BIO-RAD

HuCAL® Antibodies Technical Manual
Immunoprecipitation

In an immunoprecipitation (IP) experiment, an antibody is cross-linked to agarose, sepharose or magnetic beads in order to capture a protein of interest present in a lysate. The technique is mainly used for the analysis of protein-protein interactions, the characterization of protein complexes and the identification of post-translational modifications. For these purposes IP samples are first run on SDS-PAGE gels, followed by transfer onto membranes for western blot analysis. Alternatively, gels are silver or coomassie stained to visualize the immunoprecipitated protein(s). Bands of interest are then extracted from the gel and prepared for analysis by mass spectrometry. With the help of this technique, both new interaction partners and modifications, such as phosphorylated residues, may be determined. The IP procedure consists of four stages:

- Immobilization of the antibody onto a matrix
- Solubilization of the antigen
- Immunoprecipitation
- Analysis of the precipitated protein(s)

Basic procedures for each step are outlined in this chapter.

Protein A or G Sepharose is often used as a matrix to which conventional antibodies are cross-linked, since these compounds bind to the Fc portion of the antibody.

For HuCAL antibodies in full immunoglobulin (Ig) format we recommend Bio-Rad SureBeads™, Protein A or G Magnetic Beads. However, for HuCAL Fab antibodies a different strategy is required, since the Fab antibodies lack the Fc region.

There are three procedures suitable for immobilizing HuCAL Fab antibodies (Table 1):

1. Covalent coupling on magnetic beads via primary amino groups.
2. Immobilization on Ni-NTA magnetic beads via the His-6 Tag or immobilization on MagStrep Strep-Tactin beads via the Strep-tag.
3. Immobilization of a capture antibody on magnetic beads and incubation with the HuCAL antibody.

Magnetic beads are preferable to non-magnetic beads because they are easier to wash. Ni-NTA beads should not be used with lysis buffer containing metal-chelating compounds, for instance EDTA. Protocols for the first two procedures are shown.

For information about HuCAL Fab formats and tags, see the HuCAL Antibodies Technical Manual chapter 'Choosing the Best HuCAL Antibody Format'.

Table 1. Methods to pulldown the immunocomplex.

Matrix	Method	Advantages	Disadvantages
Primary antibody coupled to beads	Covalent coupling of Fab antibody	Low background, little non-specific reaction since no secondary antibodies are used	Coupling required for every antibody; high primary antibody consumption
Beads coupled to Anti-Human IgG F(ab')2 Antibody (catalog #STAR126)	Capture Fab	Good for testing many different antibodies	Pulldown of intrinsic antibodies in the sample
Beads coupled to Mouse Anti-Strep-Tag Immo Antibody (#MCA2488)	Capture Fab via StrepX tag	Excellent antibody affinity, good for testing many different antibodies	None
Beads coupled to Mouse Anti-Histidine Tag Antibody (#MCA1396)	Capture Fab via His-6 tag	Good for testing many different antibodies	None
Beads coupled to Mouse Anti-V5 Tag Antibody (#MCA1360GA)	Capture Fab via V5 tag	Good for testing many different antibodies	None

Table 1. Methods to pulldown the immunocomplex continued.

Matrix	Method	Advantages	Disadvantages
Beads coupled to Rat Anti-DYKDDDDK Tag Antibody (#MCA4764)	Capture Fab via DYKDDDDK tag	Good for testing many different antibodies	None
MagStrep type 3 XT beads (IBA)	Capture Fab via Strep tag	Ready-to-use beads, good for testing many different antibodies	None
Dynabeads His-Tag Isolation & Pulldown (Thermo Fisher)	Capture Fab via His-6 tag	Ready-to-use beads, good for testing many different antibodies	None
SureBeads Protein A or G Magnetic Beads (#1614013, #1614023)	Capture IgG via the Fc	Ready-to-use beads, good for testing many different antibodies	None

Protocols

Methods that use a bead coupling step were tested with Dynabeads M-450 Epoxy.

Covalent immobilization on Dynabeads M-450 Epoxy

1. Wash 300 µl magnetic Dynabeads M-450 Epoxy (Thermo Fisher #14011) twice (2x) using 0.5 ml phosphate buffer; 0.1 M, pH 7.4.
2. Resuspend beads in phosphate buffer and add 100 µg HuCAL Fab antibody to give a final volume of approximately 350 µl.
3. Incubate on a rotator for 16-20 hr at RT.
4. Place the tube in a magnetic holder for 1 min and remove the supernatant.
5. Wash the beads 3x with 1 ml of PBS.
6. Resuspend beads in 700 µl blocking solution (3% BSA in PBS).
7. Block the beads on a rotator for 30 min at RT.
8. Wash the beads 1x with 700 µl PBS.
9. Resuspend the beads in 300 µl PBS.

Note: This protocol can also be used for the immobilization of capture antibodies. In that case, add 10 µg of HuCAL antibody to 100 µl capture antibody coupled beads. Incubate for 1 hr at RT on a shaker or rotator and wash beads with PBST.

Immobilization on Dynabeads His-Tag Isolation & Pulldown via the His-6 tag

1. Wash 50 µl Dynabeads His-Tag Isolation & Pulldown (Thermo Fisher, #10103D) 2x with 700 µl PBS.
2. Resuspend the beads in 100 µl PBS and add 20 µg HuCAL Fab antibody. Add PBS to a final volume of 700 µl.
3. Incubate on a rotator for 10 min at RT.
4. Place the tube in a magnet holder for 1 min and remove the supernatant.
5. Wash the beads 4x with 700 µl PBS.
6. Resuspend beads in 700 µl blocking solution (3% BSA in PBS).
7. Block the beads on a rotator for a further 30 min at RT.
8. Wash the beads 1x with 700 µl PBS.
9. Resuspend the beads in 100 µl PBS.

Solubilization

Various techniques can be used for this step, depending on the origin and nature of the antigen. Below is a basic protocol suitable for extracting proteins from mammalian cells.

1. Use either a fresh or frozen cell pellet (containing 0.5-2.0 x 10^7 cells) thawed on ice.
2. Resuspend the cells in 1 ml ice-cold non-denaturing lysis buffer (Tris/NaCl/NP 40/EDTA/protease inhibitor [TNEC]) by gentle agitation on a vortex mixer for 30 sec. Use medium speed to avoid foaming.
3. Store the suspension on ice for 15-30 min, then transfer to a microfuge tube.
4. Clear the lysate by centrifugation for 15 min at maximum speed, at 4°C.
5. Transfer the supernatant to a fresh microfuge tube. To avoid disturbing the pellet, leave 20-40 µl supernatant in the original tube.
6. Store the supernatant on ice until used for immunoprecipitation.

Immunoprecipitation

This step involves incubation of the immobilized antibodies with the solubilized antigen, followed by extensive washing to remove unbound protein.

1. Add 100 µl bead-coupled antibodies to 1 ml lysate.
2. Incubate on a rotator for 12 hr at 4°C.
3. Wash 4x with 0.5 ml ice-cold TNEC buffer for a total wash time of approx 30 min. Store samples on ice for 3-5 min between washes, if necessary.
4. Wash 1x with PBS.

Analysis

This step is performed using standard techniques, such as coomassie-stained SDS-PAGE, western blotting or mass spectrometry.

1. Resuspend the pellet in 10 µl reducing SDS gel loading buffer (sample buffer).
2. Heat to 95°C for 5 min, and then cool on ice.

Run the entire sample on SDS-PAGE and electrotransfer onto a PVDF membrane; refer to the HuCAL Antibodies Technical Manual chapter 'Western Blotting' for protocols for analysis.

SureBeads Magnetic Beads standard immunoprecipitation protocol

Preparation and binding

1. Thoroughly resuspend the SureBeads in their solution and transfer 100 µl (1 mg at 10 mg/ml) of SureBeads to 1.5 ml tubes. Magnetize beads and discard supernatant.
2. Wash with 1,000 µl PBS-T (PBS + 0.1% Tween 20):
   a. Resuspend the beads thoroughly
   b. Magnetize beads and discard supernatant repeat 3x

   Tip: Resuspension can be achieved by vortexing or pipetting while the magnet slider is outside of the tube holder. If vortexing, spin down the tubes before magnetization to bring down drops from the tube cap.
3. Add 1-10 µg antibody in final volume of 200 µl and resuspend the beads.
4. Rotate 10 min at RT.
5. Magnetize beads and discard supernatant.
6. Wash with 1,000 µl PBS-T:
   a. Resuspend the beads thoroughly
   b. Magnetize beads and discard supernatant repeat 3x
7. Add the antigen-containing lysate, 100-500 µl.
8. Rotate for 1 hr at RT.
9. Magnetize beads and discard supernatant.
10. Wash with 1,000 µl PBS-T:
    a. Resuspend the beads thoroughly
    b. Magnetize beads and discard supernatant, repeat 3x. Before the last magnetization, transfer the resuspended beads to a new tube
11. Spin down all tubes for several seconds.
12. Magnetize beads and aspirate residual buffer from the tubes.

Elution

Elution strategy 1:

a. Add 20 µl glycine 20 mM pH 2.0 and incubate 5 min at RT
b. Magnetize beads and move eluent to a new vial
c. Neutralize eluent with 2 µl (10% eluent volume) 1 M phosphate buffer pH 7.4

Elution strategy 2:

a. Add 40 µl 1x Laemmli buffer and incubate for 10 min at 70°C
b. Magnetize beads and move eluent to a new vial

Example

Granta cells (~1.3 x 10^7 cells per immunoprecipitation) were used for precipitation of vimentin with HuCAL Fab mini-antibodies, Figure 1. Different formats of the same clone were tested with appropriately matching precipitation methods. Precipitated proteins were detected using western blot analysis with the same Anti-Vimentin Antibody (Fab-dHLX-MSx2 format) conjugated to HRP. Direct detection without a secondary antibody has the advantage that the Fab bands of the precipitating antibody are not visible on the blot.

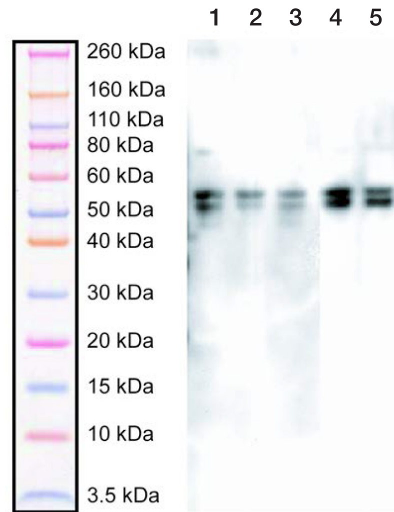

Fig. 1. Immunoprecipitation of vimentin. Western blot analysis using HRP conjugated Anti-Vimentin Antibody, clone AbD08866 (#HCA111), and ECL detection. Different Anti-Vimentin Antibody formats and beads were used for the immunoprecipitations. **Lane 1**: Fab-dHLX-MSx2 format coupled to Dynabeads M450 Epoxy. **Lane 2**: Fab-dHLX-MH format on Dynabeads TALON (Thermo Fisher, #10101D). **Lane 3**: Fab-A-V5Sx2 format on Mouse Anti-V5 Tag Antibody coupled beads. **Lane 4**: Fab-dHLX-MSx2 format on Mouse Anti-Strep-Tag Immo Antibody coupled beads. **Lane 5**: Fab-A-V5Sx2 format on MagStrep beads.

Example using SureBeads

Jurkat cells were used for precipitation of GAPDH with a HuCAL antibody in IgG format coupled to SureBeads Protein G Magnetic Beads, as shown in Figure 2.

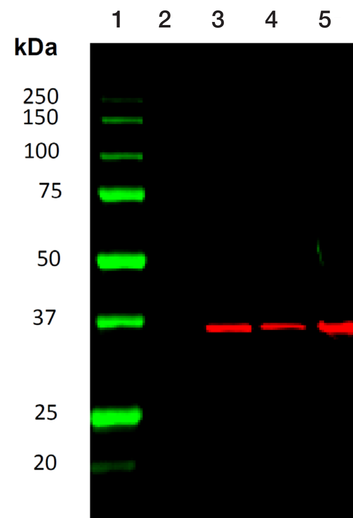

Fig. 2. Western blot analysis of GAPDH immunoprecipitation samples. IP was performed on Jurkat cell lysates using Human Anti-GAPDH Antibody, clone AbD22549_hIgG1 (#HCA272) 10 µg/ml (lane 3) and 1 µg/ml (lane 4); 10 µg/ml Human Anti-HSPA4 Antibody, clone AbD23625_hIgG1 (#HCA273) was used as a negative control (lane 2); HuCAL antibodies were coupled to SureBeads Protein G Magnetic Beads. For the western blot, Precision Plus Protein™ Prestained Standards were run in lane 1 and Jurkat whole cell lysate was run in lane 5 as positive control. Rabbit Anti-GAPDH Antibody (VPA00187) was used at 1/1000 in lanes 1-5, followed by TidyBlot™ Western Blot Detection Reagent:HRP (#STAR209P) at 1/200; results were visualized on the Bio-Rad Chemidoc™ Touch Imaging System. The results in lanes 3 and 4 demonstrate the enrichment of the GAPDH protein after immunoprecipitation.

Troubleshooting

Problem	Possible Cause and Course of Action
No bands on western blot	1. Insufficient antigen in sample. Increase amount of source material used for immunoprecipitation. 2. Antigen not expressed in the source. Test for antigen expression in source by western blotting with concentrated lysate. 3. Insufficient antibody used. Use more immobilized antibody. 4. Antigen not properly solubilized. Try different lysis buffers. 5. Antibody not suitable for immunoprecipitation. Test immunoprecipitation with pure antigen. 6. Antibody does not work in western blotting. Test western blot with pure antigen (and lysate). 7. Antibody stored at 4°C for several weeks or subjected to several freeze-thaw cycles. Use fresh aliquot of antibody that has been stored at -20°C or below.
High background signal due to non-specific binding of unrelated protein	1. Use additional washing steps (e.g. 15x with 0.5 ml for at least 1 hr). 2. Optimize lysis buffer for your cells. 3. Use covalently immobilized antibody or other secondary antibody for precipitation. 4. Try a different blocking agent for bead blocking step. 5. Increase number of washing steps.

Technical Assistance

Experienced technical advisors are available to help with any questions regarding HuCAL antibodies and their applications, both before project start and after antibody delivery. Since HuCAL technology provides many opportunities for optimizing the antibody generation process according to customer needs, we encourage the discussion of your antibody project with one of our scientists at an early stage. They can help with project design, antigen preparation, and provide information about the many options that are simply not available with typical animal based technologies. Once the antibodies are delivered, they can also provide valuable advice about protocols and detection systems. Contact us at **antibody_sales_muc@bio-rad.com** or visit **bio-rad-antibodies.com/HuCAL** for more information. General technical support requests related to catalog antibodies and accessory products can also be addressed to **antibody_tech_uk@bio-rad.com**.

Confidentiality

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Bio-Rad Laboratories, Inc.

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