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## Immunoprecipitation Protocol

### Research Use Only

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#### Introduction

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Immunoprecipitation is a procedure that results in the enrichment of a specific protein from a heterogeneous mixture, cell lysate or culture supernatant. This enrichment is accomplished by binding the protein of interest with a specific antibody, followed by precipitation of the immune complexes with Protein G or Protein A immobilized onto beads such as agarose. The precipitated immune complexes are then denatured and resolved by SDS-PAGE for further analysis. Immunoprecipitation can be used to confirm the identity of a protein, to quantify expression levels, to study the biochemical characteristics such as protein:protein interactions, or to evaluate post-translational modifications. Immunoprecipitation can be divided into the following steps:

- Sample preparation
- Preclearing
- Antibody incubation/formation of antibody-antigen complexes
- Precipitation and washing
- Analysis by SDS-PAGE and/or other methods

The protocol below offers a general guideline for immunoprecipitation with the caveat that optimization is often required for individual antigen/antibody combinations. The abundance of a given protein within a sample is highly variable and a critical factor for obtaining desired results. In this protocol, a cell concentration of approximately  $10^7$  cells/ml of lysate is recommended as a starting point. Another critical parameter is the composition of the lysis buffer used to prepare the lysate for immunoprecipitation. NP-40, a non-ionic detergent, is the most commonly used detergent in cell lysis buffers, but alternatives include Triton X-100, Saponin, Digitonin, and CHAPS. Additionally, increasing the salt concentration or decreasing the detergent concentration are steps that can be taken to optimize specificity of the immunoprecipitation.

The pre-clearing step is incorporated into the procedure to reduce the amount of non-specific contaminants in the cell lysate and to remove proteins with high affinity for Protein G or Protein A prior to the specific immunoprecipitation. Many investigators choose to skip this step in cases where the protein of interest is abundant in the sample.

The success of immunoprecipitation depends on the affinity of the antibody for its antigen as well as for the immobilized-Protein G or Protein A beads. In general, while polyclonal antibodies are best, purified monoclonal antibodies (mAb), ascites fluid, or hybridoma supernatant can also be used. Some mAbs work very well, whereas others may give unsatisfactory results. The eBioscience antibodies that have been reported in the literature to work for immunoprecipitation are listed for this application. It is recommended that the specified experimental conditions be followed as published; however, optimization of conditions by the investigator may be necessary.

Finally, choosing immobilized Protein A or Protein G for the precipitation step is critical to the success of the procedure. Protein G binds well to mouse IgG1 and most subclasses of rat and human IgGs, whereas Protein A has a much higher affinity for mouse IgG2a, IgG2b, and IgG3.

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#### Useful websites

##### **Molecular Station** (<http://www.molecularstation.com/protein/immunoprecipitation/>)

Molecular Station is a website that was started by a group of graduate students and scientists to provide a site of compiled resources for researchers in molecular biology and science in general.

##### **Protocol Online** ([http://www.protocol-online.org/prot/Molecular\\_Biology/Protein/Immunoprecipitation\\_IP\\_/index.html](http://www.protocol-online.org/prot/Molecular_Biology/Protein/Immunoprecipitation_IP_/index.html))

Protocol Online is a database of research protocols contributed by researchers worldwide.

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## Immunoprecipitation

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#### Materials

- Cell lysate
- Immobilized Protein A or Protein G (eBioscience Immobilized Protein G (Cat. No. [23-0120](#))
- Immunoprecipitation antibody

#### Buffers

- Ice-cold Lysis buffer
- Ice-cold PBS
- SDS-PAGE sample buffer

#### Instruments

- Centrifuge
- Rocking platform or rotator
- SDS-PAGE and Immunoblotting equipment and reagents

## Experimental Procedure

### Step I: Cell Lysate Preparation

1. Remove culture media and wash cells with ice-cold PBS and decant.
2. Harvest approximately  $10^7$  cells and transfer to a conical tube. If working with adherent cells you can skip this step and lyse directly on the plate (see Step 5).  
*Note: The total number of cells per mL and the cell equivalent loaded per lane on the gel should be optimized specifically for each protein and antibody. Alternatively, protein concentration can be determined using Bradford/Lowry or other protein assay.*
3. Wash cells with ~10 mL of ice-cold PBS and centrifuge at 400xg for 10 minutes at 4°C.
4. Discard supernatant and repeat step 3.
5. After the second wash, remove the supernatant completely and resuspend the cell pellet in 1mL of ice-cold Lysis Buffer containing protease or phosphatase inhibitors (see

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cocktail recipe below). The final concentration of cells should be approximately  $10^7$  cells/mL.

*If using adherent cells, the ice-cold Lysis Buffer can be added directly to the plate and put on a rocker at 4°C. Harvest by either scraping or collecting just the supernatant and proceed to Step 8.*

6. Gently vortex/mix and then transfer to a fresh 1.5 mL tube.
7. Place the tube on ice for 30 minutes, with occasional mixing.
8. Centrifuge the cell lysate at 10,000xg for 15 to 30 minutes at 4°C.
9. Carefully collect the supernatant without disturbing the pellet, and transfer to a clean tube. The pellet can be discarded.
10. The protein concentration can be determined by Bradford or another assay. Samples can be diluted to ~1 µg/µL.
11. The cell lysate can be frozen at this point for long-term storage at -80°C.

#### Step II: Cell Lysate Preclearing

1. Resuspend the immobilized Protein A or G bead slurry by gently vortexing.
2. Add 50 µL of prepared Protein A or G slurry to 500 µL of cell lysate (~ $5 \times 10^6$  cells or ~500 µg protein) and incubate on a rotator for 30 to 60 minutes at 4°C.
3. Centrifuge at 2,500xg for 2 to 3 minutes at 4°C and transfer the supernatant to a fresh 1.5 mL tube. If any of the bead slurry has been transferred, centrifuge again and carefully transfer the supernatant to another fresh 1.5 mL tube.

#### Step III: Immunoprecipitation

1. Add 1-10 µg of antibody to the pre-cleared cell lysate.  
*Note: This concentration of monoclonal antibody is suggested as a starting point. Each investigator may titrate the concentration of antibody and volume of cell lysate in preliminary experiments to determine the optimal conditions.*
2. Incubate at 4°C for 1 to 2 hours or overnight on a rotator.
3. Add at least 50 µL of Protein A or G slurry (pre-equilibrated in the corresponding IP buffer/Lysis buffer) to capture the immune complexes.
4. Incubate for 1 hour or overnight at 4°C on a rotator.  
*Note: Step 1 and 3 can be combined for a single incubation.*
5. Centrifuge the tube at 2,500xg for 30 seconds at 4°C.
6. Carefully remove the supernatant completely. Wash the beads three to five times with 500 µL of ice-cold Lysis Buffer. Centrifuge to pellet the beads in between each wash. In order to minimize background, care should be given to remove the supernatant completely after each wash.
7. After the last wash, carefully aspirate the supernatant and add 50 µL of 1X Laemmli sample buffer (or any equivalent SDS-PAGE sample loading Buffer) to the bead pellet.  
*Please take into consideration composition of the Loading buffer. Reducing agents can be added.*
8. Vortex and heat at 90-100°C for 10 minutes.

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*Note: Some incubation steps can be done at lower temperatures for longer time periods depending on the protein and protein complex of interest.*

9. Centrifuge at 10,000xg for 5 minutes to pellet the beads. Collect the supernatant carefully and load onto an SDS-PAGE gel. Alternatively, the supernatant samples can be collected, transferred to clean tube and frozen at -80°C for later use.
10. Follow the manufacturer's instructions for SDS-PAGE. Stain the gel for visual analysis of the immunoprecipitated protein. If performing immunoblotting after this step, follow the accompanying Immunoblotting (WB) Protocol.

#### Buffer Recipes

##### NP-40 Cell Lysis Buffer:

- 50mM Tris-HCl pH 8.0
- 150mM NaCl
- 1% NP-40

##### RIPA Buffer:

- 50mM Tris-HCl pH 7.4
- 1% NP-40
- 0.25% Na-deoxycholate
- 150mM NaCl
- 1mM EDTA

##### Protease Inhibitor Cocktail (100X):

- PMSF, 5mg (50 µg/mL)
- Aprotinin, 100 µg (1 µg/mL)
- Leupeptin, 100 µg (1 µg/mL)
- Pepstatin, 100 µg (1 µg/mL)

##### Phosphatase Inhibitor Cocktail (100X):

- 1mM Na<sub>3</sub>VO<sub>4</sub>
- 1mM NaF

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## References

Bonifacino JS, Dell'Angelica EC, Springer TA. 2001. Immunoprecipitation. Curr. Protoc. Mol. Biol. Chapter 10:Unit 10.16.