Western Blotting

Western Blot Detection of Immunoprecipitation Samples

BIO-RAD

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1. Select an IP Tested Antibody

When selecting an antibody for an IP experiment it is important to confirm that the antibody has been tested in IP (Figure 1).

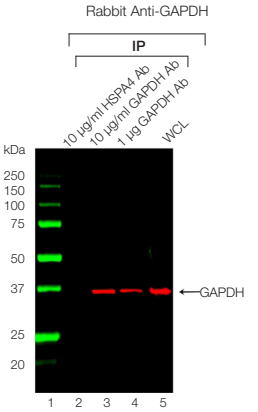


Fig. 1. Western blot analysis of GAPDH IP samples. IP was performed on Jurkat cell lysates using Human Anti-GAPDH Antibody (Cat. #HCA272) 10 µg/ml (lane 3) and 1 µg/ml (lane 4); 10 µg/ml Human Anti-HSPA4 Antibody (#HCA273) was used as a negative control (lane 2); Precision Plus Protein Prestained Standards (#1610373) were run in lane 1 and Jurkat whole cell lysate WCL was run in lane 5 as positive control. Western blot bands (red) and protein standards (green) have been pseudocolored. Visit **bio-rad.com/GetStandards** for more information on protein standards.

In contrast to other commonly performed antibody applications, such as western blotting (WB), antibodies used in an IP experiment have to recognize native rather than denatured proteins. Depending on protein folding and conformation, 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 polyspecific nature increases the probability of containing one antibody that recognizes the protein of interest.

2. Review Antibody Binding Affinities

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

Fig. 2. SureBeads Protein G (#1614023) and Protein A (#1614013) are designed for quick and easy IP experiments. SureBeads Magnetic Beads contain a superparamagnetic core, which enables rapid and highly efficient immunoprecipitation without the need for centrifugation. Visit **bio-rad.com/surebeads** for more information.

3. Include Appropriate Controls

Appropriate controls are essential for every experiment, 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 nonspecific binding of your protein of interest.

Mock-IP is generally performed with an unrelated antibody of the same isotype and host species as the antibody used during the IP procedure (Figure 3, p7). Input/whole cell lysate controls are also frequently included in experimental set-ups (please refer to Tip 10, p12).

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 should result in efficient enough lysis while preserving the native conformation 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 nonionic 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 and phosphatase inhibitors to the lysates. This ensures that your protein of interest and potential post-translational modifications remain intact (Table 1).

Table 1. Overview of commonly used protease inhibitors.

Inhibitor	Protease Inhibited
Aprotinin	Serine proteases
EDTA	Metalloproteases
Leupeptin	Serine and cysteine proteases
Pepstatin	Aspartic proteases
PMSF	Serine proteases

5. Concentrate 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, for example 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 the addition of insufficient amounts of antibody may result in an inability to immunoprecipitate your protein of interest (Figure 3). However, using excess quantities of antibody may increase the risk of nonspecific binding as well as the likelihood of antibodies being eluted off 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 target protein.

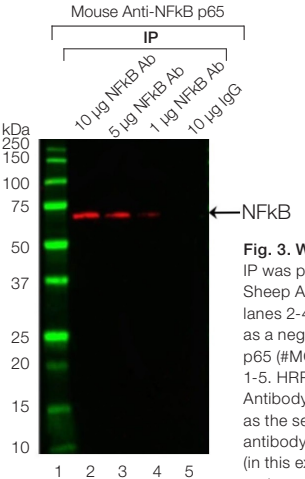


Fig. 3. WB analysis of NFkB p65 IP samples. IP was performed on Jurkat cell lysates using Sheep Anti-NFkB p65 Antibody (#AHP394; lanes 2-4) and Sheep Polyclonal IgG was used as a negative control (lane 5). Mouse Anti-NFkB p65 (#MCA6021) was used at 1/1,000 in lanes 1-5. HRP conjugated Goat Anti-Mouse IgG (H/L) Antibody (#STAR117P) was used at 1/10,000 as the secondary antibody. Using 10 µg antibody results in the best NFkB p65 recovery (in this experiment). Western blot bands (red) and protein standards (green) have been pseudocolored.

7. Wash Efficiently

Sufficient washing is essential to reduce the risk of your IP sample containing proteins other than your protein of interest and its interaction partners. High background staining on your WB 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 in the test tube.

8. Select the Best Elution Method

Elution is a critical step in the IP procedure as poor elution may result in an inability to detect your protein of interest by WB. However, too stringent elution conditions can result in large amounts of antibody being eluted off the beads. Keep also in mind that antibody heavy and light chains, seen on western blots of IP samples, originate from the antibody used during IP.

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 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 eluted off the beads.

As well as performing direct elution into sample buffer, glycine (pH 2-pH 3; for example, pH 2.5) containing elution buffers are frequently used. Elution with these buffers is less stringent and therefore reduces the risk of eluting antibodies off the beads.

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 those described, may include urea containing elution buffers (Sousa et al. 2011, Cheeseman Lab 2007).

9. 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 but also results in insufficient denaturing of the eluted antibodies. This creates a problem when performing WB analysis with secondary detection reagents specific for native antibodies. Poor denaturing will render reagents, like TidyBlot Reagent (#STAR209P), ineffective as these reagents are only able to distinguish between native and denatured antibodies when the IP sample has been sufficiently denatured.

10. Run an 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 WB experiment. You can then determine whether your selected primary antibody detects your protein of interest (Figure 4). The input sample is also often used for the quantification of IP samples and provides information on the efficiency of enriching your protein by IP.

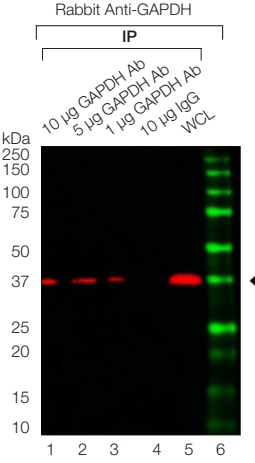
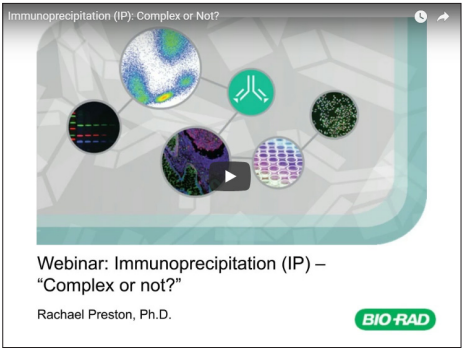


Fig. 4. WB analysis of GAPDH IP samples. IP was performed on Jurkat cell lysates using Mouse Anti-GAPDH Antibody (#MCA4739) and 10 µg Mouse IgG1 Negative Control (#MCA1209). Rabbit Anti-GAPDH Antibody (#VPA00187) was used at 1/1,000 in lanes 1-5. HRP conjugated TidyBlot Reagent (#STAR209P) was used at 1/200 and visualized on the ChemiDoc MP Imager. Western blot bands (red) and protein standards (green) have been pseudocolored.

On Demand Webinar: “Complex or not?” — learn how to use immunoprecipitation to analyze protein-protein interactions and protein complexes

Presented by: Dr Rachael Preston (Applications Scientist at Bio-Rad Laboratories).

Duration: 1 hr



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References

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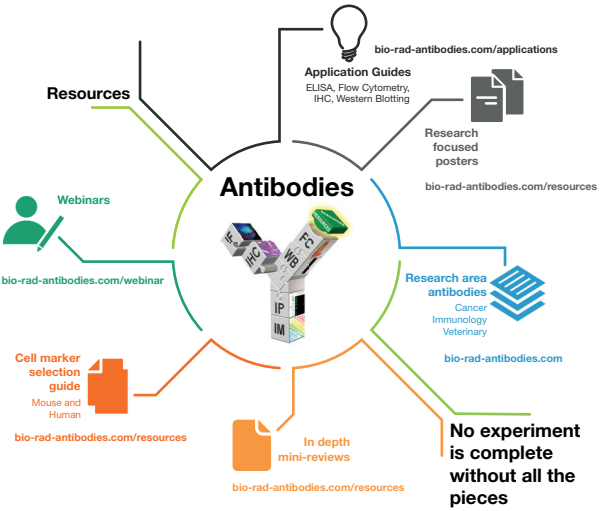

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