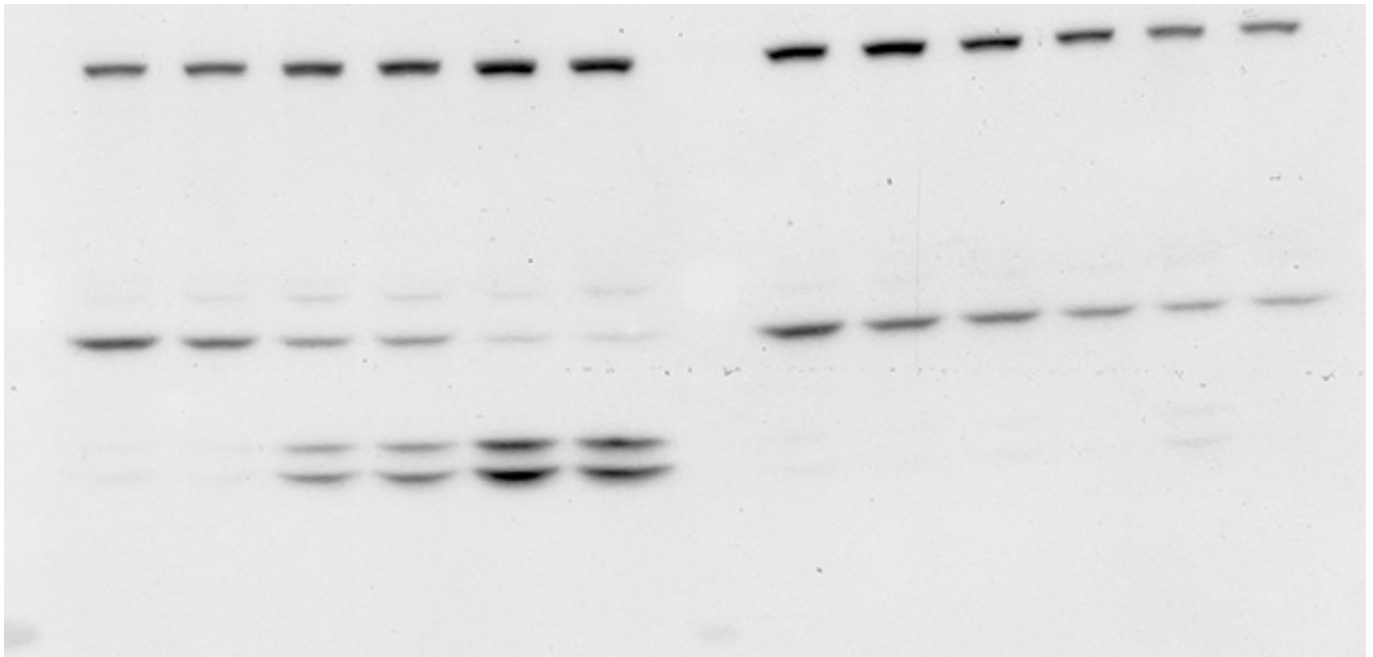


Primary antibodies are used to detect the protein of interest. Part 5 of the Western blotting guide details appropriate methods to encourage the primary antibody to perform optimally and that your Western blot performs efficiently.



Overview

- [Considerations for Selecting a Primary Antibody](#)
- [Host Species of the Primary Antibody](#)
- [Monoclonal and Polyclonal Primary Antibodies](#)
- [Primary Antibody Diluent](#)
- [Reprobing and Stripping for Additional Target Proteins](#)
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- [Reproducibility & Controls](#)
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The primary antibody is the reagent used to detect the protein of interest. Added after membrane blocking, the primary antibody binds specifically to the protein of interest. The membrane is incubated with the primary antibody at room temperature (1 hr is commonly used) on a rocker or shaking platform, although overnight incubations at 4°C may also be effective. Following incubation, with the primary antibody the membrane is washed to remove any excess and unbound antibody. This maximizes the sensitivity of this technique and increases the signal-to-noise ratio.

Considerations for Selecting a Primary Antibody

There is a wide range of primary antibodies available commercially in different formats, clonality and specificity, which are important factors to consider when selecting an appropriate reagent.

Specificity - Immunogens

The type of immunogens used to produce the antibody dramatically influences the specificity of the antibody for the target protein. Choosing an antibody raised against an unsuitable or poor quality immunogen will lead to unreliable and potentially irreproducible results. Care should be taken to select antibodies raised against well-characterized immunogens.

Peptides

Synthetic peptide immunogens rarely fold into the comparable tertiary structure of the native protein. In addition, unless added to the peptide during synthesis, they do not exhibit any post-translational modifications. Therefore, antibodies generated using peptides can be useful reagents for western blotting as target proteins have linearized epitopes due to denaturation and reduction during sample processing. Peptides can also be useful as immunogens when an antibody needs to differentiate between two proteins having highly conserved amino acid sequences. In those cases, peptides generated to the regions of difference increase the possibility of making a differentiating antibody.

Native Proteins

If purified proteins are used as an immunogen, the antibodies generated against them may not recognize the linearized epitopes of the denatured proteins in western blotting. Some antibodies raised using native protein will only recognize the epitopes on the surface of a protein in its native, oxidized conformation. This is especially true of monoclonal antibodies.

Host Species of the Primary Antibody

Ideally, the primary antibody should be raised in a different species than the sample to be analyzed. This is to avoid the secondary antibody detecting immunoglobulins from the sample if present. For example, a sample from mouse may contain mouse immunoglobulins. If the primary antibody is also made in mouse, an anti-mouse secondary antibody will detect not only the primary antibody binding to the protein of interest but also to the heavy and light chains of the immunoglobulin in the mouse sample. This can be particularly problematic when the protein of interest has a molecular weight that is similar to either that of the heavy chain (50 kDa) or the light chain (25 kDa), as differentiation between the protein of interest and endogenous mouse IgG becomes impossible.

Monoclonal and Polyclonal Primary Antibodies

Both monoclonal and polyclonal antibodies can be used for western blotting. Monoclonal antibodies are

produced from a single clone of B cells and produce antibodies specific to one epitope. Whereas polyclonal antibodies are produced from differing B-cell lineages and so contain multiple clones recognizing different epitopes on the antigen. Both types have benefits and limitations (which are shown in the table below).

Table 1. Advantages and disadvantages of Monoclonal and Polyclonal antibodies.

	Description	Advantages	Disadvantages
Monoclonal	Produced from a single B-cell lineage – specific to one epitope on an antigen.	High specificity, batch-to-batch consistency and are often well characterized.	Possible lower sensitivity due to reduced binding affinity for epitopes compromised by sample preparation/electrophoresis.
Polyclonal	Produced from differing B-cell lineages – recognize multiple epitopes on the antigen.	High sensitivity from signal amplification and detection of multiple epitopes.	Specificity relies on quality immunogens, and may suffer from batch-to-batch variability.

Primary Antibody Diluent

The working solution of the primary antibody is usually made by diluting it using either TBS (Tris-Buffered Saline) or PBS (Phosphate-Buffered Saline). It is essential that the buffer maintains the antibody's biological activity; therefore, the manufacturer's recommendations should be followed. Buffers containing detergents (TBS-Tween or PBS-Tween) which are used in washing steps, are also suitable as antibody diluents. As mentioned in Section 4, avoid PBS when detecting phosphorylated proteins because phosphate ions can interfere with the antibody binding.

Caution: Primary antibody diluent buffer

Avoid adding proteins such as powdered milk or BSA to antibody diluent solutions to prevent non-specific interactions that may lead to background signal.

The concentration of the working antibody solution should be optimized for efficient detection of target protein. Dilute solutions risk not saturating target protein, resulting in low signal. Overly high concentration solutions are not only wasteful but can lead to non-specific bands, high background, or excessive signal intensity.

Titrate Your Antibodies

It is good practice to titrate antibody concentrations to determine optimal working concentration. Use two-fold dilutions either side of the manufacturer's recommendations to identify the optimum antibody concentration for the specific western blot that is being performed.

Higher temperatures, and longer incubation times, increase the incidence of binding events, both specific and non-specific. Conventionally the membrane is typically incubated with the primary antibody solution for one hour at room temperature or 4°C overnight on a rocker or shaking platform.

Reprobing and stripping for additional target proteins

Membranes may be stripped to remove the primary and secondary antibody complex and reprobed with a second pair of antibodies to allow the detection of a second target protein. The membrane is stripped with

a low pH buffer such as 1M glycine to remove the primary and secondary antibody complex from the antigen bound to the membrane. The membrane is then blocked and incubated with a new primary antibody, washed and incubated with a compatible secondary antibody and substrate before being reimaged.

The success of stripping can be highly variable, and must be carefully performed to prevent either being too harsh and damaging the antigen. Alternatively, conditions too mild to strip the antibody complex from the membrane may lead to continued detection.

The results of a reprobed blot should only be used for confirmation, not for quantitation. Choosing a second primary antibody raised in a different host to the initial antibody can help improve specificity of the reprobing. Stripping and reprobing membranes for additional targets are typically applied in chemiluminescent detection, as fluorescent conjugates offer a more reliable method for detecting multiple targets, see Section 8 for more information about multiplex detection.

Considerations for Antibody Validation

Each primary antibody must perform as expected, detecting the protein of interest under the experimental conditions. Ideally, suitability for the experiment would be validated for each primary antibody and protein pair for each new experimental setup. Four aspects of primary antibody performance should be addressed during the validation processes. These are:

1. Specificity to the antigen/protein of interest
2. Specificity to the antigen under experimental conditions (native or denatured)
3. Affinity for the antigen
4. Reproducibility

Specificity to the antigen/protein of interest

Use negative and positive controls to establish that a primary antibody is specific to the protein of interest, ideally using purified and/or control material. It is important to include a blank sample or negative sample control to establish if the primary antibody detects endogenous proteins.

Specificity to the antigen under experimental conditions (native or denatured)

It is important to establish if a primary antibody is suitable for the detection of the target protein under the experimental conditions. This may be because a primary antibody that is suitable for use in IHC, where antigens are in their native forms, may not be able to detect the linearised antigens of proteins in western blotting and vice versa.

Affinity for the antigen

Although a primary antibody may detect the protein of interest specifically or across a range of techniques, it may not have sufficient affinity to produce a signal brighter than background.

Reproducibility

One of the problems that can be encountered with primary antibodies is the difficulty in replicating results. This may be because of batch-to-batch variability or poorly characterized specificity.

A recent movement in life-science research has been to encourage manufacturers to validate their antibodies and confirm detection of stated target antigens in specific applications. In an effort to improve and promote research resource identification, discovery, and reuse, the Resource Identification Portal was created in support of the Resource Identification Initiative. Research Resource Identifiers (RRIDs) are assigned to research materials and are unique identifiers allowing researchers to trace materials.

Specific to antibodies, the [Antibody Registry](#) catalogs antibody RRIDs to clarify their use in published literature.

Ideally, a researcher should confirm the product performs reliably, preferably using a positive control in the technique desired.

Controls

Positive control

Most primary antibodies are available with a positive control from purified protein. These controls can be run in parallel with the sample and the migration of the protein compared to the bands detected from the sample.

Tissue controls

If possible, a positive tissue control is used to determine antibody specificity and performance in detecting processed proteins. The specificity of the antibody is confirmed using cells or tissue samples known to express the protein of interest. A band observed at the expected weight provides a positive indication of antibody specificity. A negative tissue control is useful to determine non-specificity of the primary antibody. A sample of tissues or cells known not to express the target protein is probed with the primary antibody. An absence of bands is desired; any non-specific detection of proteins in the sample can indicate detection of endogenous proteins.

Negative or secondary antibody control

A secondary antibody control is simply the probing of the membrane without the primary antibody. This can be used to confirm if blocking/washing of membrane has been adequate and identify any background signal generated by the secondary antibody.

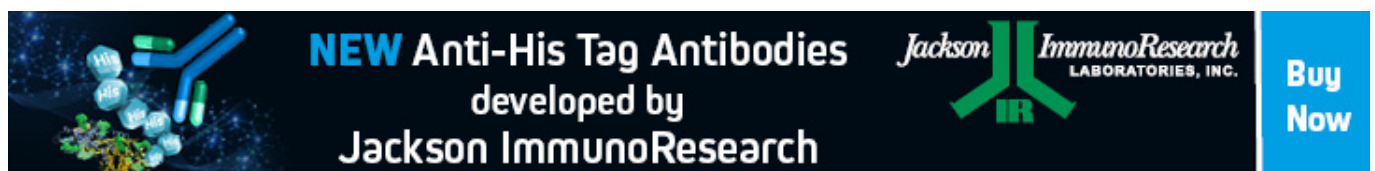
Storage

Manufacturer's guidelines should be followed for Primary antibody storage. Generally speaking, Primary antibodies should be aliquoted and frozen in single-use volumes, and working solutions should be made up fresh on the day of intended use to avoid multiple freeze-thaw cycles.

Found Part 5 of our Western blotting guide useful? sign-up for part 6 [here](#)

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