Protocol

Immunoprecipitation

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This immunoprecipitation protocol details individual steps for the enrichment and purification process of specific proteins from a complex cell lysate using an antibody bound to a solid matrix. Purified antigen(s) can be eluted by various methods, and the resultant protein target can be analyzed and/or identified by numerous assays, including the enzyme-linked immunosorbent assay (ELISA), western blotting, or mass spectrometry.

MATERIALS

It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous materials used in this protocol.

RECIPES: Please see the end of this protocol for recipes indicated by <R>. Additional recipes can be found online at <http://cshprotocols.cshlp.org/site/recipes>.

Reagents

Antibody
Ascites fluid
Hybridoma tissue culture supernatant
IgG, purified
Immune serum
Anti-immunoglobulin antibody (optional secondary antibody)
Control antibody
Laemmli sample buffer (2×) <R>
Lysate sample (e.g., prepared from Protocol: **Detergent Lysis of Tissue Culture Cells for Immunoprecipitation** [DeCaprio and Kohl 2017a] or Protocol: **Detergent Lysis of Animal Tissues for Immunoprecipitation** [DeCaprio and Kohl 2017b])
Lysis buffer (optional and dependent on cell lysate preparation protocol)
Protein A beads (50% slurry)
Wash buffer for immunoprecipitation <R>

Equipment

Aspirator
Eppendorf tubes
Ice
Microcentrifuge

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Pipetman with 200- μ L tip
Rotating platform
SDS-PAGE gels and equipment

METHOD

1. Add the antibody to the lysate sample. Typically 0.5–1.0 μ g of purified target-specific antibody is added to 0.5–1.0 mL of cell lysate prepared from 10^6 – 10^7 cells.

One microgram of immunoglobulin is typically contained within 0.5–5 μ L of serum, 10–100 μ L of hybridoma tissue culture supernatant, or 0.1–1.0 μ L of ascites fluid; however, these values serve as a guideline only.

2. Incubate on ice for 1–4 h.

High-affinity antibody–antigen reactions will be completed considerably sooner than the recommended minimum 1-h time period; however, this 1-h incubation time provides a reasonable compromise for most antibodies. Overnight reactions as suggested by some protocols are of little advantage and will increase the background.

3. (Optional) If the primary antibody displays a low affinity for Protein A or G and the inclusion of a secondary rabbit anti-immunoglobulin antibody is required, add 0.5–1.0 μ g of anti-immunoglobulin antibody after 30 min.

Use an anti-immunoglobulin antibody if there is weak affinity between the primary antibody and Protein A or G agarose. For example, IgM and chicken IgY antibodies will not bind Protein A or G beads; however, antibodies raised against IgM or IgY will facilitate binding to Protein A or G beads. Similarly, mouse IgG1 binds poorly to Protein A and sometimes not well to Protein G, thus requiring the incorporation of an antimouse immunoglobulin to improve IgG1 capture on Protein A agarose. Rabbit antibodies display a high affinity for Protein A and are commercially available against most species of immunoglobulin.

4. Always include a control antibody when performing immunoprecipitations. Ideally, use a control antibody preparation identical to the specific antibody. For example, use preimmune serum harvested from the same animal used for immune serum. When incorporating a specific antibody, use purified antibody, hybridoma tissue culture supernatant, or ascites of the same isotype as the primary antibody. When incorporating a secondary antibody, use two controls: one consisting of only the secondary antibody and one in combination with the control primary antibody.

5. Add 20–50 μ L of Protein A beads to 1 mL of antibody–antigen lysate, and gently incubate it on a rocking platform for 1 h at 4°C.

Use the smallest amount of beads permissible to reduce nonspecific binding. Protein A beads display a large antibody binding capacity that is rarely exceeded during the performance of immunoprecipitations. Be aware that the use of <20 μ L of beads presents visualization difficulties.

6. Collect the beads by centrifugation at 10,000g for 15 sec at 4°C, and wash the immune complexes using Wash buffer to remove nonspecific binding. Lysate and Wash buffers are easily removed by aspiration using a 200- μ L Pipetman tip inserted into a vacuum tube. The tip should be advanced along the side of the Eppendorf tube until just above the top surface of the beads.

Alternatively, Lysis buffer with protease inhibitors used during the cell lysate preparation (protocol specific) can be used during this washing step.

7. Wash the immune complex three to six times, and remove the final wash as completely as possible. Allow the remaining Wash buffer from the sides of the tube to collect at the bottom of the tube for 1 min before aspirating it a second time. The captured antigen can be eluted by various methods, including:

- i. *Peptide competition elution:* The excessive addition of synthetic peptide specific to the immune complex will allow for gentle elution of the target protein by competition for antibody binding sites.

- ii. *Glycine buffer (pH 2.7) elution:* The low pH of this buffer weakens the antibody–bead interaction and allows for the reuse of the beads upon extensive washing. The eluted sample should immediately be neutralized with an equal volume of Tris (pH 8.0–8.5) to prevent protein degradation.
- iii. *Urea buffer (6–8 M) elution:* The chaotrope urea is commonly used for the release of purified immune complexes from the beads into the supernatant and is highly advantageous for subsequent mass spectrometry analysis following proteolytic digestion.
- iv. *SDS buffer elution:* This buffer represents the harshest elution condition, releasing both noncovalently bound antibodies and antibody fragments along with the protein of interest. This highly efficient method is advantageous because resulting samples are more concentrated and can be directly analyzed by SDS-PAGE or stored at -20°C for subsequent use.

For Troubleshooting, see Table 1.

DISCUSSION

An immunoprecipitation, commonly referred to as an IP, commences by addition of a specific antibody to a prepared cell lysate, allowing for the formation of antigen–antibody complexes (Taylor and Best 2011; Ueki et al. 2011; Alexandru 2012; Cho et al. 2012; Conlon et al. 2012; Jain et al. 2012; Poulsen et al. 2012; Schmollinger et al. 2012; Smaczniak et al. 2012; Spiro 2012; Becker et al. 2013; Cordeiro and Bidere 2013; Li et al. 2013; Sarkar et al. 2013; You et al. 2013). Upon completion of the incubation period, Protein A, G, or L agarose or magnetic beads are added. Should a secondary antibody be required to promote more efficient binding between the primary antibody and Protein A, it is added after the primary antibody and before the Protein A beads. Alternatively, antibody and beads can be added simultaneously to a cell lysate. If, however, primary antibodies have been directly cross-linked to beads, these can be directly added to a cell lysate to establish antigen–antibody–bead complexes.

The rate of immune complex formation is dependent on the final concentration of antibody and antigen in the Lysis buffer as well as the strength of antibody–antigen and antibody–bead association. Typically, the antibody is added to the cell lysate and allowed to form the antibody–antigen complex before the addition of beads. Formation of the antigen–antibody complex will occur quickly at the liquid–liquid interface, but a slower rate of formation occurs at the liquid–solid interface between the antibody–antigen immune complexes and beads.

Immunoprecipitations are generally performed in 0.5–2.0 mL conical tubes, ensuring easy handling and convenient centrifugation using a microcentrifuge. Larger 5-, 15-, or 50-mL tubes are typically used for preparative-scale immunoprecipitations, followed by the transfer of the bead-captured immune complex to smaller tubes for the final wash steps. Polypropylene tubes are preferred over polystyrene tubes to reduce interactions between proteins and beads with the plastic. Immunoprecipitations should be constantly mixed by placing tubes on a rotating platform. All immunoprecipitations are to be performed at 4°C in a cold room or cabinet to minimize proteolytic degradation of the protein target.

Immunoprecipitations can be processed using a wide variety of techniques. Immobilized immune complexes can be washed once or twice in buffer facilitating and optimizing conditions for an enzymatic reaction, thus permitting direct performance of an enzyme reaction on the beads. Alternatively, immune complexes can be eluted in a variety of ways for further analysis including immunoblotting or mass spectroscopy.

The performance of an immunoprecipitation is not always easy. The Troubleshooting guide (Table 1) provides helpful insight into the many factors contributing to the success of an immunoprecipitation. Coimmunoprecipitation assays (commonly referred to as Co-IPs) are very similar to immunoprecipitations in that the basic technique uses a matrix-immobilized antibody for the precipitation and enrichment of a specific protein target. Although the purpose of an immunoprecipitation is the purification of a single antigen of interest, the coimmunoprecipitation focuses primarily

TABLE 1. Troubleshooting

Problem	Possible cause	Solution
Nonspecific background	Nonspecific sample binding to agarose beads or antibodies	Preclear the lysate with Protein A or G beads. Incubate the beads with lysate for 30 min at 4°C, and then pellet the beads by centrifugation. Transfer the lysate to a new tube and add primary antibody. Clarify the lysate before addition of the specific antibody by centrifugation at 10,000g for 10 min at 4°C to remove chromatin and debris or at 100,000g for 30 min at 4°C to remove membranes and other polymers. After centrifugation, transfer the supernatant to a clean tube without disturbing the pellet, and repeat the centrifugation.
	Aggregated proteins in lysate	Remove aggregates and polymerized proteins by centrifugation of the antibody–antigen binding reaction for 10 min at 10,000g and immediately transfer it to a new tube before adding the solid phase.
	Nonspecific binding to Protein A or G	Prepare Protein A or G beads in buffers containing 0.5%–2% BSA. Reduce the amount of Protein A or G beads used to the minimum to capture the antibody–antigen complex maximally. Use as little as 10 µL of beads (20 µL of 50% slurry) for each immunoprecipitation.
	Secondary antibody	Determine the minimum amount of secondary antibody required to immunoprecipitate the primary antibody completely by titration, and investigate the possible background created by this reagent alone.
	Insufficient washing	Increase the number of washes from three to five or six. Leave the solid phase in the Wash buffer for 5–10 min during each washing step. Use more stringent washes. Try using high-salt washes such as 1.0 M NaCl, 0.5 M LiCl, or 1 M KSCN or higher concentrations of detergent such as 0.2% SDS or 1% Tween 20. In addition, try alternating Wash buffers from high salt to low salt, or different detergents may help. Try washing with distilled water for one wash.
	Frozen lysates	Prepare cell lysates before use and avoid freezing lysates.
	The antibody concentration is too high.	Reduce the amount of antibody to the minimum required to immunoprecipitate the antigen maximally.
	Precipitated antibody	Centrifuge the antibody at 100,000g for 30 min to remove any particulate matter.
	Low antibody target and bead affinity	Try a different primary or secondary antibody. Transfer the Protein A beads to a fresh tube before the last wash and elution of the antibody–antigen complexes.
Specific background	Antigen consists of more than one polypeptide chain.	If specific proteins remain, remember that the antigen may consist of more than one polypeptide chain or that the primary antibody may specifically recognize more than one target. Use a monoclonal antibody recognizing a different epitope.
	Monoclonal or affinity-purified polyclonal antibody recognizes homologous epitope.	
	Polyclonal antiserum is forming complexes with additional proteins.	Use a monoclonal or affinity-purified antibody.
	Immunoblot bands are actually Ig light or heavy chains.	IgG heavy chains appear at ~55 kDa, whereas IgG light chains appear at ~28 kDa.
Specific antigen not detected	Low target protein level	Use a different cell line that expresses higher levels of the target protein. Keep the volume of lysate to a minimum. Concentrate the target protein by lysing cells in the minimum amount of Lysis buffer. Increase the amount of antibody. Use a different Lysis buffer to improve extraction or solubility. Try denaturing lysis conditions. Add protease inhibitors or use different inhibitors. Overexpress an epitope-tagged version of the target protein.
	Antibody is not suitable for immunoprecipitation.	Use a different antibody with higher affinity to the target. Use rabbit polyclonal antibodies specific to the target. Use secondary antibodies if the primary antibody is not completely captured by the Protein A or G resin.
	Incorrect bead type is used.	Verify that the correct bead type was used. Use Protein A resin if Protein G resin does not perform. Use Protein G resin if Protein A resin does not perform.
	Antibody binds weakly to either Protein A or G.	Change the bead/resin manufacturer. Explore the use of magnetic beads.
	Wash buffer stripped the immune complex from agarose beads.	Use milder Wash buffer. Reduce the salt and detergent concentration, or use a different detergent and reduce the number of washes.

(continued)

TABLE 1. Continued

Problem	Possible cause	Solution
	Incubation times are too short.	Incubate with precipitating antibody for several hours at 4°C.
	Antibody concentration is too low.	Increase the antibody concentration.
	Antigen of interest is degraded or lost.	Prepare fresh lysates in the presence of appropriate protease inhibitors and prevent freezing.
	Interfering substances in sample	Reducing agents including DTT or β -mercaptoethanol may destroy antibody function. Extreme pH and excessive detergent concentrations may interfere with antibody–antigen interactions.

on the isolation of an antigen along with any other associated proteins or ligands bound to it. Here the antigen is called the bait protein, and the bait-interacting proteins are called prey proteins, which can consist of complex partners, signaling molecules, structural proteins, or cofactors and can display highly transient or very stable interactions with the bait protein.

Although the above protocol details an immunoprecipitation, it should principally work for a coimmunoprecipitation upon optimization of the binding and wash conditions during the coimmunoprecipitation to negate possible effects on the bait–prey interactions as well as interactions between the antibody and the bait protein. The protocol does not, however, address the use of commercially available magnetic beads displaying Protein A, G, A/G, or L as a substitution for cross-linked Protein A, G, A/G, or L agarose or Sepharose beads to represent the solid-phase matrix. The use of magnetic beads may aid in the immunoprecipitation of fragile protein complexes because handling of the immunoprecipitated antigen(s) is much gentler, foregoing the repeated centrifugation steps required for the collection of agarose or Sepharose beads. In conjunction, the recovery and purification of larger antigen–antibody immune complexes might be assisted by the use of these nonporous magnetic beads, because complexes that might otherwise be limited in their purification efficiency given the porous structure of the agarose or Sepharose beads might be alleviated. The application of magnetic bead technology for the automated immunoprecipitation and purification of protein targets represents a valuable alternative for the consistent performance of immunoprecipitation of antigens according to the protocol above.

RECIPES

Laemmli Sample Buffer (2×)

Reagent	Amount to add	Final concentration (2×)
10% (w/v) SDS	4 mL	4%
Glycerol	2 mL	20%
1 M Tris-Cl (pH 6.8)	1.2 mL	120 mM
H ₂ O	2.8 mL	—

Add bromophenol blue to a final concentration of 0.02% (w/v). Store the 2× Laemmli sample buffer at room temperature.

Wash Buffer for Immunoprecipitation

Reagent	Volume per 100 mL of solution (v/v)	Final concentration
NaCl (1 M)	15 mL	150 mM
EDTA	0.2 mL	1 mM
Triton X-100 (10%)	1 mL	1%
Tris (50 mM, pH 7.4)	20 mL	10 mM
ddH ₂ O	Add to 100 mL	

Store at 4°C. Add protease inhibitor cocktail immediately before use.

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