3050 Spruce Street
Saint Louis, Missouri 63103 USA
Telephone (800) 325-5832 (314) 771-5765
Fax (314) 286-7828
email: techserv@sial.com
sigma-aldrich.com

SIGMA

ProductInformation

GELATIN BLOCKING BUFFER
for Direct Detection Southern or Western Blotting

Product No. **G7663**

TECHNICAL BULLETIN

Product Description

In order to specifically detect an antigen or target molecule immobilized on a solid support, unoccupied binding sites on the support must be blocked against binding by probe and detection molecules. Otherwise nucleic acid probes, antibodies, and detection enzymes will bind randomly on the membrane as well as to the target molecules. Blocking these sites is one way background noise is minimized.

Blocking of the non-specific protein binding sites may be accomplished by a variety of protein and detergent solutions; however, the blocking solution must be compatible with the detection system. Gelatin Blocking Buffer is compatible with a variety of detection systems, including biotin, fluorescein, and DIG detection systems. Target molecules can be immobilized on nitrocellulose, nylon, and charge-modified nylon membranes. Gelatin blocking buffer is **not** suitable for blocking PVDF membranes.

Reagents

Gelatin Blocking Buffer is provided as dry powder which on reconstitution provides 1 liter of blocking buffer.

Reagents Required but not Provided

Sigma product numbers are given where appropriate.

- Phosphate Buffered Saline (PBS) with 0.05% (v/v) Tween-20 (PBS-T), Product No. P3563
- Protein probe or antibody
- 0.1 M Tris, 0.1M NaCl, pH 9.5 (for Southern blotting)

Preparation Instructions

Dissolve the contents of one container of the gelatin blocking buffer in 800 ml deionized water. Once the contents are dissolved, add deionized water to 1000 ml and stir to mix.

Storage/Stability

Store powder at room temperature. After reconstitution, store the gelatin blocking buffer at 2-8 °C to avoid bacterial contamination. Solutions may be kept up to one week at 2-8 °C following reconstitution.

Procedure

A. Recommended procedure for Southern blot blocking, probing, and detection:

Note: In sensitive systems, fingerprints will show up on the membrane. Use powder-free gloves at all times. Avoid the use of forceps with ridges, as contact points also tend to show up.

1. Following the transfer of labeled nucleic acid to a nitrocellulose, or cross-linking to nylon or positively charged nylon membrane, incubate the membrane with gelatin blocking buffer for 60 minutes (0.6 ml/cm^2) at ambient temperature or for 30 minutes at 37 °C with gentle agitation. Also, note that blocking can be accomplished overnight at 2-8 °C.

   Note: If a labeled nucleic acid probe is to be used to detect an unlabeled nucleic acid target, the membrane must first be blocked with a suitable single-stranded DNA according to a preferred procedure.

2. The membrane may now be probed with either protein conjugates or antibodies specific to the label on the nucleic acid.

   The protein conjugate or antibody should be diluted with phosphate buffered saline with 0.05% (v/v) Tween-20 (PBS-T). An initial concentration of 1-10 ng/ml is suggested when using streptavidin-alkaline phosphatase (Product No. S2890) or streptavidin-peroxidase (Product No. S5512) as the protein conjugate.

3. Incubate the membrane with protein conjugate or antibody solution (0.6 ml/cm^2) for 30-60 minutes at ambient temperature with gentle agitation.

4. Wash the membrane, with gentle agitation, three to five times for five minutes each with PBS-T.

5. If the protein conjugate utilizes alkaline phosphatase as the enzyme, it will be necessary to follow the PBS-T washes with three washes for three minutes each with 0.1 M Tris, 0.1 M NaCl, pH 9.5. The Tris washes remove any residual phosphate and equilibrate the membrane to an alkaline pH for assaying alkaline phosphatase. If the protein conjugate utilizes horseradish peroxidase as the enzyme, the membrane is ready to be exposed to substrate after the PBS-T washes.

6. The membrane may now be exposed to chromogenic or chemiluminescent substrate following the manufacturer's instructions.

B. Recommended procedure for Western blot blocking, probing, and detection:

1. Following the transfer of the protein of interest to a nitrocellulose or nylon membrane, incubate the membrane with gelatin blocking buffer for 60 minutes (0.6 ml/cm^2) at ambient temperature or for 30 minutes at 37 °C with gentle agitation. Also, note that blocking can be accomplished overnight at 2-8 °C.

2. Dilute the primary antibody in gelatin blocking buffer. A common dilution for primary antibodies is 1:1000, but may vary. Dilutions may vary from 1:100 to 1:100,000 or higher. The researcher must determine the optimum dilution factor.

3. Incubate the membrane with the primary antibody (0.6 ml/cm^2) for 1-16 hours at 2-8 °C with gentle agitation.

4. Wash the membrane, with gentle agitation, three to five times for five minutes each with PBS-T.

5. Dilute the secondary antibody-enzyme conjugate in gelatin blocking buffer and incubate the membrane for 30-120 minutes. After the incubation, wash the membrane with gentle agitation, five to six times for five minutes each with PBS-T.

6. The membrane may now be exposed to a chromogenic or chemiluminescent substrate following the manufacturer's instructions.

C. Suggestions for colorimetric detection of labeled nucleic acid:

1. The membrane should be exposed to the colorimetric substrate until a positive signal is seen but as background begins to develop, the reaction should be stopped. The membrane should be exposed to substrate for no longer than 60 minutes.

2. For colorimetric peroxidase substrates, the reaction may be stopped by removal of substrate and transfer of the membrane to a solution of 0.1% sodium azide with 1% SDS in either PBS or TBS (Tris buffered saline).

3. For alkaline phosphatase substrates, the reaction maybe stopped by removal of substrate and transfer of the membrane to a solution of 0.3 M sodium phosphate, pH 5.5.

D. Suggestions for chemiluminescent detection of labeled nucleic acid:

1. Following exposure to substrate, the excess substrate should be blotted off and the membrane transferred to a solid support. Transferring the membrane to a "page protector," slightly larger than the membrane itself, is recommended. The supported membrane should then be placed within a heat sealable bag. Using gentle pressure, smooth out air bubbles between the membrane and the plastic bag by rolling a glass test tube or pipet over the contained membrane. Seal the bag and wipe off any excess substrate from the outside of the bag. Place the contained membrane into a film cassette and expose to film.

2. Initially, an exposure of 1 minute should be used, however, if no signal is seen, expose the membrane to film for longer times. If excess signal is seen, use as short an exposure as technically possible. See the troubleshooting guide for other hints.

E. Suggestions for Western blot detection of immobilized protein:

1. Dilutions of the enzyme-antibody conjugate depend on the substrate used for subsequent detection and should be optimized by the researcher. General guidelines for dilutions are 1:5000 for chromogenic substrates and 1:50,000 for chemiluminescent substrates. The dilution ratios are based on an initial concentration of ~1 mg/ml enzyme-antibody conjugate.

2. To verify the quality of the secondary antibody, perform a "blank" membrane, in which the primary antibody is omitted. If background is present without the primary antibody, either use a different blocking reagent or dilute the conjugate further.

3. The antibodies that are used for the detection of antigens can usually be removed by immersion of the membrane in a buffer containing 100 mM glycine, pH 2.3 for 30 minutes with agitation. This is not a universally applicable procedure; some antigens are dissociated from the membrane, so subsequent probing will be faint or non-existent. Many researchers prefer to prepare membranes in parallel if possible, thereby avoiding the uncertainty of this "stripping" step.

References

For Southern blotting:

1. Sambrook, J. F., et al. *Molecular Cloning: A Laboratory Manual,* 2nd Edition, p. 9.47-9.50, (Cold Spring Harbor Laboratory Press, 1989).

For Western blotting:

1. Bjerrum, O. J. and Heegaard. N. H. H. *CRC Handbook of Immunoblotting of Proteins,* **Vol. I,** Technical Descriptions, p. 229-236, (CRC Press, 1988).

2. Dunbar, B. S. (ed.) *Protein Blotting: A Practical Approach,* p. 67-70, (IRL Press, NY, 1994).

3. Fortin, A., et al., A 56- to 54-kilodalton non grata signal in immunoblot analysis using the horseradish peroxidase chemiluminescence system. Biochem. Cell Biol., **72,** 239-43 (1994).

Troubleshooting Guide

Problem	Possible Cause	Remedy
No Signal	No target present	If the labeled nucleic acid is not present on the membrane, it cannot be detected. Include a positive control with the next assay.
	No target protein present	Verify transfer by visualizing proteins on the membrane using a Ponceau S solution (Product No. P7170). If possible, a positive control should always be run to insure components are functioning.
	Overblocking	Masking of a signal can occur if the blocking reagent is used at too high a concentration. A dilution of 1:1 to 1:3 may be done to decrease the concentration. If the problem persists, different blocking reagents should be tried.
	Inadequate exposure time using chemiluminescence system	First exposure should be 1 minute. If no signal is seen, expose for longer times. Sigma recommends trying 5 minutes, 10 minutes, etc. If excess signal is seen, try as short an exposure as practical (down to 1 second) without using a cassette
	Enzyme conjugate may have lost enzymatic activity	Determine if the enzyme conjugate is active.
High Background	Too much antibody or protein conjugate	Perform a titer of the antibody or protein conjugate until an acceptable signal to noise ratio is obtained.
	Inappropriate blocking reagent	Increase the concentration of the blocking reagent by preparing the reagent with one-half the recommended volume of water. In addition, some antibodies may cross-react with certain blocking reagents. To test for this possibility, prepare a "blank" membrane that does not contain the primary antibody.
	Inappropriate blocking protocol	Increase the blocking time and increase the blocking temperature to 37 °C.
	Inappropriate wash protocol	Increase the number of washes.
	Overincubation in colorimetric substrate solution	Decrease the staining time. The membrane should be exposed to the colorimetric substrate until a positive signal is seen, but as the background begins to develop, the reaction should be stopped. For colorimetric substrate: Incubate for 5-10 minutes or whenever bands are visible. The time required may be increased or decreased, but should not be longer than 60 minutes. To stop reactions: For alkaline phosphatase substrates, use a solution of 0.3 M sodium phosphate, pH 5.5. For horseradish peroxidase substrates, wash the membrane with 0.1% sodium azide with 1% SDS in either TBS (Tris buffered saline) or PBS (phosphate buffered saline).
	Inappropriate film	Switch to film designated for chemiluminescent detection such as Kodak Biomax Light, MS, and MR.
Extraneous spots	Aggregated protein or antibody conjugate	Filter the conjugate through a 0.2 micron cellulose acetate filter or centrifuge the conjugate solution at 10,000 x *g* for 10 minutes and use the supernatant.

JWM 7/11/03

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