Immunoaffinity purifications of protein complexes

Necessary reagents and instrumentation:

- Magnetic beads (Dynal, NY, USA) conjugated with the antibodies to be used for the immunoisolation (see protocol for conjugation of magnetic beads)
- Optimized lysis buffer
- Protease inhibitor cocktail, used as 1/100 (v/v)
- Freshly made aqueous 0.5 N NH4OH, 0.5 mM EDTA solution
- Polytron
- Centrifuge that can spin to 3000 rpm at 4 °C
- Rotor placed at 4 °C
- Round-bottom tubes for immunoisolations (e.g. culture tubes for small volumes or 50 mL Falcon tubes for larger volumes)
- Magnets for larger tubes and for eppendorf tubes

Protocol:

The protocol described here uses as a starting sample cells that have been cryogenically disrupted with a Ball Mill (e.g. Retch MM301) or a Mortar Grinder. However, the same protocol can be used for cells that were disrupted using other methods, such as incubation with lysis buffers, glass beads, or passage through needles of various gauges. Although we found the cryogenic cell disruption to be preferred, as it significantly increases the extraction efficiency and it proved absolutely critical in most of our studies, for some specific studies that require gentle handling of the cells, the other mentioned techniques could prove to be favorable.

1. Prepare lysis buffer; the buffer should be optimized to give efficient extraction of the tagged protein, while being mild enough to maintain protein-protein interactions intact. Examples of detergents to test are: Triton, Tween, Deoxycholate, Octylglucoside, Digitonin etc. Various concentrations of salt should also be tested. Examples of buffers are indicated in Cristea et al., MCP 2005.

2. Resuspend the frozen cell powder in the optimized lysis buffer. Do not let the cell pellet thaw before adding the buffer containing the protease inhibitors. Use 5 mL buffer per 1g cells and gently mix to ensure resuspension. If the sample were derived from cells that were cryogenically grinded inside round-bottom eppendorf tubes, add the buffer in small aliquots and shake the tube. In this way, the stainless steel grinding ball is used to wash the tube several times.

3. Use a 10-15 s Polytron step to improve the extraction. Keep the cell lysate cold. This step will generate foam. It is advisable to use a container in which the cell lysate will occupy less than 1/3 of the volume before the Polytron step. This will ensure that sample will not be lost during the procedure.

4. The cell lysate can be placed on a rotor (gentle rotation) at 4 °C for 10 min to allow for a decrease in the foam level.

5. Centrifuge the cell lysate at 3000 rpm, 4 °C, for 10 min. Even if some foam is apparent prior to this step, it will disappear after the centrifugation.

6. Measure the necessary amount of conjugated beads (the volume corresponding to the amount of beads necessary for the IP), place next to a magnet to draw the beads to the side of the tube (not to the bottom). Remove the PBS, 0.02% NaN3 solution, and wash the beads three times with 1mL of lysis buffer. After the third wash, resuspend the beads in a small volume of lysis buffer (e.g. 50-100 mL).

7. After the centrifugation, transfer the supernatant of the cell lysate to a clean container. Use preferably a round-bottom or wide-bottom tube (e.g. culture tubes for smaller volumes or 50 mL Falcon tubes for larger volumes).

8. Check carefully the supernatant of the cell lysate for any particles that might have not pelleted. If any particles are present, remove them, as these can bind to the beads and effect the specificity and efficiency of the isolation.

9. Add the washed beads to the cell lysate.

10. Incubate at 4 °C by gentle rotation. Ensure that beads are in contact with the cell lysate and do not get trapped on the walls or cap of the container.

11. Incubate for 5 min to 3 hours. Do not use longer incubation times, as this promotes the accumulation of non-specific binding and the loss of weak interacting partners.

12. After this incubation time, place the tube next to a magnet. Transfer the flowthrough to another tube to use it for Western blot analyses to test for the efficiency of protein recovery.

13. Wash beads six times with 1 mL lysis buffer. During the 1st wash, transfer the beads to a clean eppendorf tube. After the 4th wash, transfer the beads to another clean eppendorf tube.

14. After the 6th wash, elute the isolated proteins from the beads with 500 mL aqueous 0.5 N NH4OH, 0.5 mM EDTA solution. Make the elution solution fresh. For elution, place the beads in the elution container on a shaker (e.g. Tomy shaker) for 20 min at room temperature. The elution can also be performed with other solutions. For example, acid elutions can be achieved with 0.1 M citrate, pH 3.1 (as recommended by Dynal), or 0.1 % TFA, pH 1.5. In our hands, the elution with citrate has not proved very efficient.

15. After elution, place the tube next to a magnet and transfer the eluate to a clean eppendorf tube. Keep the beads to test for the efficiency of elution.

16. Snap freeze the eluate in liquid nitrogen and let it dry in a speed vac overnight (or for minimum 4 hours). Ensure that the sample is fully dried before proceeding further.

17. Add SDS sample buffer (e.g. 20 mL) to the dried sample. Shake on a Tomy shaker for 10 min, and place the sample at 70 °C (if possible with shaking) for 10 min. Keep 2-10 % of sample for Western blot analysis to test for IP efficiency.

18. Run the sample on a 1-D SDS-PAGE gel and stain the gel with a stain compatible with mass spectrometric analysis (e.g. Colloidal Coomassie Blue stain, Zinc stain).

Reference:

I.M. Cristea, R. Williams, B.T. Chait, M.P. Rout **"Fluorescent proteins as proteomic probes"** *Mol Cell Proteomics* (2005) 4, 1933-1941.

I.M. Cristea, J.W. Carroll, M.P. Rout, C.M. Rice, B.T. Chait, M.R. Macdonald **"Tracking and elucidating alphavirus-host protein interactions"** *J Biol Chem*, (2006) Aug 8.