Immunoprecipitation Protocol

Immunoprecipitation is a general method to obtain the enrichment of a specific protein from tissue lysate and cell lysate. It can be used to purify a specific protein, to identify a novel protein, and to determine protein-protein interaction. Its major component contains the binding of a target protein with a specific antibody and precipitation of the immune complexes with Protein G or Protein A immobilized onto beads such as agarose. The precipitated immune complexes are denatured and resolved for further analysis. The procedure can be divided into the following steps: Sample preparation, immunoprecipitation procedure, and analysis by Western blot analysis and/or other methods.

1. Reagents

PBS Buffer

8.5 g	NaCl
1.4 g	Na_2HPO_4
0.2 g	NaH_2PO_4
1000 ml	ddH_2O

Adjust to pH 7.4

Store at 4 °C

RIPA Buffer

0.60 g	Tris base
0.88 g	NaCl
1 ml	NP40
0.5 g	Sodium deoxycholate
0.1 g	SDS
100 ml	ddH_2O

Adjust to pH 7.6

Store at 4 °C

Note: RIPA buffer (Lysis buffer) is particularly useful for nuclear membrane disruption for nuclear extracts. It gives low background but can denature kinases.

Protease Inhibitor Cocktail (100X)

5 mg	PMSF
100 µg	Aprotinin

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100 µg	Leupeptin
100 µg	Pepstatin
1 ml	PBS Buffer

Store at -20 °C

Note: This buffer is used to prevent the degradation of protein in cell/tissue lysate caused by protease.

Phosphatase Inhibitor Cocktail (100X)

184 mg	Na_3VO_4
42 mg	NaF
1 ml	PBS Buffer

Store at -20°C

Note: This buffer is recommended for phospho-specific protein immunoprecipitation. It is used to prevent dephosphorylation of protein caused by phosphatase.

2X SDS-PAGE Sample Buffer

1.5 g	Tris base
4 g	SDS
20 ml	Glycerol
1.5 g	DTT
0.02 g	Bromophenol blue
100 ml	dd H_2O

Adjust to pH 6.8

Store at -20 °C

Protein A or G Agarose Beads

Note: Please prepare Protein A or G Agarose beads according to manufacturer's instructions.

2. Procedure

2.1 Sample preparation

Note: It is advisible to pre-cold all reagents and tubes on ice.

a. Suspension cells lysate preparation

1. Gently transfer the cell suspension into a pre-cooled microfuge tube.
2. Spin down 10 minutes at 1000x g at room temperature and discard supernatant.
3. Washes the cell with cold PBS Buffer and spin down 10 minutes 1000x g at room temperature and discard supernatant.

b. Adherent cells lysate preparation

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1. Remove culture media and wash cells with ice-cold PBS Buffer and drain the PBS Buffer.
2. Add ice-cold PBS Buffer again and scrape adherent cells off the dish using a cold plastic cell scraper and then gently transfer the cell suspension into a pre-cooled microfuge tube.
3. Centrifuge at 1000x g in a microcentrifuge at 4 °C for 10 minutes, and carefully remove the supernatant.

The following step is used for both suspension cells and adherent cells

4. Add 10 µl Protease Inhibitor Cocktail (100X) to the tube containing 1ml RIPA Lysis buffer.
5. Gently discard supernatant and add ice-cold RIPA Lysis buffer.

Note: The amount of lysis buffer is suggested as follows:1ml per 10^7 cells/100mm dish/150cm^2 flask; 0.5ml per $5x10^6$ cells/60mm dish/75cm^2 flask. And 10 µl Phosphatase Inhibitor Cocktail is added in 1ml RIPA buffer when Phopho- protein is tested in the immunoprecipitation.

6. Place the tube on ice for 30 to 60 minutes with occasional mixing.
7. Centrifuge the cell lysate in a microcentrifuge at 10,000x g for 15 to 30 minutes at 4 °C.
8. Carefully collect the supernatant without disturbing the pellet, and transfer to a clean tube. The pellet can be discarded.
9. The protein concentration can be determined by Bradford or another assay. Samples should be diluted to 1µg/µl.
10. The cell lysate can be frozen at this point for long-term storage at -80 °C.

c. Tissue lysate preparation

1. Dissect the tissue of interest with clean tools on ice as quickly as possible to prevent degradation by proteases.
2. Place the tissue in round bottom microfuge tubes and immerse in liquid nitrogen to “snap freeze”. Keep on ice for immediate homogenization.
3. Add 10 µl Phosphatase Inhibitor Cocktail (100 x) to the tube containing 1 ml Lysis buffer. Mix and then add it to the tissue.

Note: 1ml Lysis buffer is used per 20 mg tissue. Phosphatase Inhibitor Cocktail should be added when phopho-specific antibody is used in the immunoprecipitation.

4. Homogenize tissue with a homogenizer on ice for 10 to 15 minutes.
5. Gently transfer the tissue suspension into a pre-cooled microfuge tube.
6. Centrifuge for 20 min at 12,000x g at 4 °C in a microcentrifuge.
7. Gently remove the tubes from the centrifuge and place on ice. Aspirate the supernatant and place in a fresh tube kept on ice; discard the pellet.
8. The protein concentration can be determined by Bradford or another assay. Samples should be diluted to ~1 ug/µl.
9. The tissue lysate can be frozen at -80 °C for long-term storage.

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2.2 Cell lysate pre-clearing

Note: this step is optional but not necessary

1. Resuspend the 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 (~5x10^6 cells or ~500 µg proteins) and incubate on a rotator for 30 to 60 minutes at 4 °C.
3. Centrifuge at 2,500x g for 2-3 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.

2.3 Immunoprecipitation procedure

1. On ice, add appropriate specific antibody and control antibody to each tube containing 10-500 µg cell/tissue lysate.

Note: These amounts of antibody should be optimized. 1-5 µg antibody is recommended for 10-500 µg cell/tissue lysate.

2. Incubate the sample with the antibody at 4 °C for 1 to 2 hours or overnight on a rotator.
3. Mix the slurry well and add 100 µl of the beads to each sample. Always keep samples on ice.

Note: Beads will tend to stick to the sides of the tip so try to minimize the movement in the pipette and use a tip cut 5 mm from the top. See following selection guide of Protein A/Protein G Beads.

4. Incubate the lysate-beads mixture at 4 °C under rotary agitation for 1 hour or overnight at 4 °C on a rotator.
5. Centrifuge the tube at 2,500x g for 30 seconds at 4 °C.
6. Remove the supernatant completely. Wash the beads three to five times with 500µl of ice-PBS.

Note: Beads will tend to stick to the sides of the tip so try to minimize the movement in the pipette and use a tip cut 5 mm from the top.

7. After the last wash, carefully aspirate the supernatant and add 50 µl of 2X SDS-PAGE Sample Loading Buffer to the bead pellet.
8. Vortex and heat at 90-100 °C for 10 minutes. It's recommended that the supernatant can be frozen for long-term storage at -80 °C.

2.4 Western blot analysis

1. Load 20 µl of supernatant onto 1 lane of SDS-PAGE mini-gel.
2. Transfer proteins from gel to blot and probe with appropriate antibodies.

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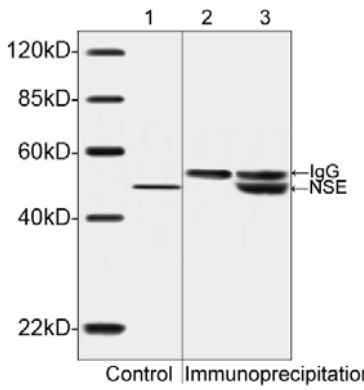
3. Protein A/Protein G Beads selection guide

Species	Isotope	Protein A	Protein G
Mouse	IgG1	+	+++
	IgG2a	+++	+++
	IgG2b	++	++
	IgG3	+	+
	IgM	-	-
Rat	IgG1	-	+
	IgG2a	-	+++
	IgG2b	-	++
	IgG2c	+	++
Chicken	All isotypes	-	++
Rabbit	All isotypes	+++	++
Goat	All isotypes	-	++

4. IP Examples

Immunoprecipitation analysis with tissue lysates



Western blot analysis of mouse brain tissue lysates and its immunoprecipitates.

1: Mouse brain lysates served as Input control

2: Immunoprecipitates of mouse brain lysates incubated with Rabbit IgG Control (GenScript, A01008) and Protein A served as negative control

3: Immunoprecipitates of mouse brain lysates incubated with NSE Antibody, pAb Rabbit (GenScript, A01406) and Protein A.

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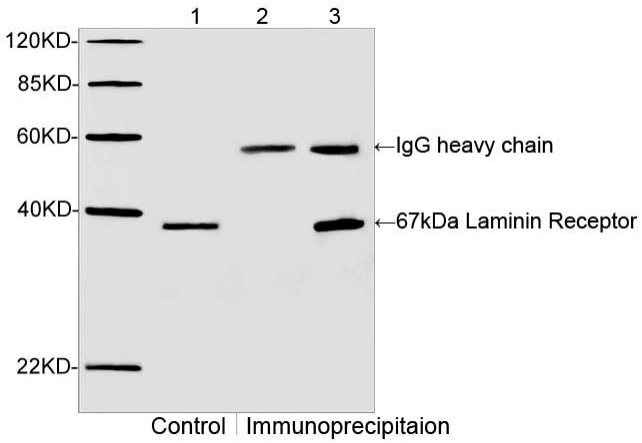
Immunoprecipitation analysis with cell lysates



Western blot analysis of extracts from MCF-7 and its immunoprecipitates.

1: MCF-7 cell lysates served as Input control

2: Immunoprecipitates of the MCF-7 incubated with Rabbit IgG Control (GenScript, A01008) and Protein A served as negative control

3: Immunoprecipitates of the MCF-7 cells incubated with 67kDa Laminin Receptor Antibody, pAb, Rabbit (GenScript, A01638) and Protein A.

5. Recommended Products

Name	Cat. No.
Human IgG Control(Whole Molecule), Purified	A01006
Mouse IgG control (Whole Molecule), Purified	A01007
Rabbit IgG Control (Whole Molecule), Purified	A01008
Goat IgG Control (Whole Molecule), Purified	A01009
Chicken IgY Control (Whole Molecule), Purified	A01010
Chicken IgY Precipitating Resin	L00405

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