Cell Lysis and Immunoprecipitation Protocol with CHAPS Buffer

Part No. CIB-1, 1X Solution; 60ml or 120ml. Protocol applies to pH 6.0 – 8.0
0.5% CHAPS in HEPES buffer.

Background
The CHAPS Immunoprecipitation Buffer (CIB-1) is formulated to maintain intermolecular interactions following transmembrane and cytosolic protein extraction. The CHAPS reagent is ideal for "pull down - co-immunoprecipitation" (i.e. Co-IP) analysis, since it maintains protein conformation as well as the assembly of protein complexes. The protocol outlined below is for cell culture cells.

Application to Culture Cells
Following cell lysis and protein solubilization into CHAPS Buffer, assembled proteins can be isolated together using co-immunoprecipitation technique. An immunoprecipitating antibody is added to bind to one of the assembled protein targets. A solid support, such as Protein-G Sepharose, is added to non-covalently link to the antibody-protein complex and make it insoluble. Following low speed sedimentation and wash, the solid support-antibody-protein complexes are isolated together. An elution approach is then used to remove the antibody-protein complex from the solid support into a supernatant.

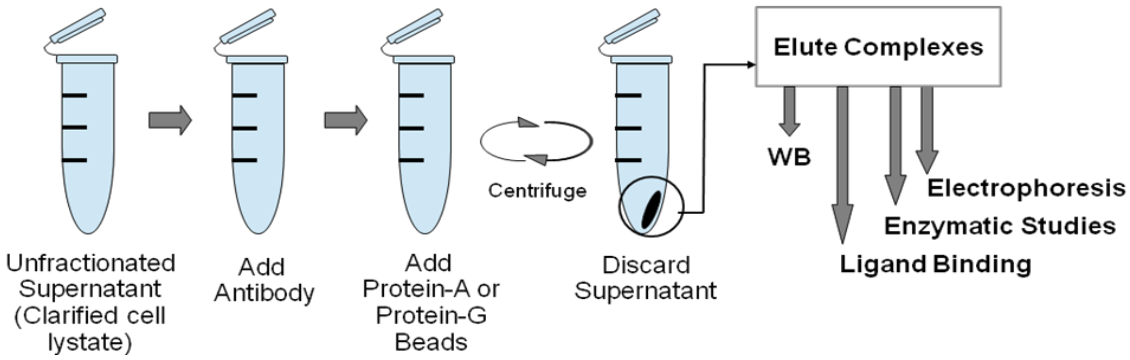
By resolving the eluted proteins in gels and developing Western blots with antibodies targeting co-assembled proteins, the researcher can quantify the interaction among two or more protein species. Functional activity assays can also be performed to characterize the biochemical properties of co-immunoprecipitated and assembled proteins.

Application to Tissues
The CHAPS Lysis and Immunoprecipitation Buffer can also be used with tissue homogenates for downstream immunoprecipitation. Use the steps noted at the end of this protocol (page 3, Section F) with tissue samples to generate an unfractionated supernatant and then proceed with the protocol at step D, page 3.

Required Equipment and Reagents:
1. Microcentrifuge at 4°C
2. Rocker table
3. Cold room or refrigerated case.
4. Antibody suitable for immunoprecipitation as specified by the vendor. Generally, the immunoprecipitating antibody must recognize the *NATIVE* conformation.
5. Antibody suitable for Western blot detection as specified by the vendor. The antibody selected for Western blot must recognize the *DENATURED* state of the targeted protein.

Suggested Controls to Verify Successful Immunoprecipitation:
1. Perform the immunopreciptation protocol with an irrelevant antibody to identify the appearance of spurious protein bands.
2. Resolve in Western blots 1) the original unfractionated cell lysate, 2) the unbound fraction (e.g. the fraction containing proteins that did not bind to the protein-A or G solid support), and 3) the immunoprecipitated materials to confirm fractionation and enrichment.
3. Develop a Western blot to detect the protein directly targeted by the immunoprecipitating antibody. This procedure confirms that immunoprecipitation occurred.

Overview



Protocol:

A. Cell Culture:
Cells should be cultured to 80-90% confluency prior to performing cell lysis and immunoprecipitation

B. Cell Lysis:
We recommend using 300µl of CHAPS Buffer solution for one to three 10cm cell culture dishes of cells. Scale accordingly for other numbers or sizes of cell culture dishes according to the surface area of the dish. Prior to lysis, prepare ice cold CHAPS Buffer with protease and phosphatase inhibitors. (Fivephoton Biochemicals offers a protease inhibitor cocktail (Part No. PI-1) and a Phosphatase Inhibitor Cocktail (Part No. PIC-1) that can be added to the cell lysis solutions).

C. Cells should be washed free of serum proteins using PBS prior to performing immunoprecipitation to prevent appearance of non-specific serum protein bands in downstream Western blots. Remove the cell culture medium and gently wash the 10cm cell culture dish with 5ml PBS, three times. Use a gentle stream of PBS to prevent excessive loss of cells from the plate. After the washes, cover the cell culture dish with the lid and place the cell culture dish on a bed of ice.

1. Lyze cells and generate a supernatant fraction rapidly as follows: Dispense 300µl ice cold CHAPS Buffer with protease/phosphatase inhibitors over the cell layer, rotate the plate by hand to cover cells with a film of CHAPS Buffer, then immediately dislodge the cells with a cell scraper. Use a transfer pipette with a wide opening to siphon the cells into a 1.5ml microcentrifuge tube. The 300µl Chaps Buffer solution can also be transferred sequentially to up to three separate 10cm plates of cells before the cell suspension is placed into a 1.5ml microcentrifuge tube.

2. Place the 1.5ml microcentrifuge tube with cell suspension on ice for 10 min: Strongly tap the tube several times during this 10 min period to facilitate cell membrane dissolution. You can also use a rocker table to rotate the cell suspension to further facilitate cell membrane dissolution. Do not vortex the cell lysate if immunoprecipitation is planned.

3. Centrifuge the cell lysate in a cooled microcentrifuge at full speed for 15 min to partition supernatant and pellet. Collect the **supernatant fraction**, which contains extracted membrane and cytosolic proteins, and dispense this supernatant into another 1.5 ml microcentrifuge tube that is placed in ice. This transferred supernatant corresponds to the **unfractionated supernatant fraction**. Set aside a small aliquot for comparisons to the immunoprecipitated materials that are generated later in the protocol. The unfractionated supernatant can be stored at -20°C, or -80°C for longer term storage.

D. Immunoprecipitation:

1. Add immunoprecipitating antibody to the unfractionated supernatant fraction, using the antibody titer recommended by the manufacturer. Place the tube with the immunoprecipitation reaction on a rocker table under refrigeration (such as a cold room, or refrigerated case) for 15-30 min to mix the antibody-supernatant mixture. You may opt to initially try the shorter 15 min period for immunoprecipitation, a time period that is more apt to maintain low affinity interactions.

2. Directly dispense 60µl of immunoprecipitation beads (e.g. Sepharose-G beads, or protein-A beads) into every 300µl to 2ml aliquot of unfractionated supernatant with immunoprecipitating antibody (from Step C1). Place this tube on a rocker table for another 30 min to 1 hr under refrigeration to generate an insoluble solid support–antibody-protein complex.

3. Use a refrigerated microcentrifuge at 3000 rpm for 5 minutes to sediment the immunoprecipitation beads. The resulting supernatant is the **unbound fraction**. Collect the unbound fraction without disturbing the immunoprecipitating bead pellet. Store the unbound fraction at -20°C or -80°C.

   Add an additional aliquot of fresh 300µl ice cold CHAPS Buffer with protease and phosphatase inhibitors to the immunoprecipitation beads. Gently rotate the tube 180° by hand three times and centrifuge again at 3000 rpm for 5 minutes. Remove and discard the wash supernatant. Repeat this wash procedure two more times. After the last wash, use a microcentrifuge to sediment the immunoprecipitation beads at 14000 rpm for 15 min. Remove as much of the wash solution as possible using a pointed plastic Pasteur pipette. The **immunoprecipitated fraction**, which is bound to the beads, becomes sedimented at the bottom of the tube.

E. Elution of the Immunoprecipitated Fraction From the Beads and Western Blot:

1. There are several methods to elute the immunoprecipitated proteins from the solid support to release a soluble **immunoprecipitated fraction**. The simplest method applicable for subsequent Western blotting is to apply Laemmli Sample Buffer (LSB; with mercaptoethanol) directly to the immunopreciptation beads: Add 100µl LSB to each 60µl of sedimented immunoprecipitation beads. Vortex the LSB – immunoprecipitate solution at full speed for 30 sec, and then heat at 60°C for 10 min. Now sediment the beads using low speed centrifugation (3000 rpm) for 5 min. Collect the supernatant; The immunprecipitated proteins (along with the immunoprecipitating antibody) will be released into the supernatant.

2. The supernatant can be resolved in subsequent Western blots. Alternatively, if your protein of interest aggregates easily when heated at high temperature in LSB, heat instead at 37°C for 30 min. and follow the same aforementioned steps. Resolve in consecutive gel lanes and Western blots, 1) the unfractionated fraction, 2) unbound fraction and 3) immunoprecipitated fraction. With successful immunoprecipitation, you should observe enrichment of your protein of interest, along with any co-assembled proteins in the immunopreciptated fraction.

F. Tissue Homogenization Protocol Prior to Immunoprecipitation (Perform on ice)

1. Pulverize approximately 90 µL of tissue. Place tissue in a 1.5 ml round bottom microcentrifuge tube.
2. Add general phosphatase and protease inhibitor cocktails to 500 µL of ice-cold CHAPS Lysis and Immunoprecipitation Buffer
3. Add 500 µL CHAPS Buffer with inhibitors to pulverized tissue.
4. Homogenize tissue with a mini pestle-homogenizer using 15 strokes, 3 seconds/stroke on ice.
5. Centrifuge 12000g for 15 min at 4°C.
6. Remove supernatant (without lipid layer) and transfer into another 1.5 ml tube.
7. Centrifuge again at 12000g for 15 min at 4°C.
8. Transfer supernatant to another tube. The supernatant fraction contains the extracted proteins that can be used for immunoprecipitation.

Troubleshooting:

1. Immunoprecipitation did not occur. Resolution: 1) You may have to empirically identify an antibody for immunoprecipitation that recognizes the native conformation of the epitope. 2) The protein complex is not maintained outside of a live-intact cell. Cross-linking procedures using membrane permeable cross-linking reagents and live cells may be required to capture the interaction and assembly among proteins.

2. The IgG heavy chain of the immunoprecipitating antibody (that was dispensed in the immunoprecipitation solution) migrates in gels at the same position as a protein of interest, and therefore masks its appearance in a Western blot. Resolution: For Western blot development, use an antibody derived from an animal host different from the immunoprecipitating antibody. In this case, the appropriate secondary-HRP conjugated antibody will not bind appreciably to the immunoprecipitating antibody that was transferred to the Western blot, preventing the appearance of an overlaying Western blot band.