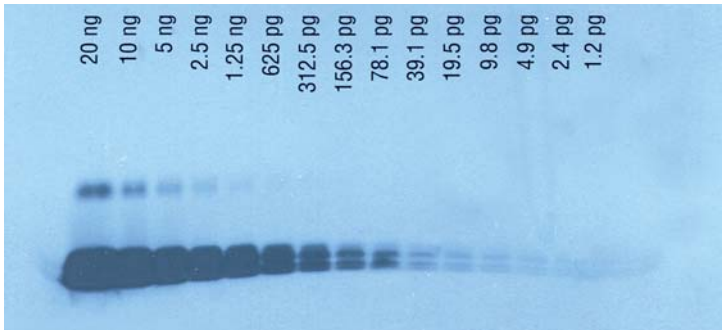

Figure 19. Serial dilutions of recombinant mouse IL-2 were separated electrophoretically on a 4-20% SDS-polyacrylamide gel. The separated protein was then transferred to nitrocellulose membrane followed by blocking. The membranes were subsequently incubated in a 1/500 (1 µg/ml) dilution of purified rat anti-mouse IL-2, followed by a 1/5,000 (200 ng/ml) dilution of AP-labeled goat anti-rat IgG. The membranes were washed and then incubated in Lumi-Phos™ WB Substrate for five minutes prior to film exposure.

Lumi-Phos™ WB Chemiluminescent Substrate overcomes all of the limitations posed by conventional chemiluminescent substrates for AP.

Lumi-Phos™ WB Substrate provides sensitivity in the low picogram range, enabling you to detect mere attomoles of your target ligand. Lumi-Phos™ WB Substrate also produces less background noise than other popular chemiluminescent substrates for AP, providing a better signal:noise ratio and a clearer image. Because signal generation is immediate, there's no need to wait 15 to 30 minutes for a measurable signal. All of these benefits are combined with a low, low price.

Highlights:

- High sensitivity – able to detect 1.2 pg or 71 attomoles of the target ligand mouse IL-2
- Low background – high signal:noise ratios produce clearer blots
- Inexpensive – less expensive than other substrates (based on 2004 U.S. list prices) and there is no need to purchase additional enhancers for nitrocellulose membranes
- Long signal duration – allows you to redevelop blots over and over
- Attomole-range detection and crystal-clear background
- Immediate strong signal – no more waiting 15 to 30 minutes for the signal to become strong enough to detect
- Ready to use – no mixing required with this one-component system

PRODUCT #	DESCRIPTION	PKG. SIZE
34150	Lumi-Phos™ WB Chemiluminescent Substrate	100 ml

References

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Liu, R.Y., *et al.* (2000). *J. Biol. Chem.* **275**, 21086-21093.
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Table 9. Quick reference substrate guide

Substrate	Product #	Measurement / Color	Dilution range of Antibody (From 1 mg/ml stock)	Approximate Sensitivity*	Enzyme
SuperSignal® West Pico Substrate	34080	425 nm chemiluminescent	1° 1:1K-1:5K 2° 1:20K-100K	1 pg	HRP
SuperSignal® West Dura Substrate	34075	425 nm chemiluminescent	1° 1:1K-1:50K 2° 1:50K-250K	250 fg	HRP
SuperSignal® West Femto Substrate	34095	425 nm chemiluminescent	1° 1:5K-1:50K 2° 1:100K-500K	60 fg	HRP
1-Step™ TMB - Blotting Substrate	34018	Dark blue PPT	1° 1:500 2° 1:2K-20K	1 ng	HRP
1-Step™ 4-CN Substrate	34012	Blue-purple PPT	1° 1:500 2° 1:2K-20K	1 ng	HRP
CN/DAB Substrate	34000	Black PPT	1° 1:500 2° 1:2K-20K	1 ng	HRP
DAB Substrate	34001	Brown PPT	1° 1:500 2° 1:2K-20K	1 ng	HRP
Metal Enhanced DAB Substrate	34065	Brown-black PPT	1° 1:500 2° 1:2K-20K	20 pg	HRP
Lumi-Phos™ Substrate	34150	440 nm chemiluminescent	1° 1:5K 2° 1:25K	15 pg	AP
1-Step™ NBT/BCIP Substrate	34042	Black-purple PPT	1° 1:500 2° 1:2.5K	30 pg	AP
1-Step™ NBT/BCIP + Suppressor Substrate	34070	Black-Purple PPT	1° 1:500 2° 1:2.5K	30 pg	AP
NBT Substrate	34035	Blue-purple PPT	1° 1:250 2° 1:2.5K	100 pg	AP
BCIP Substrate	34040	Blue-purple PPT	1° 1:250 2° 1:2.5K	100 pg	AP
Fast Red Substrate	34034	Red PPT	1° 1:250 2° 1:2.5K	320 pg	AP

* Actual sensitivity is unique to each antibody-antigen pair. The approximate sensitivities listed are conservative amounts that should be easily detectable for most antigens.
1°= Primary, 2°= Secondary, PPT = precipitate, HRP = horseradish peroxidase, AP = alkaline phosphatase

Data Imaging

There are several methods for capturing data generated from chemiluminescent Western blots including X-ray film, cooled CCD cameras and phosphorimagers that detect chemiluminescence. Cooled CCD cameras, which offer the advantages of instant image manipulation, higher sensitivity, greater resolution and a larger dynamic range than film, also eliminate the need for a darkroom and film processing equipment. However, this technology has one drawback – it requires a substrate that produces an intense signal of long enough duration to be captured by the camera. To meet the needs required by this new technology, Pierce introduced SuperSignal® West Dura Extended Duration Substrate (Product # 34075). Its 24-hour light emission and ultra-intense signal combine to allow researchers to take full advantage of the benefits offered by digital imaging equipment. Most instrument companies know and recommend SuperSignal® West Substrates over other chemiluminescent substrates for use in their instruments. The high signal intensity and long signal duration make them ideal – and sometimes essential – for use in these instruments.

Although electronic data capture with digital cameras and imagers is growing in popularity as the technologies improve and equipment prices decline, most of the data obtained from Western blotting with chemiluminescence is still captured on film. Often, it is necessary to expose several films for different time periods to obtain the proper balance between signal and background. The goal is to time the exposure of the membranes to the film so that the desired signal is clearly visible while the background remains low. This is difficult to accomplish because the process cannot be observed and stopped when the desired endpoint is reached. If the film is not exposed long enough (underexposed), the signal will not be visible. If the film is exposed too long (overexposed), the signal may be lost in the background or separate bands may become blurred together. An overexposed film can be "fixed" by incubating it in Erase-It® Background Eliminator Solution (Product # 21065), which effectively decreases the initial exposure time without altering the integrity of the data. This is done at the lab bench while watching the film and the process can be halted when the signal is clearly visible and background is at a minimum. For more information on this method, see page 44.

Most instrument companies know and recommend SuperSignal® West Substrates over other chemiluminescent substrates for use in their instruments.

Troubleshooting tips for chemiluminescence and cooled CCD cameras

- SuperSignal® West Dura and SuperSignal® West Femto Substrates are the recommended substrates for use in imaging instruments.
- SuperSignal® West Pico Substrate will work in imaging instruments, but sensitivity may not be as good as that which is obtained with film.
- Imagers sometimes require longer exposure times than required by film to obtain similar images.
- Background is less of an issue in many of these instruments; therefore, higher antibody concentrations may be used to achieve the best image in the shortest exposure time.
- No darkroom is necessary when using imaging instruments. The instruments have their own light-proof boxes.
- Refer to the instrument manufacturer's instructions for more information on an individual instrument.

Featured Product

CL-XPosure™ Film

Save 60-75% on film!

Pierce CL-XPosure™ Film | Kodak BioMax™ MR-1 Film | Kodak X-Omat™ Blue (XB) Film

Figure 20. CL-XPosure™ Film vs. Kodak Film. Three types of X-ray film were tested using identical Western blotting conditions (2 blue, 1 grey). The results showed no appreciable difference between any of these films. The only significant difference is the cost-per-sheet of film as indicated in the table below.

Cost Comparison of 5" x 7" Sheets

Product	Cost-per-sheet (U.S. Price)
Pierce CL-XPosure™ Film (Blue X-ray Film)	$0.79
Kodak X-Omat™ Blue (XB) Film (Blue X-ray Film) (Perkin Elmer)	$2.10
Kodak BioMax™ MR-1 (Gray X-ray Film) (Amersham)	$3.36

Source: 2004 Online Catalogs

Highlights:

- Up to one-third the price of competitive products
- Provides the same detection sensitivity as other commercially available films
- Available in either 5" x 7" or 8" x 10" sheets, in packages of 25, 50 or 100 non-interleaved sheets

Reference

Tikhonov, I., *et al.* (2003). *J. Virol.* **77**, 3157-3166.

PRODUCT #	DESCRIPTION	PKG. SIZE
34090	CL-XPosure™ Film (5" x 7" sheets)	100 sheets
34091	CL-XPosure™ Film (8" x 10" sheets)	100 sheets
34092	CL-XPosure™ Film (5" x 7" sheets)	25 sheets
34093	CL-XPosure™ Film (8" x 10" sheets)	50 sheets

SuperSignal® West Pico HisProbe™ Kit

Specific detection of histidine-tagged fusion proteins.

This chemiluminescent system uses HisProbe™-HRP chemistry to overcome the limitations of anti-histidine antibodies and other detection strategies. HisProbe™-HRP is more specific for polyhistidine tags, reducing background problems. Unlike anti-His antibodies, HisProbe™-HRP can recognize polyhistidine tags independent of adjacent tags.

Highlights:

- Specific – more specific for the detection of histidine-tagged fusion proteins than anti-His antibodies
- Fast – one-step probe incubation eliminates the need to run a lengthy two-step primary/secondary antibody sequential reaction protocol
- Sensitive – when used in combination with SuperSignal® West Chemiluminescent Substrates, kit allows the detection of even low-expression histidine-tagged clones
- More versatile than anti-polyHis antibody-based systems; the HisProbe™ Kit detects poly-histidine fusion proteins that are undetectable using some monoclonal anti-polyHis antibodies
- Sufficient reagents for fifty 7.5 x 10 cm blots

Comparison of Polyhistidine-tagged (PHT) Fusion Protein Detection Methods

A. B.

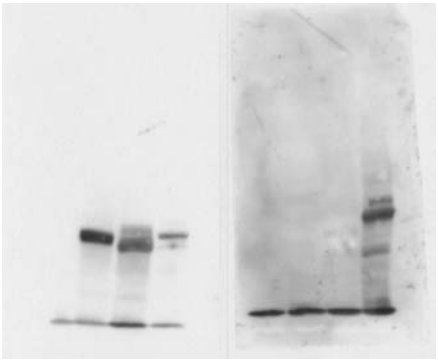

Figure 21. Panel A using HisProbe™-HRP shows high specific binding and low background. Panel B using anti-polyHis failed to recognize two of the three fusion proteins.

PRODUCT #	DESCRIPTION	PKG. SIZE
15165	HisProbe™-HRP	2 mg
15168	SuperSignal® West Pico HisProbe™ Kit	Kit
	Includes: HisProbe™-HRP	2 mg
	SuperSignal® West Pico Chemiluminescent Substrate	500 ml
	Blocker™ BSA in TBS (10X)	1 x 125 ml
	BupH™ Tris Buffered Saline Packs	10 x 500 ml
	Surfact-Amps® 20 (10%)	6 x 10 ml ampules
15166	PhosphoProbe™-HRP	2 mg
23031	Ethylenediamine Dihydrochloride	10 g
22980	EDC	5 g
22981	EDC	25 g
15167	PhosphoProbe™ Phosphorylated Protein Detection Kit	Kit
	Includes: PhosphoProbe™-HRP	2 mg
	EDC	5 g
	Ethylenediamine	10 g
	Tween®-20	1 vial

References

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Kanaya, E., *et al.* (2001). *J. Biol. Chem.* **276**, 7383-7390.

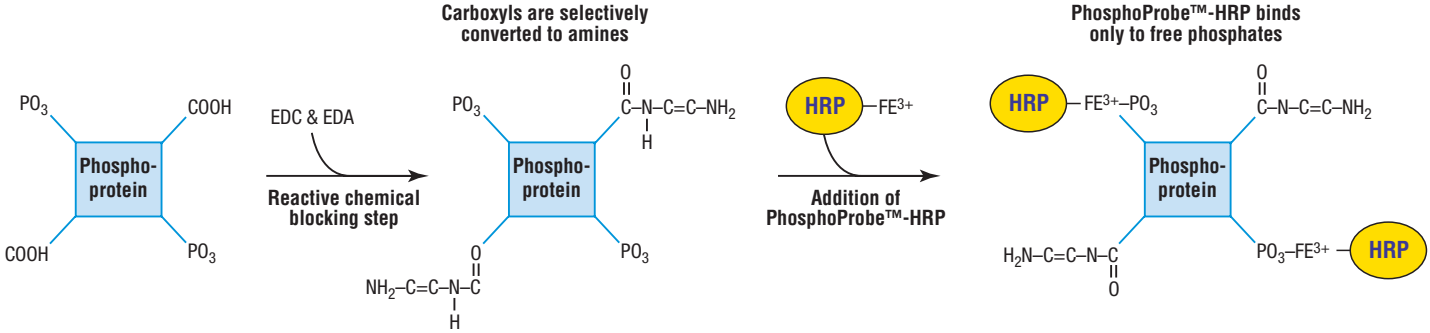
Kiick, K.L., *et al.* (2002). *PNAS* **99**, 19-24.

Sylvester, S.R. and Roy, A. (2002). *Biol. Reprod.* **67**, 895-899.

Phosphoprotein Detection Reagent and Kit

Novel chemistry enables specific detection of phosphorylated protein.

PhosphoProbe™-HRP is an iron (Fe^{3+})-activated derivative of horseradish peroxidase (HRP). PhosphoProbe™-HRP exhibits two distinct binding specificities, one of which is phosphate ($R\text{-}PO_3$)-specific. The other binding specificity is related to a carboxyl-containing binding motif that is common to most proteins and some peptides. This carboxyl motif binding specificity can be used in a total protein detection application. A novel treatment, reactive chemical blocking (RCB), may be used to eliminate this carboxyl-binding motif, thus imparting exclusive specificity toward phosphate groups. PhosphoProbe™-HRP, in conjunction with RCB, is a universal phosphate detection probe. PhosphoProbe™-HRP has been optimized for direct detection of phosphoester molecules such as nucleotides or protein/peptides containing phosphoserine, phosphothreonine and phosphotyrosine.



O-GlcNAc Western Blot Detection Kit

High specificity monoclonal against O-GlcNAc

The Pierce *O*-GlcNAc Western Blot Detection Kit contains the most highly specific mouse monoclonal antibody available for the detection of the *O*-GlcNAc posttranslational modification on a membrane. Reaction of the monoclonal antibody in this Western blotting kit is confined to the β-*O*-linked serine or threonine GlcNAc modification. There is no cross-reactivity with the α-*O*-GlcNAc linkage, the α/β-*O*-GalNAc modification or the other *N*-linked oligosaccharides.

Speed and sensitivity of chemiluminescent detection

Chemiluminescent detection with Pierce SuperSignal® West Dura Extended Duration Substrate allows visualization of *O*-GlcNAc-modified proteins in less than one minute after exposure of the blot to X-ray film. In addition to speed, this kit is sensitive to the low picomole range.

Performance validated on Jurkat cell lysates

This Western blot kit also includes our popular M-PER® Mammalian Cell Lysis Reagent; an HRP-labeled, anti-IgM antibody conjugate; blocking buffer; and wash buffer components all validated to perform as specified.

Highlights:

Kit includes MAb CTD 110.6, the most specific monoclonal antibody for the detection of β-*O*-linked *N*-acetylglucosamine (*O*-GlcNAc)

- Detect only the β-*O*-GlcNAc modification on the proteins within the sample
- Detection of the target modification confined to only β-*O*-linked serine or threonine
- No cross-reactivity with the α-*O*-GlcNAc linkage

Kit includes M-PER® Mammalian Protein Extraction Reagent

- Convenient, efficient eukaryotic cell lysis

Kit includes super-sensitive, patented SuperSignal® West Dura Chemiluminescent Substrate

- Provides maximum sensitivity from the antigen-primary-secondary-HRP complex formed at the site of a β-*O*-GlcNAc modification
- Sensitivity to low picomole level

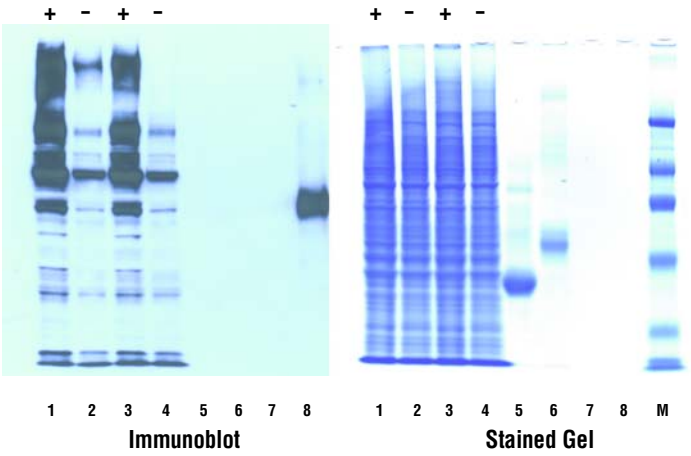
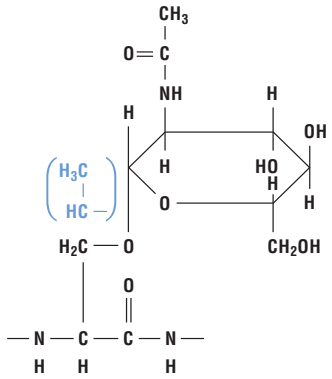


Figure 22. Western blot detection of *O*-GlcNAc-modified proteins after SDS-PAGE. Lanes 1-4 are proteins from the Jurkat cell extract. Lanes 5, 6 and 7 are the negative controls: ovalbumin (5 µg), fetuin (5 µg) and *O*-β-GalNAc-modified BSA (10 ng). Lane 8 is *O*-β-GlcNAc-modified BSA (5 ng, positive control). The (+) and (-) refer to plus and minus treatment with PUGNAc and glucosamine, and M represents the molecular weight marker (BlueRanger® Prestained Protein Molecular Weight Marker Mix, Product # 26681).



β-O-GlcNAc Modified Serine/ Threonine in Peptide Linkage

PRODUCT #	DESCRIPTION	PKG. SIZE
24565	***O*-GlcNAc Western Blot Detection Kit**	Kit
	Sufficient material to develop up to 10 mini-blots	
	Includes: M-PER® Mammalian Protein Extraction Reagent Dilution	25 ml
	Buffer (10X) – Blocking Buffer	2 x 50 ml
	BupH™ Phosphate Buffered Saline	17 packs
	Surfact-Amps™ 20 (10% Tween®-20 solution)	3 x 10 ml
	Anti *O*-GlcNAc Monoclonal Antibody (MAb CTD 110.6) in ascites	100 µl
	Goat anti-Mouse IgM(µ), HRP Conjugate	75 µg
	SuperSignal® West Dura Extended Duration Substrate	100 ml

Note: This Western blot kit is shipped in a single box as a two-part kit. **Part A** contains some components that require storage at 4˚C upon arrival. **Part B** contains only the *O*-GlcNAc-specific monoclonal antibody. This MAb is shipped on dry ice to ensure it maintains integrity during transit. Upon its arrival, store it at -20˚C.

Studying Protein Interactions by Far-Western Blotting

Far-Western blotting was originally developed to screen protein expression libraries with ^{32}P-labeled glutathione S-transferase (GST)-fusion protein. Far-Western blotting is now used to identify protein:protein interactions. In recent years, far-Western blotting has been used to determine receptor:ligand interactions and to screen libraries for interacting proteins. It is possible to study the effect of post-translational modifications on protein:protein interactions, examine interaction sequences using synthetic peptides as probes and identify protein:protein interactions without using antigen-specific antibodies with this method of analysis.

Far-Western blotting vs. Western blotting

The far-Western blotting technique is quite similar to Western blotting. In a Western blot, an antibody is used to detect the corresponding antigen on a membrane. In a classical far-Western analysis, a labeled or antibody-detectable "bait" protein is used to probe and detect the target "prey" protein on the membrane. The sample (usually a lysate) containing the unknown prey protein is separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) or native PAGE and then transferred to a membrane. When attached to the surface of the membrane, the prey protein becomes accessible to probing. After transfer, the membrane is blocked and then probed with a known bait protein, which usually is applied in pure form. Following reaction of the bait protein with the prey protein, a detection system specific for the bait protein is used to identify the corresponding band (Table 10).

Specialized Far-Western Analysis

By creative design of bait protein variants and other controls, the far-Western blotting method can be adapted to yield very specific information about protein:protein interactions. For example, Burgess, *et al.* used a modified far-Western blotting approach to determine sites of contact among subunits of a multi-subunit complex. By an "ordered fragment ladder" far-Western analysis, they were able to identify the interaction domains of *E. coli* RNA polymerase β´ subunit. The protein was expressed as a polyhistidine-tagged fusion, then partially cleaved and purified using a Ni^{2+}-chelate affinity column. The polyhistidine-tagged fragments were separated by SDS-PAGE and transferred to a nitrocellulose membrane. The fragment-localized interaction domain was identified using a ^{32}P-labeled protein probe.

Importance of Native Prey Protein Structure in Far-Western Analysis

Far-Western blotting procedures must be performed with care and attention to preserving as much as possible the native conformation and interaction conditions for the proteins under study. Denatured proteins may not be able to interact, resulting in a failure to identify an interaction. Alternatively, proteins presented in non-native conformations may interact in novel, artificial ways, resulting in "false-positive" interactions. The prey protein in particular is subjected to preparative processing steps for far-Western blotting that can have significant effects on detection of protein:protein interactions. This is not to imply that identification of valid interactions is not possible, but only to stress the importance of appropriate validation and use of controls.

Table 10. Comparison of Western blotting and far-Western blotting methods

Step	Western Blotting	Far-Western Analysis
Gel Electrophoresis	Native or denaturing (usually)	Native (usually) or denaturing
Transfer System	Optimal membrane and transfer system determined empirically	Optimal membrane and transfer system determined empirically
Blocking Buffer	Optimal blocking system determined empirically	Optimal blocking system determined empirically
Detection (several possible strategies)* [Arrows designate sequence of steps in the detection strategy]	Unlabeled primary antibody→ Enzyme-labeled secondary antibody→ Substrate reagent	Unlabeled bait protein→ Enzyme-labeled bait-specific antibody→ Substrate reagent
	Enzyme-labeled primary antibody→ Substrate reagent	Radiolabeled bait protein→ Exposure to film
	Biotinylated antibody→ Enzyme-labeled streptavidin→ Substrate reagent	Biotinylated bait protein→ Enzyme-labeled streptavidin→ Substrate reagent
		Fusion-tagged bait protein→ Tag-specific antibody→ Enzyme-labeled secondary antibody→ Substrate reagent

* Labeled antibodies generally are enzyme-labeled (either horseradish peroxidase or alkaline phosphatase). By contrast, bait proteins generally are not enzyme-labeled because a large enzyme label is likely to sterically hinder unknown binding sites between bait and prey proteins. Other labeling and detection schemes are possible.

Critical Steps in Far-Western Analysis

Gel Electrophoresis

Separation of proteins by SDS-PAGE (i.e., denaturing conditions with or without a reducing agent) offers more information about molecular weight, presence of disulfides and subunit composition of a prey protein, but may render the prey protein unrecognizable by the bait protein. In these cases, the proteins may need to be subjected to electrophoresis under native conditions; i.e., nondenaturing and without reducing agent.

Transfer to Membrane

After separation on the gel, proteins are electrophoretically transferred from the gel to a membrane in two to 16 hours. The type of membrane (e.g., nitrocellulose or PVDF) used for the transfer of proteins is critical, as some proteins bind selectively or preferably to a particular membrane. The efficiency and rate of protein transfer is inversely proportional to the molecular weight of the protein. In some cases, transfer conditions alter the shape of the protein and destroy or sterically hinder the interaction site on the protein. For Far-Western analysis, it is essential that at least the interaction domain of the prey protein is not disrupted by the transfer or is able to re-fold on the membrane to form a three-dimensional (3-D) structure comprising an intact interaction site. Generally, a significant percentage of the protein population renatures upon removal of SDS. When SDS is eliminated during the transfer process, transferred proteins generally renature with greater efficiency and are, therefore, more easily detected by far-Western blotting. In the event that the protein is unable to re-fold to create an intact binding site, it may be necessary to add a denaturation/renaturation step to the procedure or to perform the protein:protein interaction in-gel without transfer. (See In-Gel Far-Western Detection section that follows.) Denaturation/renaturation is typically accomplished using guanidinium hydrochloride.

Blocking Buffer

After transferring proteins to the membrane, Western blotting procedures require that unreacted binding sites on the membrane be blocked with a non-relevant protein solution. In addition to blocking all remaining binding sites on the membrane, a blocking buffer reduces nonspecific binding and aids in protein renaturation during the probing procedure. A variety of different protein blockers may be used, and no one blocking protein solution will work for all blotting experiments. Any given protein blocker may cross-react or otherwise disrupt the specific probing interaction being studied. Determination of an effective blocking buffer must be made empirically. Often, bovine serum albumin (BSA) is used as a starting point for many membrane-probing reactions. Insufficient blocking may result in high background, whereas prolonged blocking could result in a weak or masked signal. Renaturation of the protein also appears to occur during the blocking step so it is important to optimize the blocking conditions to obtain the best signal-to-noise ratio for each application and then not deviate from the method.

Binding and Wash Conditions

Protein:protein interactions vary, depending on the nature of the interacting proteins. The strength of the interactions may depend on the pH, salt concentrations and the presence of certain co-factors during incubation with the bait protein. Some protein:protein interactions may also require the presence of additional proteins. Whatever the necessary conditions, they will need to be maintained throughout the procedure to maintain the interaction until it can be detected. This may influence the formulation of washing buffer used between probing steps.

Controls

When identifying protein:protein interactions by the far-Western technique, it is important to always include appropriate controls to distinguish true protein:protein interaction bands from nonspecific artifactual ones. For example, experiments involving detection with recombinant GST fusion proteins should be replicated with GST alone. A bait protein with a mutation in the predicted interaction domain can be processed as a control to determine specificity of the protein:protein interaction.

A non-relevant protein can be processed alongside the prey protein sample to act as a negative control. Ideally, the control protein would be of similar size and charge to the protein under investigation and would not interact nonspecifically with the bait protein.

In approaches that use a secondary system for detection of the prey protein, such as enzyme-labeled streptavidin with a biotinylated bait protein, it is important to include a duplicate control membrane that is probed only with the labeled streptavidin. This would reveal any bands resulting from endogenous biotin in the sample or nonspecific binding of the labeled streptavidin. When a fusion tag is used with a corresponding antibody, it is critical to probe one of the control membranes with the labeled antibody alone. This control helps to confirm that the relevant band is not due to nonspecific binding of the labeled secondary antibody. To obtain meaningful results, appropriate test and control experiments should be subjected to gel electrophoresis, transfer and probing in parallel.

In-Gel Far-Western Detection

Advantages of In-Gel Detection

Because of restrictions associated with the transfer process, blocking and the possibility of non-specific binding of bait proteins to unrelated bands on the membranes, it is sometimes advantageous to perform far-Western detection within the gel. In this procedure, the gels are pre-treated with 50% isopropyl alcohol and water to remove SDS from the gel and to allow the prey protein to renature. The gel is then incubated with the bait protein. The bait protein is then detected with an HRP-tagged antibody or biotin-binding protein.

The same controls and experimental conditions necessary for optimization of membrane-based far-Westerns apply to in-gel detection. With in-gel detection, the blocking step can be eliminated but the "bait" protein and the labeled detection protein must be diluted in the blocking buffer to reduce nonspecific binding. Also, higher amounts of prey and bait proteins are often required for detection compared to membrane detection with the equivalent chemiluminescent substrate.

ProFound™ Far-Western Protein:Protein Interaction Kits

Pierce provides two kits for far-Western analysis. These kits are optimized for detection both on-membrane or in-gel. One kit allows the detection of biotinylated bait proteins (Product # 23500) and the other allows for the detection of GST-tagged bait proteins (Product # 23505). Both kits include blocking and wash buffers, HRP-labeled detection protein (Streptavidin-HRP or Anti-GST-HRP), and an extremely sensitive formulation of UnBlot® Chemiluminescent Substrate optimized for both on-membrane and in-gel use.

References

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Featured Product

ProFound™ Far-Western Protein:Protein Interaction Kits

Highlights:

- On-membrane or in-gel detection options – on-membrane detection offers greater sensitivity; in-gel detection method offers speed and prevents problems associated with incomplete or inefficient transfer
- Nonradioactive alternative for far-Western analysis – reliable and sensitive biotin/streptavidin-HRP or anti-GST-HRP chemistry combined with chemiluminescent detection offers a practical and safe alternative to radiolabeling the bait protein
- Useful interaction range – kit targets moderate to strong associations between a prey and the biotinylated bait protein or GST-tagged probe protein
- Primary antibody-free detection – kit uses a biotinylated or GST-tagged protein as the probe, eliminating the need for antibody production
- Compatible with both SDS-PAGE and native gels – provides option to probe for prey proteins in a more native environment because reduced or denaturing systems may not always present an interface that promotes the intended interaction
- Reduced nonspecific binding – biotin/streptavidin-HRP systems demonstrate less nonspecific binding compared to antibodies directed against the bait protein; the anti-GST antibody conjugate is highly specific for the GST tag
- Compatible with protein staining – can be used for total protein staining after the chemiluminescent detection step, eliminating the need to run two gels

PRODUCT #	DESCRIPTION	PKG. SIZE
23500	**ProFound™ Far-Western Biotinylated-Protein:Protein Interaction Kit**	10 mini gels
	Materials and methods for the discovery, in-gel or on-membrane, of protein interactions using a biotinylated bait protein as the probe.	
	Includes: Streptavidin-HRP	0.1 mg
	Dilution Buffer (10X)	50 ml
	BupH™ Phosphate Buffered Saline	17 packs
	10% Tween®-20	6 x 10 ml ampules
	UnBlot® Stable Peroxide	55 ml
	UnBlot® Luminol Enhancer	55 ml
	Cellophane Exposure Sheets	10 pack
23505	**ProFound™ Far-Western GST-Protein:Protein Interaction Kit**	10 mini gels
	Materials and methods for the discovery, in-gel or on-membrane, of protein interactions using a GST-tagged bait protein as the probe.	
	Includes: Anti-Glutathione S-Transferase (GST)-HRP	0.25 mg
	Dilution Buffer (10X)	50 ml
	BupH™ Phosphate Buffered Saline	17 packs
	10% Tween®-20	6 x 10 ml ampules
	UnBlot® Stable Peroxide	55 ml
	UnBlot® Luminol Enhancer	55 ml
	Cellophane Exposure Sheets	10 pack

Detection of Difficult-to-transfer Proteins

The major reason that proteins are blotted or adsorbed onto a membrane for detection with an antibody is that the proteins on a membrane are more accessible to immunochemical reagents (antibodies, etc.) than are proteins within polyacrylamide gels. A recent advance in the field of Western blotting involves immunodetection of proteins directly in the gel. This technique, Pierce UnBlot® In-Gel Detection, circumvents the transfer and blocking steps entirely, allowing immunoblotting techniques to be applied to proteins that cannot be transferred efficiently from a gel to a membrane. Because there is no transfer of proteins from gel to membrane, no protein is lost in the process and no artifacts are introduced into the data. This makes UnBlot® Detection an ideal control experiment to confirm results obtained by Western blotting and to study proteins that cannot be transferred to a membrane.

Another feature of the UnBlot® System is that it does not require any blocking step. If there is no blocking, then there is no chance of cross-reactivity with the blocking buffer. This saves time because no blocking buffer optimization is necessary and background is often lower than with traditional Western blotting.

Protein left in a gel after transfer to a nitrocellulose membrane

Figure 23. Pure GFP/6xHis-tagged protein and *E. coli* bacterial GFP/6xHis-tagged lysate were separated by SDS-PAGE (Novex® 10-20% Tris-Glycine gels). Gels were transferred to nitrocellulose membrane using the Bio-Rad® Mini Gel Transfer Unit. Following the transfer, the protein left in the gel was detected using the UnBlot® System with a 1:500 dilution of anti-Penta His antibody followed by a 1:250 dilution of HRP-labeled goat anti-mouse antibody. **Lanes 1-5:** *E. coli* bacterial GFP/6xHis-tagged lysate diluted 1:100, 1:250, 1:1,000, 1:2,000 and 1:4,000, respectively. **Lanes 6-13:** pure GFP/6xHis-tagged protein at 12.5, 6.25, 3.12, 1.56, 1.0, 0.5, 0.1 and 0.05 ng, respectively. **Lane 14:** 6xHis-tagged ladder (1:16 dilution).

UnBlot® In-Gel Chemiluminescent Detection

Highlights

- Uniform representation of antigen(s) not skewed by inefficient transfer
- Compatible with stripping and reprobing protocols
- Compatible with protein staining
- Sensitive to 1 ng – comparable to ECL™ Substrate

Benefits

- Many proteins, such as membrane proteins, do not transfer well to membranes; the use of UnBlot® Technology prevents any problems associated with incomplete transfer
- When performing transfers, low molecular weight (MW) proteins transfer more efficiently than higher MW proteins, often skewing results
- Transfer units, buffers, membranes and filter paper are eliminated
- Procedure can be optimized by stripping and reprobing without running another gel
- After immunodetection, the gel can be used for total protein staining; there's no need to run two gels
- The blocking step is omitted because the antibodies bind only specific antigens on the gel

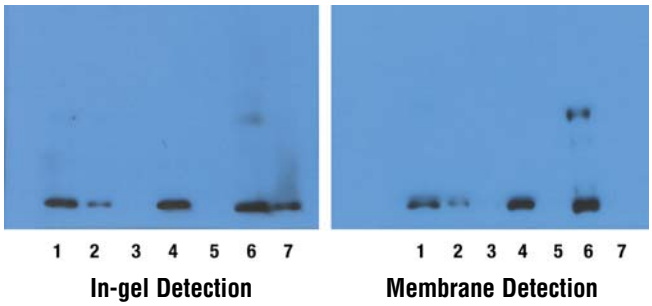

Figure 24. High sensitivity using UnBlot® Detection. Pure GFP 6xHis-tagged and yeast GFP extract were separated by SDS-PAGE. One gel was transferred to nitrocellulose membrane. After transfer, the membrane was blocked overnight in 1% BSA. The other gel was pre-treated with 50% isopropanol. Antigens were detected using a 1:1,000 dilution of polyclonal Anti-Living Color Peptide Antibody, Rabbit (Clontech) and the UnBlot® In-Gel Chemiluminescent Detection Kit – Rabbit (Product # 33500). Signal was detected using UnBlot® Substrate. The lanes on the gel and in the membrane are as follows: Lanes 1, 2 and 3 correspond to 10, 5 and 1 ng pure GFP/6xHis-tagged, respectively. Lanes 4 and 5 correspond to *E. coli* bacterial GFP lysate diluted 1:100 and 1:1,000, respectively. Lanes 6 and 7 correspond to yeast GFP Lysate diluted 1:10 and 1:100, respectively.

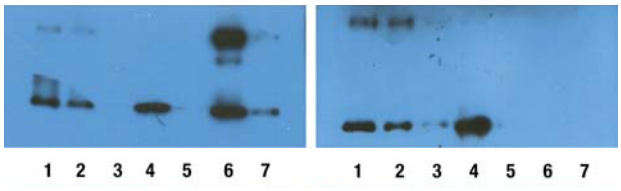

Figure 25. Versatility and specificity of the UnBlot® System. *E. coli* bacterial GFP/6xHis-tagged lysate. Pure GFP/6xHis-tagged and yeast GFP extract were separated by SDS-PAGE. Both gels were pre-treated with 50% isopropanol. The antigens were detected using a 1:1,000 dilution of GFP Monoclonal, Mouse (Gel #1) or of a 1: 500 dilution of anti-Penta his, mouse antibody (Gel #2) and the UnBlot® In-Gel Chemiluminescent Detection Kit – Rabbit (Product # 33500). Signal was detected using UnBlot® Substrate. Lanes 1, 2 and 3 correspond to 10, 5 and 1 ng pure GFP/6xHis-tagged, respectively. Lanes 4 and 5 correspond to *E. coli* bacterial GFP lysate diluted 1:100 and 1:1,000, respectively. Lanes 6 and 7 correspond to yeast GFP lysate 1:10 and 1:100, respectively.

NOTE: The UnBlot® In-Gel Chemiluminescent Detection Kit has been tested successfully with Novex®, FMC-BioWhittaker and Bio-Rad Criterion™ brand gels.

- The UnBlot® In-Gel Chemiluminescent Detection Kit does not perform well with Bio-Rad Ready Gels, Precise™ Protein Gels or Gradipore iGels. Studies showed 25 times lower sensitivity and require individual optimization.
- The recommended gel thickness for use with this kit is 0.75-1.5 mm.
- The recommended cross-linking of gel is 8-18%, 4-20% and 10-20% gradient.

When using UnBlot® Technology with homemade gels, the glass plates must be siliconized prior to pouring the gel. Please visit the Pierce web site to review the protocol and see other tips on optimizing UnBlot® Technology.

PRODUCT #	DESCRIPTION	PKG. SIZE
33500	**UnBlot® In-Gel Chemiluminescent Detection Kit – Rabbit** *Sufficient reagents to perform 10 mini-gel detections.* Includes: UnBlot® Substrate Stabilized Goat anti-Rabbit-HRP Dilution Buffer BupH™ Pack PBS Buffer Tween®-20 Hands-Off™ Incubation Colander Pre-cut Cellophane CL-XPosure™ Film (5" x 7")	Kit 110 ml 10 µl 50 ml 17 packs 5 x 10 ml 1 unit 10 sheets 25 sheets
33505	**UnBlot® In-Gel Chemiluminescent Detection Kit – Mouse** Includes same components as Product # 33500 except it contains Goat anti-Mouse-HRP instead of Goat anti-Rabbit-HRP	Kit 10 µl
33510	**UnBlot® In-Gel Chemiluminescent Detection Kit for Biotinylated Antibody Probes** Includes: Streptavidin-HRP Dilution Buffer Phosphate Buffered Saline 10% Tween®-20 UnBlot® Substrate Cellophane Exposure Sheets	Kit 0.1 mg 50 ml 17 packs 6 x 10 ml 110 ml 10 pack
33515	**UnBlot® In-Gel Chemiluminescent Detection Kit for GST-Tagged Proteins** Includes: Anti-Glutathione S-transferase (GST)-HRP Dilution Buffer Phosphate Buffered Saline 10% Tween®-20 UnBlot® Substrate Cellophane Exposure Sheets	Kit 0.25 mg 50 ml 17 packs 6 x 10 ml 110 ml 10 pack
33550	**UnBlot® Chemiluminescent Substrate**	110 ml
33499	**Hands-Off™ Incubation Colander**	1 unit

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Desai, S., Dworecki, B. and Cichon, E. (2001). *Anal. Biochem.* **297**, 94-98.

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Optimizing the Signal-to-Noise Ratio

Signal-to-noise ratio (S/N ratio) refers to how much relevant content (signal) something has as opposed to non-relevant content (noise). The term is from radio, but is often applied to Western blotting. In Western blotting, the signal is the density of the specific protein band being probed for; the noise is the density of the background. Optimizing the S/N ratio is often more important than increasing the sensitivity of the system. The sensitivity of the system is irrelevant if the signal cannot be distinguished from the noise.

The General Troubleshooting Guide in the next section contains many tips on optimizing the S/N, including a method of increasing the signal and lowering the background by optimizing antibody concentration. This process is made much easier by stripping and reprobing the membrane instead of starting from the beginning.

Stripping and Reprobing a Membrane

One of the major advantages offered by chemiluminescent detection is the ability to strip reagents from a blot and then reprobe the same blot. This is possible with chemiluminescence because all of the reagents can be removed from the membrane because the product detected is light rather than a colored precipitate on the membrane. A blot may be stripped and reprobed several times to visualize other proteins or to optimize detection of a protein (i.e., antibody concentrations) without the need for multiple gels and transfers. The key to this process is to use conditions that cause the release of antibody from the antigen without causing a significant amount of antigen to be released from the membrane. Various protocols have been proposed to accomplish this purpose and they generally include some combination of detergent, reducing agent, heat and/or low pH. During the stripping procedure, some amount of antigen is inevitably lost from the membrane. It is important to minimize this loss by stripping the antibody under gentle conditions. Because each antibody-antigen pair has unique characteristics, there is no guaranteed method to remove every antibody while preserving the antigen. Restore™ Western Blot Stripping Buffer (Product # 21059) was designed to achieve maximum removal of antibodies from a membrane while preserving the integrity of the antigen. It is unique among stripping buffers because it is odor-free and can often strip a membrane in as little as 15 minutes.

Stripping and reprobing a Western blot instead of running an entirely new blot may be preferable because it:

- **Conserves sample**

  When the protein mixture is rare or valuable, reprobing conserves the sample and allows the membrane to be analyzed with the same or different antibodies.
- **Saves time**

  It is time-consuming to run an SDS-polyacrylamide gel and then transfer the proteins to a membrane. By using the same blot for several different detections, you save time.
- **Makes it easy to optimize**

  The increased light emission intensity of SuperSignal® West Pico Substrate, along with the increased sensitivity of SuperSignal® West Dura and SuperSignal® West Femto Substrates, often require antibody concentration optimization to achieve the highest quality blot. Optimization is achieved easily by stripping the membrane and reprobing with a different antibody concentration.
- **Saves money**

  By reusing the same blot, you save money on the costs of blots, membrane, buffers and protein sample.
- **Makes it easy to confirm atypical results**

  When immunoblot results are not expected, reprobing allows the use of the same protein sample without going back to gel electrophoresis.
- **Makes it easy to correct mistakes**

  Immunoblotting requires many steps, providing ample opportunity for mistakes to occur. By stripping the membrane, the blot can be reused.

Following any stripping procedure, the blot should be tested to ensure that all of the detection reagents were removed. The membrane should be washed several times with blocking agent, incubated with secondary antibody, then reincubated with chemiluminescent substrate. If the primary antibody was effectively removed by the stripping procedure, no secondary antibody should bind to the membrane and no signal should be produced. If bands are still visible on the blot, the stripping conditions must be intensified. Often a simple increase of the reaction time or temperature will complete the stripping process. However, it is sometimes necessary to alter the composition of the stripping buffer or change methods entirely.

Protocol for Stripping an Immunoblot

Note 1: Optimization of both incubation time and temperature is essential for best results.

Note 2: If the blot cannot be stripped immediately after chemiluminescent detection, the blot can be stored in PBS at 4°C until the stripping procedure is to be performed.

1. Place the blot to be stripped in Restore™ Western Blot Stripping Buffer and incubate for 5-15 minutes at RT. Use a sufficient volume of buffer to ensure that the blot is completely wetted (i.e., approximately 20 ml for an 8 x 10 cm blot). Alternatively, the blot can be incubated with a solution of 2% w/v SDS, 62.5 mM Tris•HCl, 100 mM β-mercaptoethanol, pH 6.8 for 30-90 minutes at 50-70°C. However, these reaction conditions are much harsher than Restore™ Western Blot Stripping Buffer and are more likely to interfere with future ligand:antibody interactions.

Note: In general, high-affinity antibodies will require at least 15 minutes of stripping and may require an incubation temperature of 37°C.

2. Remove the blot from the Restore™ Western Blot Stripping Buffer and wash in Wash Buffer.

3. Test for the removal of the immunodetection reagents.

   A. To test for complete removal of the HRP label, incubate the membrane as described above with fresh SuperSignal® West Working Solution and expose to film. If no signal is detected with a 5-minute exposure, the HRP conjugate has been successfully removed from the antigen or primary antibody.

   B. To test for complete removal of the primary antibody, incubate the membrane with the HRP-labeled secondary antibody, followed by a wash in wash buffer. Apply fresh SuperSignal® West Working Solution as described above. If no signal is detected with a 5-minute exposure, the primary antibody has been successfully removed from the antigen.

   C. If signal is detected with experiment A or B, place the blot back into Restore™ Western Blot Stripping Buffer for an additional 5-15 minutes. Some antigen/antibody systems require an increase in temperature and/or longer incubation periods. Analysis of the successful removal of immunoprobes is recommended to prevent removal of the antigen or the unsuccessful removal of the antibodies.

After it has been determined that the membrane is free of immunodetection reagents, a second immunoprobing can begin.

Note 1: The Western blot can be stripped and reprobed several times, but it may require longer exposure times or a more sensitive chemiluminescent substrate. Subsequent reprobings may result in a decrease in signal if the antigen is labile in Restore™ Western Blot Stripping Buffer. Analysis of the individual system is required.

Note 2: Reblocking of the membrane is not critical, but it may be required in some applications.

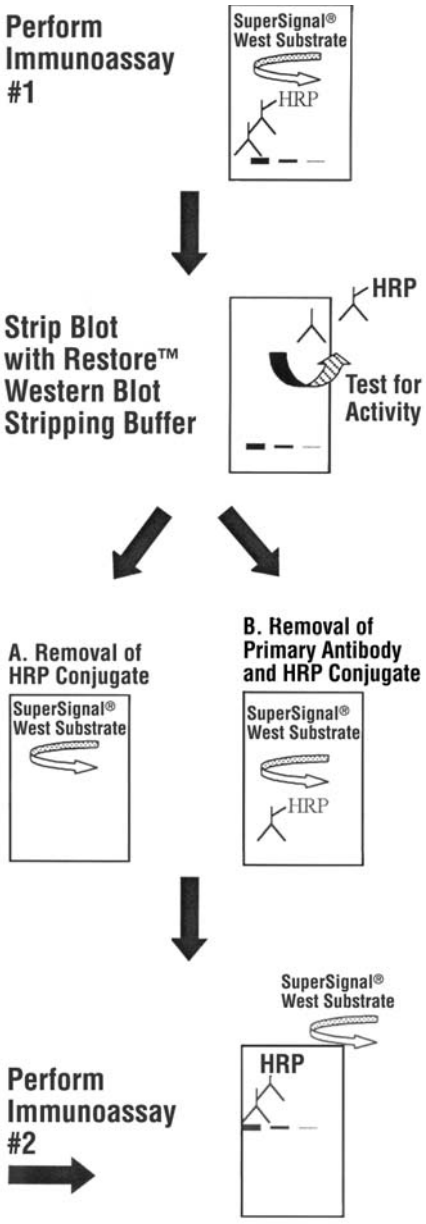

Figure 26. Restore™ Western Blot Stripping Buffer Protocol.

Restore™ Western Blot Stripping Buffer

Strip time off your research with Restore™ Stripping Buffer.

Tired of re-running electrophoresis gels and waiting to see your results? Although optimizing assay conditions is the best way to achieve optimum results, re-performing the gel electrophoresis process to test each new primary antibody or antibody concentration is time-consuming and expensive. You can forget about starting over when you use new Restore™ Western Blot Stripping Buffer!

Optimize Assay Conditions

Using Pierce SuperSignal® West Substrates, Figure 27 shows how the secondary antibody concentrations are optimized after a single stripping and re-probing cycle. A chemiluminescent detection system is the most sensitive method to detect any reagent still bound after the stripping procedure.

Test Different Primary Antibodies

There's no need to waste precious sample and re-run a gel to test different primary antibodies. Simply strip the membrane with Restore™ Stripping Buffer to remove the first primary antibody and set of reagents. It takes only 5-15 minutes, depending on the affinity of the primary antibody. After stripping, re-probe with a new primary antibody. Figure 28 shows how the SuperSignal® Chemiluminescent Detection System can analyze two different proteins using the same blot.

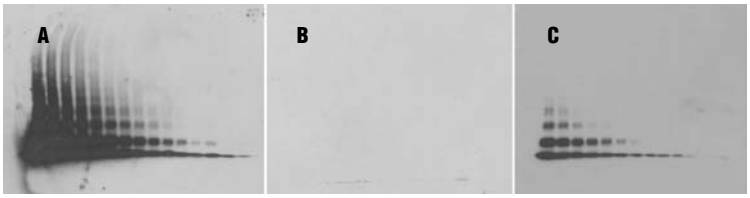

Figure 27. Antibody optimization study. Western blots of Interleukin-2 (diluted 20-0.156 ng) were detected using SuperSignal® West Pico Chemiluminescent Substrate. The first blot (A) used the primary antibody diluted to 1/1,000 (0.5 µg/ml) of Rat anti-Mouse IL-2 (BD PharMingen) and the horseradish peroxidase (HRP)-labeled Goat anti-Rat secondary antibody (Product # 31470) diluted 1/5,000. The same blot was stripped with Restore™ Western Blot Stripping Buffer (B) for 5 minutes at room temperature and re-probed (C) with the primary antibody at 1/5,000 and the HRP-secondary conjugate at 1:20,000. SuperBlock® Blocking Buffer was used for blocking.

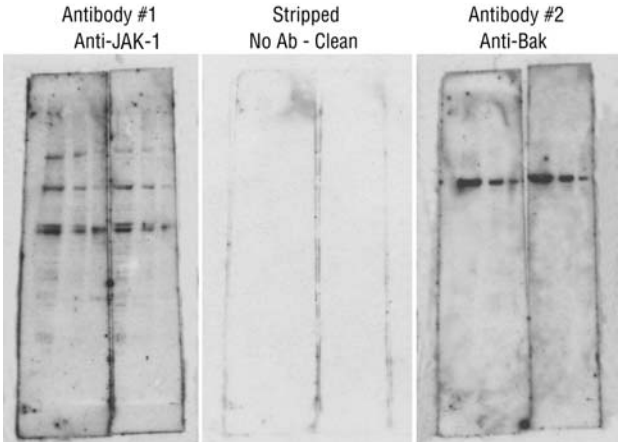

Figure 28. Re-probing with different antibodies. Western blots of HeLa cell lysate protein (diluted from 750-83.3 ng) were detected with SuperSignal® West Dura Chemiluminescent Substrate. The first blot used polyclonal rabbit anti-JAK-1 primary antibody (BD PharMingen) at 1:2,000 dilution with an HRP-secondary conjugate diluted at 1:350,000. The same blot was stripped for 5 minutes at room temperature in Restore™ Western Blot Stripping Buffer and then re-probed with purified mouse anti-human Bak monoclonal primary antibody at 1:1,000 with the HRP-secondary conjugate at 1:100,000. Five-percent nonfat milk with 0.05%Tween®-20 was used for blocking.

Highlights:

- Saves time – no need to re-run gels
- Saves precious sample – re-probe the membrane using the same target sample
- Provides efficient removal – proprietary formulation works better than "homemade" buffers
- Gentle formulation – does not damage target protein after stripping and re-probing
- Odor-free – no mercaptans means no acrid odors with reducing agents
- Economical – less-expensive than other competing stripping buffers

References

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PRODUCT #	DESCRIPTION	PKG. SIZE
21059	**Restore™ Western Blot Stripping Buffer** *Sufficient for stripping 25 (8 cm x 10 cm) blots.*	500 ml
21062	**Restore™ Western Blot Stripping Buffer** *Sufficient for stripping 1 (8 cm x 10 cm) blot.*	30 ml

Qentix™ [ken´-tiks] Western Blot Signal Enhancer

It's like intensifying screens in a bottle.

There are many ways to increase the sensitivity of a Western blot. Some methods are as simple as switching substrates or blocking buffers, while others are more time-consuming such as optimizing antibody titer or checking for proper protein transfer. Those solutions are covered in the troubleshooting section of this handbook.

One of the more certain and easiest ways to increase the sensitivity of any Western blot is to use the new Qentix™ Western Blot Signal Enhancer.

Qentix™ Western Blot Signal Enhancer does for enzyme-/substrate-based blotting what intensifying screens do for radioactive blotting – it increases the signal up to 10-fold (or one order of magnitude) in only 15 minutes.

The Qentix™ Western Blot Signal Enhancer membrane treatment is a simple, 15-minute procedure that can be added to your current Western blotting protocol. The result is an increase in the intensity of target protein bands on the Western blot or detection of target proteins at a level that could not previously be detected. Some protein targets have resulted in a 10-fold increase in band intensity after treatment with the Western Blot Signal Enhancer compared to the typical detection protocol without treatment.

Highlights:

Enhances chemiluminescent, fluorescent and colorimetric detection up to 10-fold

- Treatment with Western Blot Signal Enhancer can boost the band intensity from three- to 10-fold, regardless of what substrate is used

Enhances detection of targets transferred to either nitrocellulose or PVDF*, independent of membrane pore size

- Works with the most commonly used Western blotting membranes
- Signal intensity has been increased with targets such as mouse IL-6, p53, NF-κB, BRCA1 and EGF

Room temperature-stable, ready-to-use reagents

- No thawing, formulating or diluting necessary

15-minute protocol

- Optimized to save time and improve detection capability of your specific analyte

** Signal enhancement of proteins on PVDF membrane has been shown to be variable from no significant enhancement for some proteins, to several-fold enhancement for others.*

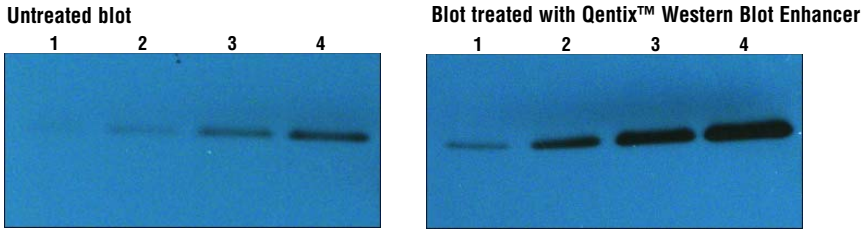

Figure 29. Enhanced chemiluminescent detection of identical serial dilutions of IL-6 before and after treatment with Qentix™ Western Blot Signal Enhancer.

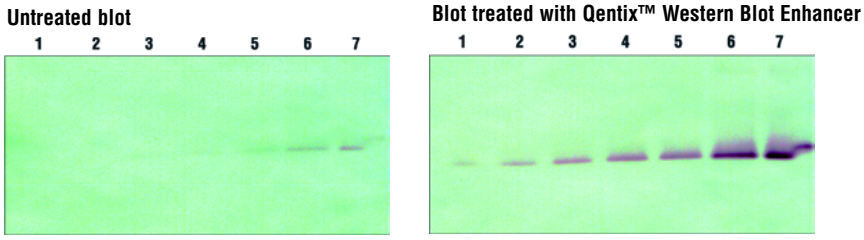

Figure 30. Enhanced chromogenic detection of identical serial dilutions of IL-6 before and after treatment with Qentix™ Western Blot Signal Enhancer.

PRODUCT #	DESCRIPTION	PKG. SIZE
21050	**Qentix™ Western Blot Signal Enhancer*** *Sufficient reagent for ten 10 cm x 10 cm blots.*	Kit
	Includes: Enhancer Reagent 1	250 ml
	Enhancer Reagent 2	250 ml

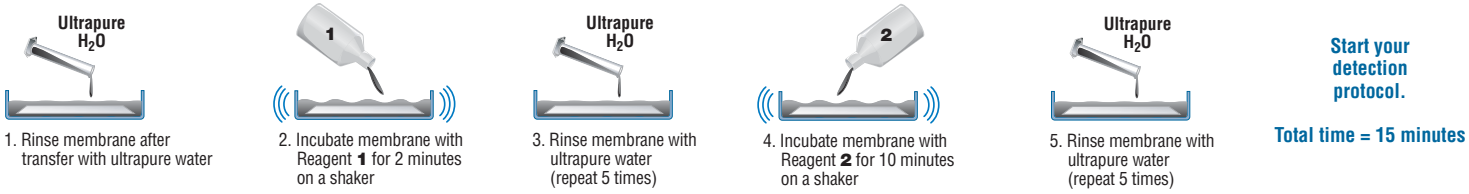

Figure 31. Qentix™ Western Blot Signal Enhancer Protocol – performed after transfer and before blocking.

Erase-It® Background Eliminator

Another method by which the S/N ratio can be improved is to "erase" the background, leaving just the signal with little to no interference. Erase-It® Background Eliminator does just that **without altering the integrity of the data.** The Erase-It® Solution works on overexposed film, lightening the entire film evenly. The end result is to effectively reduce the initial exposure time. This is done directly in the lab while viewing the film. No darkroom is required. The process can be halted when the signal is clearly visible and the background is at a minimum, thereby increasing the S/N ratio without altering the data's integrity (Figure 32).

Erase-It® Background Eliminator provides fast, easy removal of background image on exposed X-ray film for Western, Northern or Southern blots, so you can see your results clearly.

High background, shading, overexposed bands and speckling are problems inherent to film exposure. High background and shading can be caused by overexposure, poor use of blocking buffer, or inappropriate enzyme-labeled probe or antibody concentration. Overexposed bands are a common occurrence when the enzyme-labeled probe or antibody concentration used is too high or if the film was exposed for too long. Speckling and shading occur when enzyme conjugates form complexes and precipitate on the blot. The Erase-It® Kit can correct all these problems without the need to re-expose your blot to film or re-do the experiment, allowing you to visualize your data within minutes (Figures 32, 33 and 35). The Erase-It® Solution can be used with newly exposed films or exposed films that have been stored for years. In addition, the Erase-It® Kit can be used with any brand of film.

For applications requiring densitometric measurement, the Erase-It® Background Eliminator reduces signal evenly over the film so that relative densitometry values are consistent (Figure 34).

The procedure is simple. Immerse your exposed film in Erase-It® Working Solution, watch for desired image and stop the reaction by rinsing the film in water. The Erase-It® Solution works quickly, with ideal signal level typically attained in just a few minutes.

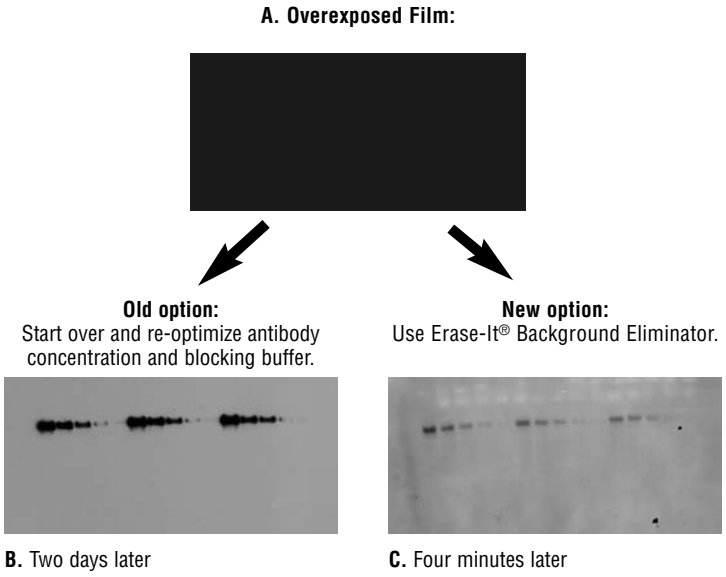

Figure 32. A431 cell lysate was electrophoresed on a 4-12% NuPage® Gel (Novex) and transferred overnight to nitrocellulose. The membrane was blocked with SuperBlock® Blocking Buffer in PBS (Product # 37515) for 1 hour and incubated with 1.25 ng/ml of HRP-labeled mouse anti-phosphotyrosine (PY20) for 1 hour. After the membrane was washed for 30 minutes, SuperSignal® West Dura Substrate (Product # 34075) was added. The blot was exposed to film for 10 seconds and resulted in a completely black image (A). Using the old option to resolve the problem of a completely dark film, another gel was prepared to optimize assay conditions. The proteins were transferred overnight and then the membrane was blocked with a 5% dry milk solution for 1 hour. The blot was detected with 2.5 ng/ml of anti-phosphotyrosine (PY20)-HRP and SuperSignal® West Dura Substrate (Product # 34075). The blot was exposed to film for 10 seconds. This optimization required a two-day procedure (B). Using the new option, the initial dark film (A) was treated with Erase-It® Background Eliminator to allow the band images to appear in 4 minutes (C).

Highlights:

- Reduces signal evenly over the film – no "altering" of results
- Fast, easy background elimination from overexposed, speckled or shaded films
- Works with any X-ray film, new or old
- No need for time-consuming re-exposures to find the optimal image
- No need to re-optimize assay reagents to obtain the optimal image

Erase-It® Technology can be used for any application using X-ray film exposures including:

- Western, Northern and Southern blots such as with SuperSignal® Substrates and the North2South® Chemiluminescent Detection Kit
- In-gel detection systems
- Gel-shift assays
- Ribonuclease protection assays (RPA)

PRODUCT #	DESCRIPTION	PKG. SIZE
21065	**Erase-It® Background Eliminator** *Sufficient reagent to prepare 3 liters of working solution.*	Kit
	Includes: Erase-It® Reagent A	100 ml
	Erase-It® Reagent B	100 ml

Lightens Overexposed Bands

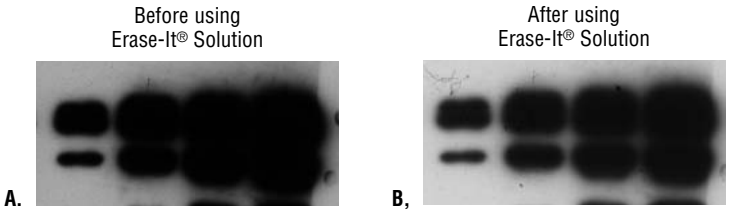

Figure 33. Recombinant human wild-type p53 baculovirus lysate was separated on a 12% SDS-polyacrylamide gel. The proteins were transferred to a nitrocellulose membrane and blocked with SuperBlock® Blocking Buffer in PBS (Product # 37515). The protein was detected with mouse anti-p53 followed by goat anti-Mouse-HRP (Product # 31434) and SuperSignal® West Pico Substrate (Product # 34080). The membrane was exposed to film for 1 minute (A). The film had overexposed bands and was treated with Erase-It® Background Eliminator for 6 minutes. The resulting image provided better visualization of the different p53 protein bands (B).

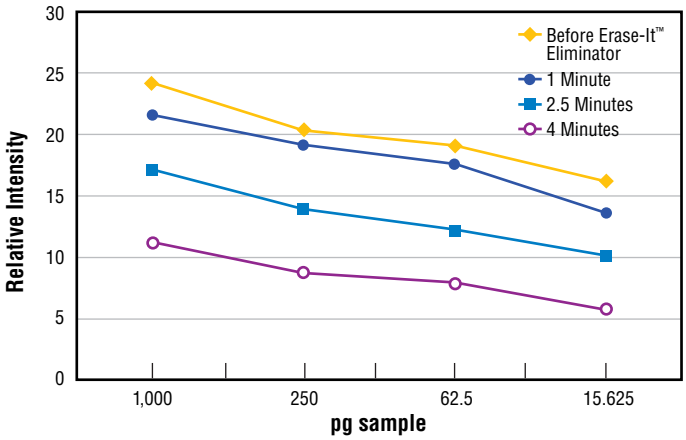

Figure 34. Densitometry data on dot blot comparing before and after use of the Erase-It® Background Eliminator. Dot blots were prepared on nitrocellulose (Product # 77010) using Biotinylated-BSA (Product # 29130) at 1,000, 250, 62.5 and 15.6 pg. The blot was blocked with SuperBlock® Blocking Buffer in PBS (Product # 37515) and incubated with a 1/50,000 dilution of SA-HRP (Product # 21126). The blot was then washed for 30 minutes, incubated in SuperSignal® West Pico Substrate (Product # 34080) and exposed to film (Product # 34092) for 5 minutes. The resulting film had high background that was cut into four strips each containing three replicates per concentration. The Erase-It® Working Solution was used on separate film strips at 1, 2.5 and 4 minutes, leaving a control strip for comparison. After scanning on a densitometer, the relative signal intensity was compared. The results showed that signal intensity decreased evenly with time when treated with the Erase-It® Solution maintaining similar slopes on a dose response curve.

Erases Speckling

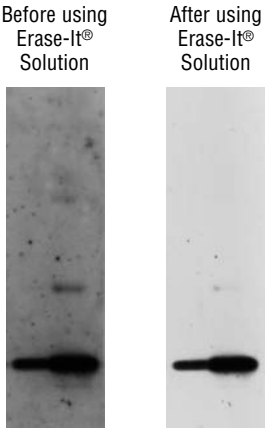

Figure 35. Recombinant Human TNFα was electrophoresed on a 4-20% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane. The membrane was blocked and detected with mouse anti-human TNFα followed by goat anti-mouse-HRP (Product # 31434) and SuperSignal® West Dura Substrate (Product # 34075). The blot was exposed to film for 30 seconds, resulting in considerable background speckling (A). The film was then treated with Erase-It® Background Eliminator for 2 minutes to eliminate the background speckling (B).

High Background that is Uniformly Distributed

Possible Causes	Precautions/Solutions
Antibody concentrations are too high	• The primary and/or secondary antibody can cause high background if the concentrations used are too high. • Decrease antibody concentrations.
Wrong blocking buffer was used	• Compare different blocking buffers.
Insufficient blocking of nonspecific sites	• Optimize blocking buffer. The best blocking buffer is system-dependent. • Increase the concentration of protein in the blocking buffer. • Optimize blocking time and/or temperature. Block for at least 1 hour at RT or overnight at 4°C. • Add Tween®-20 to blocking buffer. Use a final concentration of 0.05% Tween®-20. Skip this step if you use StartingBlock™ T20 Blocking Buffer in PBS (Product # 37539) or TBS (Product # 37543) or SuperBlock® T20 Blocking Buffer in PBS (Product # 37516) or TBS (Product # 37536). These buffers already contain Tween®-20 Detergent at optimized concentrations. • Prepare antibody dilutions in blocking buffer with 0.05% Tween®-20.
Cross-reactivity of antibody with other proteins in blocking buffer	• Use a different blocking buffer. • Do not use milk to block membranes when using an avidin-biotin system. Milk contains biotin. • Test for cross-reactivity. Block a clean piece of membrane, incubate with antibodies and then detect with SuperSignal® Chemiluminescent Substrate. • Reduce the concentration of the HRP conjugate.
Insufficient washing	• Increase number of washes and the volume of buffer used. • Add Tween®-20 to wash buffer if it's not already included. Use a final concentration of 0.05% Tween®-20. *(Caution: If the concentration of Tween®-20 is too high, it can strip proteins off the membrane.)* Skip this step if you use StartingBlock™ T20 Blocking Buffer in PBS (Product # 37539) or TBS (Product # 37543) or SuperBlock® T20 Blocking Buffer in PBS (Product # 37516) or TBS (Product # 37536). These buffers already contain Tween®-20 Detergent at optimized concentrations.
Exposure time is too long	• Reduce the time the blot is exposed to film.
Membrane problems	• Make sure membranes are wetted thoroughly and according to the manufacturer's instructions. • Use new membranes. • Ensure the membrane is adequately covered with liquid at all times to prevent it from drying. • Use agitation during all incubations. • Handle membranes carefully – damage to the membrane can cause nonspecific binding. • Do not handle membrane with bare hands. Always wear clean gloves or use forceps.
Contamination or growth in buffers	• Prepare new buffers.

High Background that is Blotchy or Speckled

Possible Causes	Precautions/Solutions
Antibody concentrations are too high	• The primary and/or secondary antibody can cause high background if the concentrations used are too high. • Decrease antibody concentrations.
Aggregate formation in the HRP conjugate can cause speckling	• Filter the conjugate through a 0.2 µm filter. • Use a fresh, high-quality conjugate.
Wrong blocking buffer was used	• Compare different blocking buffers.
Insufficient blocking of nonspecific sites	• Optimize blocking buffer. The best blocking buffer is system-dependent. • Increase concentration of protein in the blocking buffer. • Optimize blocking time and/or temperature. Block for at least 1 hour at RT or overnight at 4°C. • Add Tween®-20 to blocking buffer. A concentration of 0.05% Tween®-20 is recommended. Skip this step if you use StartingBlock™ T20 Blocking Buffer in PBS (Product # 37539) or TBS (Product # 37543) or SuperBlock® T20 Blocking Buffer in PBS (Product # 37516) or TBS (Product # 37536). These buffers already contain Tween®-20 Detergent at optimized concentrations. • Make up antibody dilutions in blocking buffer with 0.05% Tween®-20.
Cross-reactivity of antibody with other proteins in blocking buffer	• Use a different blocking buffer. • Do not use milk to block membranes when using an avidin-biotin system. Milk contains biotin. • Test for cross-reactivity. Block a clean piece of membrane, incubate with antibodies and then detect with SuperSignal® Chemiluminescent Substrate. • Reduce the concentration of the HRP conjugate.
Membrane was not wetted properly	• Wet membrane according to the manufacturer's instructions. • Do not handle membrane with bare hands. Always wear clean gloves or use forceps. • Use a new membrane. • Make sure the membrane is covered with a sufficient amount of liquid at all times to prevent it from drying. • Use agitation during all incubations. • Incubate membranes separately to ensure that membrane strips are not covering one another during incubations. • Handle membranes carefully – damage to the membrane can cause nonspecific binding.
Contamination in buffers	• Use new buffers. • Filter buffers before use.
Contaminated equipment	• Make sure electrophoresis equipment, blotting equipment and incubation trays are clean and free of foreign contaminants. • Make sure there are no pieces of gel left on the membrane after transfer. Proteins can stick to the pieces of gel and cause background.

Blotting with Chemiluminescence – Troubleshooting Guide

Weak Signal or No Signal

Possible Causes	Precautions/Solutions
Proteins did not transfer properly to the membrane	• After transfer is complete, stain the gel with a total protein stain to determine transfer efficiency. *(Note: Total protein stains may not be able to detect low quantities of antigen.)* • Use MemCode™ Stain to check membrane for transfer efficiency. • Make sure there is sufficient contact between the gel and membrane during transfer. • Make sure the transfer sandwich is assembled correctly. • Be sure to follow the membrane manufacturer's instructions for wetting the membrane. • Make sure transfer unit does not overheat during electroblotting procedure. • Use positive control and/or molecular weight markers. • Optimize transfer time and current. • Use Pierce Lane Marker Sample Buffer. The tracking dye transfers to the membrane. • Make sure sample preparation conditions prior to blotting of the protein have not destroyed antigenicity of the sample. *(Caution: Some proteins cannot be run under reducing conditions.)*
Insufficient binding to membrane	• Adding 20% methanol to the transfer buffer helps binding. Low M.W. antigen may pass through the membrane. Use a membrane with a smaller pore size.
Insufficient amount of antibodies	• Increase antibody concentrations. Antibody may have poor affinity for the protein of interest. • Antibody may have lost activity. Perform a dot blot to determine activity.
Insufficient amount of antigen present	• Load more protein onto the gel.
The antigen is masked by the blocking buffer	• Try different blocking buffers. • Optimize blocking buffer protein concentration.
Buffers contain azide as a preservative	• Azide is an inhibitor of HRP; do not use azide as a preservative.
Exposure time is too short	• Lengthen the film exposure time. *(Note: SuperSignal® Chemiluminescent Substrates will continue to glow for a minimum of six hours.)*
Substrate incubation is too short	• A five-minute substrate incubation is required when using SuperSignal® Substrates.
The wrong membrane was used	• Nitrocellulose is the recommended membrane when using SuperSignal® West Pico Chemiluminescent Substrate for Western Blotting. PVDF can be used, but may require further optimization. • SuperSignal® West Dura Chemiluminescent Substrate and SuperSignal® West Femto Chemiluminescent Substrate can be used with nitrocellulose, PVDF, nylon and charge-modified nylon membranes.
Substrate has lost activity	• SuperSignal® West Pico Chemiluminescent Substrate and SuperSignal® West Dura Chemiluminescent Substrate are stable for up to 12 months at RT. SuperSignal® West Femto Chemiluminescent Substrate is stable for at least six months at RT. • To determine if the substrate has lost activity, prepare a small amount of working solution. In a darkroom, add a small amount of HRP conjugate. A blue light should be observed. If no glow is observed, either the substrate or the HRP conjugate has lost activity. • Ensure that there is no cross-contamination between the two bottles. Contamination between the two substrate reagents can cause a decline in activity.
Membrane has been stripped and reprobed	• There may be some antigen loss or denaturation during membrane stripping procedures. Optimize stripping procedure. • Reprobe only when necessary. • Avoid repeated reprobing of the same membrane.
Digestion of antigen on the membrane	• Blocking substance may have proteolytic activity (e.g., gelatin).
Protein degradation from blot storage	• Prepare a new blot.

Blotting with Chemiluminescence – Troubleshooting Guide

Nonspecific Bands

Possible Causes	Precautions/Solutions
Antibody concentrations are too high	• Reduce antibody concentrations.
SDS caused nonspecific binding to immobilized protein bands	• Wash blots after transfer. • Do not use SDS during immunoassay procedure.

Diffuse Bands

Possible Causes	Precautions/Solutions
Antibody concentrations are too high	• Reduce antibody concentrations.
Too much protein is loaded onto the gel	• Reduce the amount of protein loaded onto the gel.

Black blots with white bands or signal that decreases quickly

Possible Causes	Precautions/Solutions
Antibody concentrations are too high	• Reduce antibody concentrations, especially the HRP conjugate. Signal that decreases quickly and the appearance of white bands are indications that there is too much HRP in the system.

Problem: Partly developed area or blank areas

Possible Causes	Precautions/Solutions
Incomplete transfer of proteins from the gel	• Make sure there are no air bubbles between the gel and membrane during transfer. • Wet membrane according to the manufacturer's instructions. • Do not handle the membrane with bare hands. Always wear clean gloves or use forceps. • Use a new membrane. • Incubate membranes separately to ensure that membrane strips are not covering one another during incubations.

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1. Make the protein solution of interest in a sample buffer and heat it to boiling for 5 minutes. The sample buffer should contain the following:
   - .03 M Tris•HCl
   - 5% SDS to denature the protein and to generate a constant anionic charge-to-mass ratio for the denatured protein chains
   - 50% glycerol to give the sample a higher density than the running buffer, allowing the sample to "sink" to the bottom of the well
   - A low-M.W. dye for dye-front determination
   - As needed, a reducing agent such as 100 mM β-mercaptoethanol, dithiothreitol or TCEP that will reduce the disulfide bonds present in the protein sample

   Adjust solution to pH 6.8.

2. Add the protein solution in the sample buffer to an SDS-polyacrylamide gel (SDS-PAGE).

3. Separate the proteins electrophoretically by M.W.

4. Transfer the protein from the gel to a membrane.

Substrate	SuperSignal® West Pico Substrate	SuperSignal® West Femto Substrate	SuperSignal® West Dura Substrate	Lumi-Phos™ WB Substrate
Recommended Membrane	Nitrocellulose or PVDF	Nitrocellulose or PVDF	Nitrocellulose or PVDF	Nitrocellulose

5. Remove the membrane blot and block the nonspecific sites with a blocking buffer for 20-60 minutes at RT with shaking. For best results, block for 1 hour at RT. Optimization of blocking buffer may be required to achieve best results. **Please see the Optimization of Blotting Buffers section, page 7.**

6. Incubate the blot with the primary antibody with shaking for 1 hour. For recommended antibody dilutions, see the table below. If desired, blots can be incubated with primary antibody overnight at 2°C-8°C. The necessary dilution will vary depending on the primary antibody used and the amount of antigen that was transferred. **Please see the Optimization of Antibody Concentration section, page 22.**

	SuperSignal® West Pico Substrate	SuperSignal® West Femto Substrate	SuperSignal® West Dura Substrate	Lumi-Phos™ WB Substrate
Recommended Primary Antibody Dilutions (from 1 mg/ml stock)	1/1,000-1/5,000 or 0.2-1.0 µg/ml	1/5,000-1/100,000 or 0.01-0.2 µg/ml	1/1,000-1/50,000 or 0.02-1.0 µg/ml	1/200-1/2,000 or 0.5-5.0 µg/ml

7. Wash the membrane with wash buffer. At least four to six changes of the wash buffer are recommended. Use as large a volume of wash buffer as possible. For each wash, suspend the membrane in wash buffer and agitate for at least 5 minutes. Increasing the wash buffer volume and/or the number of washes may reduce background. Tris buffered saline (TBS), phosphate buffered saline (PBS) or another suitable wash buffer can be used. The addition of 0.05% Tween®-20 to the wash buffer may also help reduce background.

Note 1: Briefly rinsing the membrane in wash buffer prior to incubation will help increase the efficiency of the wash step.

Note 2: If using an enzyme-conjugated primary antibody, proceed directly to Step 10.

8. Incubate the blot with enzyme-conjugated secondary antibody or avidin for 1 hour with shaking at RT. For recommended antibody- or avidin-conjugate dilutions, see the table below. The necessary dilution will vary depending on the enzyme conjugate used, the primary antibody used in Step 6 and the amount of antigen that was transferred.

	SuperSignal® West Pico Substrate	SuperSignal® West Femto Substrate	SuperSignal® West Dura Substrate	Lumi-Phos™ WB Substrate
Recommended Secondary Antibody Dilutions (from 1 mg/ml stock)	1/20,000-1/100,000 or 10-50 ng/ml	1/100,000-1/500,000 or 2.0-10 ng/ml	1/50,000-1/250,000 or 4.0-20 ng/ml	1/5,000-1/25,000 or 40-200 ng/ml

9. Repeat Step 4 to wash away any unbound enzyme-conjugated secondary antibody. It is crucial to thoroughly wash the membrane after the incubation with the enzyme conjugate.

10. If the working solution has not been prepared, prepare it now. For SuperSignal® West Substrates, mix equal volumes of the Luminol/Enhancer Solution and the Stable Peroxide Solution. Prepare a sufficient volume to ensure that the blot is completely wetted with substrate and the blot does not dry out. Lumi-Phos™ WB Substrate is provided in a ready-to-use format, but it should be brought to room temperature. **Recommended volume: 0.125 ml/cm^2 of blot surface**.

11. Incubate the blot with SuperSignal® Substrate Working Solution for 5 minutes or with Lumi-Phos™ WB Substrate Working Solution for 3 minutes.

12. Remove the blot from the substrate working solution and place it in a plastic membrane protector. (A plastic sheet protector works very well, although plastic wrap may also be used.) Remove all air bubbles between the blot and the surface of the membrane protector.

13. Place the wetted blot against the film and expose. Standard autoradiographic film can be used. A recommended first exposure time is 60 seconds. Exposure time can be varied to obtain optimum results. The use of enhanced or pre-flashed autoradiographic film is unnecessary.

Note: If a cooled CCD Camera (e.g., Alpha Innotech Corporation's ChemiImager™ Camera) is used, longer exposure times may be necessary.

14. Develop the film using appropriate developing solution and fixative for the type of film used.

15. On an optimized blot, the light generated should last a minimum of six hours. The blot can be re-exposed to film, as needed, to obtain the optimal results. Longer exposure times may be necessary as the blot ages.

Recommended Reading

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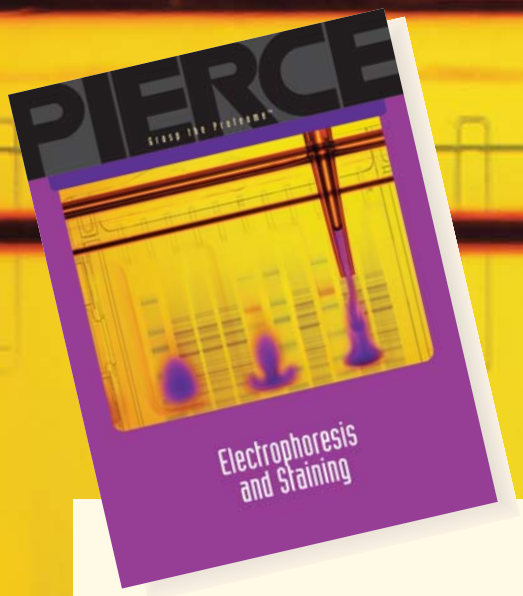

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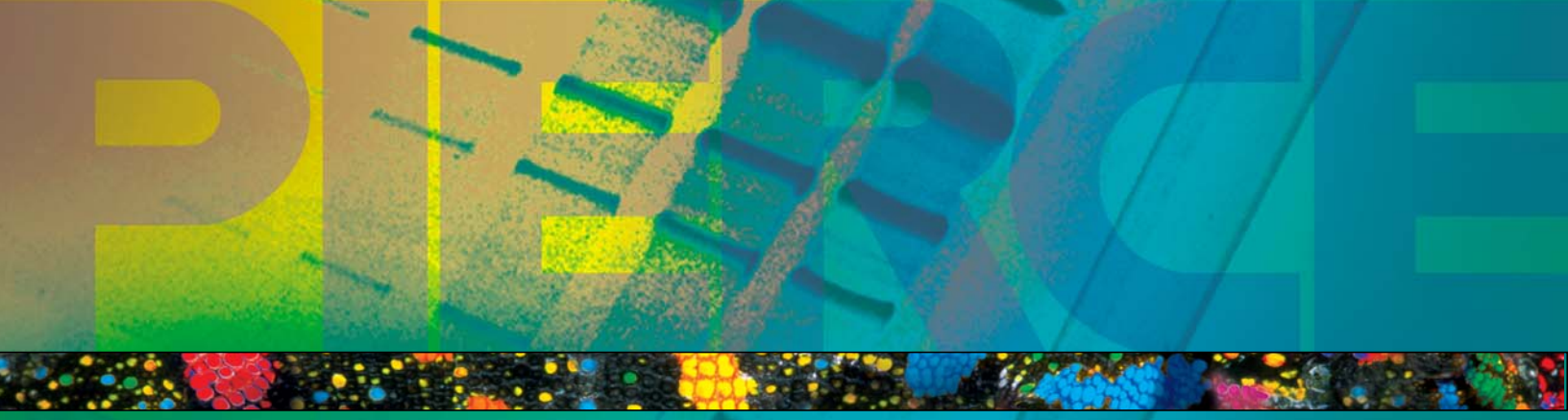

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