Immunoprecipitation Protocol V.3

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Sam Li[1]

[1]BioLegend

BioLegend
Tech. support email: **tech@biolegend.com**

Sam Li
BioLegend

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Abstract

Immunoprecipitation is a procedure by which proteins or peptides that react specifically with an antibody are removed from solution and examined for quantity or physical characteristics. Immunoprecipitation can also be used to “enrich” a protein population prior to Western Blotting. For example, one can perform immunoprecipitation with a pan-specific antibody against a protein of interest followed by Western blotting with a modification-specific antibody (such as a phospho-specific antibody or an acetylation-specific antibody).

Guidelines

Tips:

1. The choice of lysis buffer depends on the location of the protein (membrane, cytosolic, nuclear).
2. Immunoprecipitates allowed to incubate overnight may have a higher background than ones processed for shorter periods of time due to time-dependent aggregation or denaturation of cellular proteins.
3. Always use an isotype-matched irrelevant control antibody (monoclonal) or same-species serum from a non-immunized animal to remove non-specific antibody binding in cellular lysates.

Materials

1X Cell Lysis Buffer:
25mM Tris-HCl pH 7.5, 150mM NaCl, 0.1% Triton X-100, 2mM EDTA, 1µg/ml leupeptin, 1µg/ml aprotinin, 1mM Na_3PO_4,1mM PMSF, 5mM NaF, 3mM $Na_4P_2O_4$

5X SDS sample Buffer:
312.5mM Tris-HCl (pH 6.8) 10% SDS (w/v), 250mM DTT, 50% Glycerol, 0.05% Bromophenol Blue (w/v). Use at 1X

10X SDS Running Buffer:
Dissolve 144g of Glycine, 30g of Tris base and 10g SDS in 800ml of distilled H_2O. Add distilled H_2O to 1 liter. Use at 1X

Transfer Buffer:
2.25g Tris base, 10.5g Glycine 1g SDS, 200ml Methanol. Add distilled water to 1.0L

Troubleshooting

Preparation of antibody-protein A, G, A/G agarose beads:

1 Wash protein A, G, A/G agarose beads with cell lysis buffer by pulsing in a microcentrifuge tube (two minutes at 5,000 rpm). Aspirate and discard supernatant. Wash the beads three times with cell lysis buffer.

2 Adjust antibody concentration to 5-10µg/ml in PBS and transfer 500µl of diluted antibody to 5-10µl of agarose beads for each sample.

3 Place the antibody-protein A, G, A/G agarose mix on a shaker and rotate at 4°C for one hour.

4 Spin down the protein A, G, A/G beads for two minutes at 5,000 rpm and wash the antibody-beads three times with cell lysis buffer.

Preparation of cell extract:

5 Collect cells and centrifuge at 1200 rpm for 5 minutes at 4°C.

6 Discard the supernatant and immediately add 800µl of ice-cold lysis buffer to the cells and vortex, then incubate for 30 minutes on ice.

7 Freeze and thaw the samples with dry ice for two more cycles or sonicate for 15 seconds to ensure the full release of the proteins from the cells.

8 Spin lysates at 14,000 rpm in a pre-cooled centrifuge for 10 minutes and keep the supernatant.

Immunoprecipitation:

9 Adjust the protein concentration of the supernatant to 1-2mg/ml with lysis buffer.

10 Mix 100-500µl of cell extract with antibody-protein A, G, A/G agarose and rotate the samples at 4°C for about two hours.

11 Collect the agarose beads by pulsing in a microcentrifuge tube (two minutes at 5,000 rpm, 4°C). Aspirate and discard the supernatant. Wash the beads 3 times with ice-cold cell lysis buffer.

12 After the final wash, remove the supernatant and add 20µl of 2X SDS sample buffer. Boil for 5 minutes at 95°C and spin down the beads at maximum speed in a microcentrifuge for 5 minutes at room temperature. Carefully pipette off the supernatant.

13 Load 30µl of sample in each well of a 1.5mm thick gel. Run the gel according to manufacturer's recommendations and continue with immunoblotting using BioLegend's Western Blotting protocol (alternately, radiolabeled proteins prepared from target cells can be used to directly visualize the immunoprecipitated protein).