Blocking: Key to Optimizing Your Western Blots

Introduction

An important key to a successful Western blot is often overlooked – selection of the blocking buffer. In the Western blotting workflow, blocking is done after proteins are transferred from the gel to the membrane, and before the membrane is incubated with the primary antibody (Figure 1). The purpose of the blocking step is to prevent or reduce nonspecific binding of the primary and secondary antibodies to proteins on the membrane or to the membrane itself.

When blocking works optimally, background is eliminated and the signal-to-noise ratio is increased, providing cleaner blots and more robust data. Researchers may spend a great deal of time optimizing many aspects of their experiment – identifying the optimal amount of sample to load on the gel, the buffer makeup and acrylamide percentage of the gel, the type and pore size of the membrane, and the dilution factor for primary and secondary antibodies – but use a standard blocking buffer without considering the potential effects of different blocking agents and blocking buffer components on their experimental system. The dramatic effect blocking buffer can have on Western blot data is demonstrated in Figure 2.

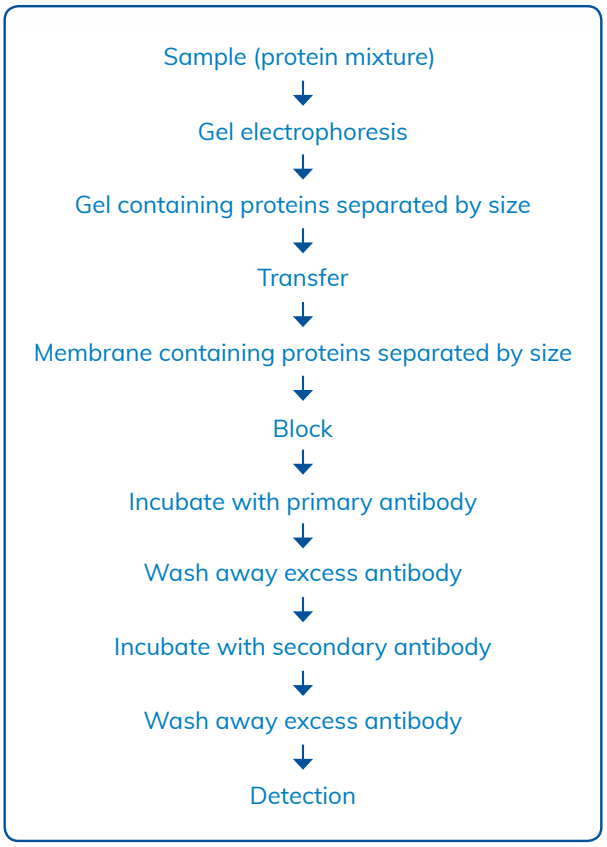

Figure 1. Overview of a Western blotting protocol using indirect antigen detection.

Blocking buffer components

Blocking buffer may be used in two places in the Western blotting workflow. First, the membrane is incubated with the blocking buffer during the blocking step. Additionally, the primary and secondary antibodies may be diluted in the same or an additional blocking buffer during the antibody incubation steps to further prevent nonspecific binding. When designing a blocking buffer, a balance must be reached between preventing nonspecific binding while not disrupting the specific antibody-antigen binding, and components must be chosen that will not interfere with downstream detection systems. Though finding the best blocking buffer may require a process of trial and error, there are some characteristics of blocking buffer components that can help guide selection of the initial blocking buffer tried.

Blocking agents

The blocking agent is the blocking buffer component that will bind to or "block" nonspecific binding sites that would otherwise adsorb primary or secondary antibody. The blocking agent may be protein or non-protein based. Some of the more commonly used blocking agents are discussed below.

Normal serum—Whole serum may be used as a blocking agent. Serum from the same species as the secondary antibody is often used, but serum can be from any species except the source of the primary antibody/target of the secondary antibody (that is, do not block with rabbit serum if using a primary antibody raised in rabbit). In practice, serum can become expensive and in some cases has been found to result in higher background than other blocking agents (Hauri 1986).

Bovine serum albumin (BSA)—A solution of 3% BSA in saline was used as the blocking buffer in one of the first papers to describe protein immunoblotting (Towbin 1979). Early studies to identify the optimal protein solution to use as a blocking agent determined that BSA provided the best combination of satisfactory blocking, availability, and expense (Burnette 1980). Purified preparations of BSA for use as a blocking agent are readily available and BSA remains a common choice for immunoblotting.

Nonfat dry milk—Though BSA performs well in many scenarios, the cost of its use as a blocking agent can become prohibitive when a large number of Western blots are performed. In search of a less-expensive option, Johnson et al identified an alternative blocking solution they called "BLOTTO", consisting of 5% nonfat dry milk in PBS (Johnson 1984). Nonfat dry milk remains a very popular blocking agent. However, milk is not recommended as a blocking agent under certain conditions. Milk contains phosphoproteins so may result in high background when the target antigen is a phosphorylated protein. In addition, milk can contain avidin which will interfere with streptavidin- and avidin-based detection systems. Membranes should not be exposed to milk solutions for long incubations since bacterial growth and precipitation of components can occur.

Casein—Casein is the most abundant protein in milk. Purified preparations of casein are readily commercially available and casein is frequently used as blocking agent. It may present a less-expensive alternative single-protein blocking agent to BSA. Because casein is a phosphoprotein, it is not recommended when the protein of interest is a phosphoprotein.

Gelatin—Gelatin consists of proteins and peptides resulting from the partial digestion of collagen. Porcine and bovine gelatins have been used as blocking agents in Western blotting, but recently gelatin from cold-water fish has become a more popular option. Because fish gelatin does not contain mammalian serum proteins, it may reduce the chance of background due to cross-reaction between buffer components and mammalian antibodies.

Polyvinylpyrrolidone (PVP-40)—PVP-40 is a water-soluble polymer that is a component of Denhardt's Solution, long used as a blocking agent used for Northern and Southern blotting. PVP-40 has been demonstrated to be an effective protein-free blocking agent for Western blots, particularly in combination with Tween 20 (Haycock 1993).

Tween 20—Nonionic detergents can prevent weak noncovalent and hydrophobic interactions between proteins, and between proteins and the membrane, without interfering with specific antigen-antibody binding. However, they can also cause loss of target protein from the membrane. Because it was found to be least likely to cause loss of antigen, Tween 20 is the nonionic detergent most frequently included in blocking buffers (Haycock 1993). Tween 20 is often added at a low percentage to buffers containing another blocking agent. Some studies, however, have found Tween 20 alone to be sufficient as a blocking agent and Haycock found Tween 20 to be superior to protein blocking agents and to PVP-40 when those agents were used alone (Haycock 1993).

Blocking agent	Considerations
Nonfat dry milk	• Inexpensive • Along with BSA, one of the most frequently used blocking agents • Contains phosphoproteins; may interfere with some antigens • Contains avidin; may interfere with some detection systems
BSA	• Along with nonfat dry milk, one of the most frequently used blocking agents • Compatible with most antigens and detection systems
Casein	• Is a phosphoprotein so may interfere with some antigens
Fish gelatin	• Does not contain mammalian proteins; may reduce potential for cross-reaction of blocker components with mammalian antibodies
PVP	• Does not contain protein; may reduce potential for cross-reaction of blocker components with antibodies
Tween-20	• Does not contain protein; may reduce potential of cross-reaction of blocker components with antibodies • Predicted to disrupt weak interactions but not stronger, specific antibody-antigen interactions • May cause loss of proteins from membrane

Other considerations—Blocking buffers are often prepared in phosphate-buffered saline (PBS) or Tris-buffered saline (TBS). For detecting phosphorylated proteins, TBS is recommended because PBS may interfere with the antibody-antigen interaction. PBS can also interfere with alkaline phosphatase detection systems.

Finally, good laboratory technique is important to achieving optimal blocking. Blocking buffers should be freshly prepared and free of particulates. During incubation in blocking buffer, the blot should be agitated to allow even distribution of the blocking agent over the surface of the blot and to prevent settling of particulates onto the blot. And, blocking time should be controlled; in some blocking buffers, long blocking times can result in loss of protein from the blot (DenHollander 1989).

Take the guessing out of choosing a blocking buffer

Azure Biosystems offers three blocking buffers to simplify Western blot optimization. Azure Chemi Blot Blocking Buffer is formulated to reduce background and increase signal with chemiluminescent blots. Azure Fluorescent Blot Blocking Buffer provides effective blocking while also stabilizing fluorescent signal. Azure Protein-Free Blot Blocking Buffer is protein-free to reduce background with antibodies that demonstrate a high degree of cross-reactivity with protein blocking agents such as nonfat dry milk, BSA or casein (Figure 2). Contact Azure for more information about which blocking buffer is best for your experiment.

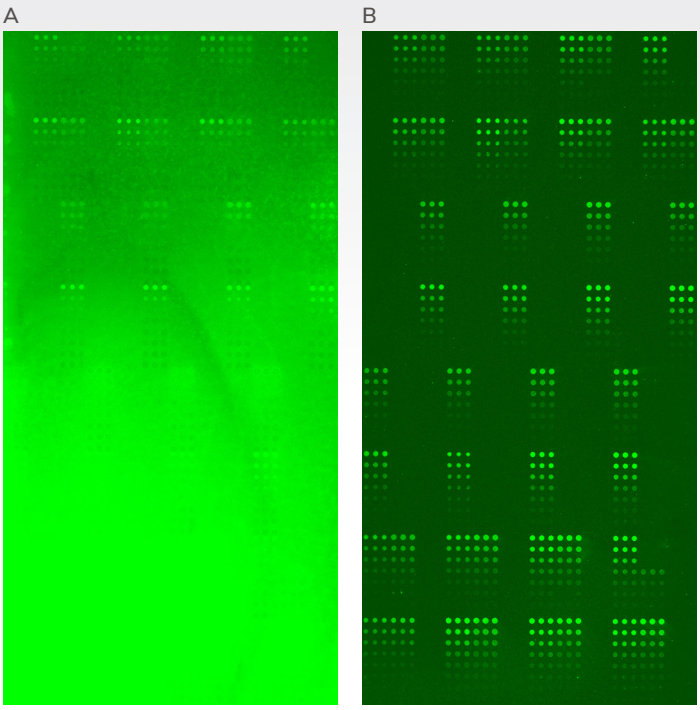

Figure 2. Dramatic effect of blocking buffer on background and signal-to-noise ratio. Duplicate dot blots were processed and imaged identically except for the blocking step which was either 5% nonfat dry milk in TBST (A) or Azure Protein-Free Blot Blocking Buffer (B).

References

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