INSTRUCTIONS

BioWest™ Extended Duration Chemiluminescent Substrate

Product Description

Description: BioWest Extended Duration Chemiluminescent Substrate

Contents: BioWest Luminol/Enhancer Solution, 50 ml; BioWest Stable Peroxide Buffer Solution, 50 ml

This kit contains sufficient substrate for 800 cm^2 of blotting membrane.

Storage: Stable for 1 year at room temperature. For long-term storage, keep product at 4°C.

Note: BioWest Substrate's light emission wavelength is 425 nm.

BioWest Substrate:

This product is an extremely sensitive enhanced chemiluminescent substrate for the development of immunoblots utilizing a horseradish peroxidase (HRP) label. This substrate requires high dilutions of the antibodies. Please read the following guidelines for best results, especially the Optimization of Reagents section.

Solutions are irritating to eyes, respiratory system and skin. Avoid contact with skin and eyes.

Preparation of BioWest Substrate Working Solution:

Mix equal parts (1:1) BioWest Luminol/Enhancer Solution and BioWest Stable Peroxide Solution prior to use. This working solution is stable for a minimum of 24 hours at room temperature and may be stored at ambient light and temperature. Mix enough working solution to adequately cover the blot. We recommend at least 0.125 ml/cm^2 .

Western Blotting Protocol

1. Transfer protein from the gel to a membrane. For best results, we recommend the use of nitrocellulose membrane. Other membrane types including PVDF and charge modified nylon can be used; however, optimization may be required.
2. Remove the membrane blot and block the nonspecific sites with a blocking buffer for a minimum of 30 minutes at room temperature with shaking. For best results, blocking for one hour at room temperature is recommended. Optimization of blocking buffer may be required to achieve best results. Please see Optimization of Reagent section.
3. Incubate the blot with the primary antibody for one hour with shaking. The recommended starting primary antibody dilution is 1:5,000 to 1:50,000 from a 1 mg/ml stock concentration. Please see Optimization of Reagents section.
4. Wash the membrane with wash buffer. Tris Buffered Saline (TBS) or Phosphate Buffered Saline (PBS) containing 0.05% Tween®-20 or other suitable wash buffer can be used.
5. Incubate the blot with HRP-conjugated secondary antibody for one hour at room temperature with shaking. The recommended starting dilution for a standard 1 mg/ml HRP-conjugate is between 1:50,000 to 1:250,000.

Recommended secondary antibodies and reagents: We recommend Goat anti-mouse HRP conjugate (Pierce No. 31430) or Goat anti-rabbit HRP-conjugate (Pierce No. 31460), depending on the primary antibody used in Step 3. For Avidin/Biotin systems, we recommend either Streptavidin-HRP (Pierce No. 21124) or NeutrAvidin™-HRP (Pierce No. 31001). These reagents may be used at 1:100,000 to 1:250,000 dilution as a starting point.

6. Wash the membrane with wash buffer. We recommend at least 4-6 changes; more washes may help to reduce background.
7. Immerse the blot in BioWest Substrate Working Solution for five minutes. Use a sufficient volume to ensure that the blot is completely wetted with substrate and it does not dry out. We recommend at least 0.125 ml/cm^2.
8. Remove the blot from the substrate solution and place it in a membrane protector. (A plastic sheet protector works well, although plastic wrap can be used.) Remove any bubbles between the blot and the surface of the membrane protector.

   Note: The substrate allows for re-exposure over a 24-hour period of time, provided that the membrane does not dry out.
9. Place your blot on the fold down tray of the Epi Chemi II Darkroom and insure that it is saturated and completely flat on the surface of the tray. Apply additional buffer if required.
10. Place a flat object with text (platic UVP BioImaging target) next to your blot and focus the camera.
11. Turn off the overhead white lights, open the lens aperture to its lowest f-stop setting, insure that the clear filter is selected on the darkroom and the door is tightly closed.
12. Select the desired camera operation ***(1) On-Chip Integration (2) Sequential Integration or (3) Dynamic Integration***, and capture images of your blot.

Note: Please consult the LabWorks™ Quick-Start or Imaging Manual for details on the camera operation, how to visually enhance the blot images and calibrate or quantitate bands.

Optimization of Reagents

For best results, it may be necessary to optimize all components of the blot detection, including the amount of sample used, the concentration of the primary antibody, and the concentration of the secondary antibody as well as membrane and blocking reagents. Due to the extremely sensitive nature of the substrate, any inherent background in the blot will be more apparent than with other substrates. ***If you are already using a chemiluminescent detection system for your blots, you should find that BioWest Substrate will allow you to dilute your sample and primary and secondary antibodies by a factor of 10-20.***

Primary Antibody Optimization: With a variety of antibodies, good signal is produced with a 1:5,000 to 1:100,000 dilution of a 1 mg/ml stock concentration. If your antigen is present in fairly high amounts, use a relatively higher dilution of primary antibody.

Secondary Antibody Optimization: The HRP-conjugated secondary antibodies against mouse (Pierce No. 31430) and rabbit (Pierce No. 31460) antibodies are routinely used at 1:100,000 to 1:250,000 dilution (stock concentration ² 1 mg/ml). For some applications, these antibodies may be used at even higher dilutions. Remember that sample concentration, primary antibody concentration and secondary antibody concentration are all interrelated.

Antigen Optimization: The high intensity BioWest Substrate allows you to dilute your sample further than you do with standard chemiluminescent substrates. Usually, the sample may be diluted 10-20 fold, depending on the amount of antigen present in the sample.

Optimization of Blocking Reagent: The most appropriate blocking buffer for use in Western Blotting with enhanced chemiluminescent detection is often system-dependent. Determining the proper blocking buffer can help to increase the sensitivity of the signal produced. Occasionally, when switching from one substrate to another, the blocking buffer that you were 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 milk as a blocking reagent for blots that rely on the avidin/biotin system because milk contains variable amounts of biotin and phosphoproteins. Although SuperBlock ® Blocking Buffer (Pierce No. 37515) often gives excellent results, we recommend testing a few blocking reagents for their suitability with your particular system. There is no blocking reagent that will be the optimal reagent for all

systems. Blocker™ Buffer Sample Packs are available in either TBS or PBS (Pierce Nos. 37540 and 37510).

Use of Detergents: The use of Tween® -20 or Triton® X-100 is often recommended in washing steps and as an additive to diluents. We recommend using only high-quality products such as Pierce Product No. 28320 and 28314 because these are purified detergents packaged in ampules. They are guaranteed to be low in peroxides and other contaminants.

Troubleshooting and Precautions for Optimal Immunoblot Results

1. BioWest is a very sensitive and intense substrate. It is essential to optimize the immunoblot conditions to achieve maximum signal and minimum background. The most common problem encountered is excess background. It is important to understand that the substrate is "reporting" the presence of HRP, therefore, the reason for the background must be found in the preceding steps prior to HRP labeling. The antibody concentration required will be inherently more dilute than that required with traditional enhanced chemiluminescent systems. To optimize the appropriate concentrations, a dot blot systematic analysis may be performed. In general, you can dilute your antibodies about 10-20 fold in comparison to conventional enhanced chemiluminescent substrates.
2. A sufficient volume of wash buffer, blocking buffer and antibody solutions should be used to cover the blot and ensure that the blot never becomes dry. Larger blocking and wash buffer volumes may result in reduced nonspecific background. Make sure the blot moves freely and is surrounded by fluid at all times. Use a shaker or manually shake the container constantly. At least four to six changes of washing solutions will be needed.
3. To further decrease background signal, include 0.05% Tween® -20 and the appropriate blocker as a carrier solution for the dilution of the HRP-conjugated antibody as well as the unconjugated primary antibody.
4. Do not use sodium azide as a preservative for buffers with this immunoperoxidase system as it is an inhibitor of HRP. The presence of azide will greatly reduce or eliminate the signal.
5. Exposure times will vary with each blot and the type of system you are using. We recommend using the following ***Sequential Integration*** preset default times for use with various UVP BioImaging Systems for Chemiluminescent Detection:

Chemi System	Western Chemi 3
BioChemi System (binned 4x4, 16-bit mode)	Western Chemi 1
BioChemi System (binned 1x1, 12-bit mode)	Western Chemi 2
OptiChemi System	Western Chemi 1

6. If the signal is too intense, you may try to reduce the exposure time to 5-10 seconds, or to give the blot a short rinse in distilled water followed by incubation in a 1:5 dilution of the substrate working reagent (dilute in water). This is an emergency procedure to "save" a blot. It is better to repeat the experiment with a much higher dilution of the antibodies. A blot with a high background may, on occasion, be "saved" in the same manner.
7. A weak signal may be the result of insufficient quantities of antigen or antibody, a poor transfer of protein, or a loss of enzyme activity. If you would like to test the substrate, mix 1 ml of each solution in a glass test tube. Then, in the dark, add 2-5 µl undiluted HRP conjugate. You should observe a blue light that will persist for several minutes and then diminish. This demonstrates that the substrate is working well.
8. Spots within the protein bands may be a result of poor protein transfer, unevenly hydrated membrane, or a bubble between the film and the membrane. If you see a multitude of small spots, these are usually due to aggregates in the HRP conjugate and may be avoided by filtering the diluted HRP conjugate through a 0.2 µm filter prior to use in the blotting procedure.

Reprobing the Immunoblot

The immunoblot can be stripped of blocking reagent and antibody and then reprobed. Caution should be taken because some antigen/antibody systems may be more sensitive to the stripping procedure and may not yield adequate results. Only actual experimentation will yield information on whether or not a given system will allow reprobing.

Stripping

1. Incubate the blot in Restore Western Blot Stripping Buffer (Pierce No. 21059) for 5-15 minutes at room temperature on a shaking platform. Note: In general, higher affinity antibodies will require at least 15 minutes of stripping and **may** require incubation at 37°C.

   Alternatively, the blot can be incubated with a stripping buffer (2% w/v SDS, 62.5 mM Tris•HCl pH 6.8, 100 mM ß-mercaptoethanol) for 30 minutes at 50°C-70°C.
2. Wash the membrane with wash buffer 2-3 times for 10 minutes per wash.
3. Incubate the stripped blot with BioWest Working Solution to determine if the blot has been sufficiently stripped. Repeat the previous steps if any signal is detected.

Reprobing

1. Wash the blot with wash buffer 2 times for 10 minutes per wash.
2. Block with blocking reagent as described in the Western Blot protocol, then continue the procedure from that point.

References

1. Mattson, D.L. and Bellehumeur, T.G. (1996). Comparison of three chemiluminescent horseradish peroxidase substrates for immunoblotting. Anal. Biochem. 240, 306-308.

2. Lapham, C.K., et al. (1996). Evidence for cell-surface association between fusin and the CD4-gp 120 complex in human cell lines. Science 274, 602-605.

Trademarks

Ordering Information and Technical Support

BioWest Kit reorder part number 98-0066-01

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For details on recommended conjugates, blocking, wash and stripping buffers, contact Pierce Chemical Company at www.piercenet.com or (800) 874-3723 or (815) 968-0747

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