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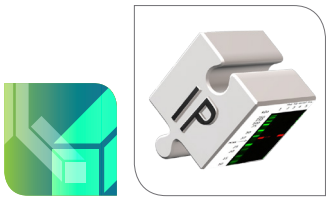
10 Tips for the western blot detection of IP samples



1 Select an immunoprecipitation (IP) tested antibody

When selecting an antibody for an IP experiment it is important to confirm that the antibody has been tested in IP. In contrast to other commonly performed antibody applications, such as western blotting, antibodies used in an IP experiment have to recognize native rather than denatured proteins. Depending on protein folding and confirmation, the specific epitope against which a monoclonal antibody was raised may not be accessible to the IP antibody. Therefore, polyclonal antibodies are often selected when first setting-up IP experiments; their poly-specific nature increases the probability of containing one antibody that recognizes the protein of interest.

2 Review antibody binding affinities to Protein A and Protein G

Once you have selected the best antibody for your experiment, it is important to review what bead type to use. Both agarose and magnetic beads are available in Protein A and Protein G formats and the choice between Protein A or Protein G is dependent on the antibody host species and isotype. For more information about antibody binding affinities to Protein A and Protein G, please refer to bio-rad-antibodies.com/binding-affinities.

3 Include appropriate controls

Appropriate controls are essential for every experiment as they assist with experimental troubleshooting and provide assurance that you have immunoprecipitated your protein of interest. For IP experiments it is important to include an isotype control (also known as mock-IP), which controls for unspecific binding of your protein of interest. One generally performs the mock-IP with an unrelated antibody of the same isotype and host species as the antibody used during the IP procedure. Input/whole cell lysate controls are also frequently included in experimental set-ups (please refer to Tip 10).

4 Carefully choose your lysis buffer

Selecting the optimal lysis buffer is essential, and different lysis methods are generally recommended for different protein types and purposes. As a general rule, the lysis buffer you select should result in efficient enough lysis while preserving the native confirmation and activity of your protein of interest. Buffers such as radioimmunoprecipitation assay (RIPA) buffer, which contain ionic detergents tend to be more stringent than those containing non-ionic detergents, like Triton™ X-100. Although RIPA is often the lysis buffer of choice when analyzing membrane proteins, the sodium dodecyl sulfate (SDS) contained in the RIPA buffer has been reported to denature kinases (Couthon et al. 1996).

It is also important to add protease, phosphatase and deubiquitinase inhibitors to the lysates. This ensures that your protein of interest and potential post-translational modifications remain intact.

5 Consider cellular fractions or concentrating your sample

If you intend to immunoprecipitate a low abundant protein, you should use the smallest volume of lysis buffer possible to concentrate your sample. Alternatively, if the subcellular location of your protein of interest is known, e.g. if your protein is localized in the nucleus, you could perform cellular fractionations and exclusively use the nuclear fraction for performing the IP.

6 Optimize the antibody amount

Determining the optimal quantity of antibody to couple to beads is important as addition of insufficient amounts of antibody may result in an inability to immunoprecipitate your protein of interest. Using excess quantities of antibody, on the other hand, may increase the risk of unspecific binding as well as the likelihood of antibodies being eluted off the beads. To mitigate the risk of background staining on your western blot, it is therefore important to perform antibody titrations to determine the optimal amount of antibody required for recovering your protein of interest.

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Wash efficiently

Sufficient washing is essential to mitigate the risk of your IP sample containing proteins other than your protein of interest and its interaction partners. High background staining on your western blot may be a sign of insufficient washing. It is therefore important to remove as much supernatant as possible during each wash step without disturbing the beads. You may also need to perform additional wash steps when using agarose beads compared to magnetic beads, as the increased risk of losing/pipetting up beads requires you to leave some supernatant.

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Select the best elution method

Elution is a critical step in the IP procedure as ineffective elution may result in an inability to detect your protein of interest by western blotting. However, too stringent elution conditions may result in large amounts of antibody being eluted off the beads and western blot detection of these antibodies, specifically their denatured forms (the antibody heavy and light chains seen on western blots of IP samples originate from the antibody used during the IP procedure).

Eluting your sample directly in Laemmli sample buffer containing SDS but no reducing agents is a commonly used and stringent elution method. Once the Laemmli buffer has been added, the sample is heat denatured. The beads are then collected and the supernatant is transferred to a new vial to which a reducing agent is added. Alternatively, the elution may also be performed with Laemmli buffer containing reducing agents. However, this method may increase the amount of antibody being eluted off the beads.

In addition to performing direct elution into sample buffer, glycine (pH 2 – pH 3; e.g. pH 2.5) containing elution buffers are frequently used. Elution with these buffers is less stringent and therefore mitigates the risk of eluting antibodies off the beads. In addition, since this technique utilizes a pH change to elute proteins off beads, rather than denaturing, the elution of native proteins is possible.

To determine the best elution method for your protein of interest you may have to compare a variety of elution methods, which in addition to the described two methods may include urea containing elution buffers (Sousa et al. 2011, Cheeseman Lab 2007).

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Denature your sample efficiently

Especially when performing direct elution in sample buffer, the duration and temperature of your heat treatment are critical. Inefficient denaturing not only reduces the amount of recovered protein, it also results in insufficient denaturing of the eluted antibodies. This creates a problem when performing western blot analysis with secondary detection reagents specific for native antibodies. Inadequate denaturing will render reagents such as TidyBlot™ ineffective as these reagents are only able to distinguish between native and denatured antibodies when the IP sample has been sufficiently denatured.

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Run a whole cell lysate/input sample on your western blot

Taking a whole cell lysate/input sample prior to the IP procedure is critical. This lysate, when run on the same SDS-PAGE gel and prepared under the same conditions as your IP samples, acts as a positive control for your western blotting experiment; it enables you to determine whether your selected primary antibody detects your protein of interest. The input sample is also often used for the quantification of IP samples and provides information on the efficiency of enriching your protein by immunoprecipitation.

References:

Cheeseman Lab (2007). LAP Protocols: One-Step IP. http://jura.wi.mit.edu/cheeseman/protocols.php, accessed September 23, 2016.

Couthon F et al. (1996). Denaturation of MM-creatine kinase by sodium dodecyl sulfate. J Protein Chem 15, 527-537. http://www.ncbi.nlm.nih.gov/pubmed/8895099.

Sousa MML et al. (2011). Antibody cross-linking and target elution protocols used for immunoprecipitation significantly modulate signal-to noise ratio in downstream 2D-PAGE analysis. Proteome Science, 9:45. http://www.ncbi.nlm.nih.gov/pubmed/21816076.

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