Protocol for Immunoprecipitation Kit (With HRP-conjugated protein A)

1. Preparation of cell or tissue lysates

a. Cell

①Pre-cool a refrigerated centrifuge to 4 °C.

②Count cells with a hemocytometer before harvesting them.

③Cells were harvested by centrifugation at 500 × g for 5 min at 4 °C.

④Wash cells 3 times with ice-cold 1 × PBS, and then add 100 μL pre-cooled IP lysis buffer to every 10^6 cells (add Protease inhibitor to 1 × right before use). To obtain protein lysates with high concentration, the amount of IP lysis buffer can be appropriately reduced.

⑤If the target protein is phosphorylated protein, an additional amount of phosphatase protein inhibitor should be added.

⑥Resuspend cells in IP lysis buffer, and then lyse on ice for 30 min with gentle inversion every 10 min.

⑦See Lysis and Storage for the next steps.

b. Tissue

①Dissect the target tissue and wash with pre-cooled 1 × PBS to remove the remaining blood as much as possible, and cut the tissue into smaller pieces whilst keeping it on ice.

②Transfer the tissue pieces to a pre-cooled homogenizer.

③Add 10-20 μL IP Lysis buffer for every 1 mg of target tissue (add Protease inhibitor to 1 × right before use).

④Thoroughly homogenize the target tissue and lyse it on ice for 30 min, inverting every 10 min.

⑤See Lysis and Storage for the next steps.

C. Lysis and Storage

①Sonicate the lysates to break the cells or tissue up further and to shear DNA. Adjust sonication time to your type of sample: 1 min for cell lysates and 2-5 min for tissue lysates at a power of about 180 watts (in rounds of 2 seconds sonication/2 seconds rest for each cycle). Keep the sample on ice during the sonication.

②Lysates were placed on ice for 20-30 min, inverting every 10 min.

③Centrifuged at 4 °C for 10-15 min at 10,000 × g, transfer the supernatant to a new EP tube for later use. Discard the sediment or store it for subsequent analysis.

④Determine protein concentration of the lysate by BCA protein assay.

⑤Lysates can be used for IP or Western blotting, and also can be stored in aliquots at -80 °C.

⑥For Western blotting, mix sample with 5 × Sample buffer to a final dilution of 1 ×. Heat the mixture to 95 °C for 5 min before loading onto an SDS-PAGE gel.

2. Preparation of rProtein A/G Beads

Invert the tubes containing rProtein A/G beads slurry, remove the required amount of rProtein A/G beads slurry, and wash the beads three times with 1 × PBS by low-speed centrifugation (1 mL PBS can be used each time, 500 × g centrifugation for 30 s). Finally resuspend the beads in PBS to the original volume.

3. Pretreatment of lysates (optional)

①Cut off the end of the sterilized pipette tip at a 45° angle, quickly pipette the resuspended rProtein A/G Beads slurry, and add it to the EP tube containing lysates. Typically 1-3 mg of total protein lysates need to be added 30 μL of resuspended rProtein A/G beads slurry.

②Incubate with rotation at 4 °C for 30-60 min (vertical rotation mixer recommended, low speed rotation).

③Centrifuge at 500 × g for 1 min at 4 °C and transfer the supernatant to a new EP tube.

4. Immunoprecipitation

①Pipette 200-350 μL of lysates containing 1-3 mg of total protein (or pretreatment), add it to Spin columns with End caps at the bottom, add 1-4 μg of specific antibody and 150-300 μL of Incubation buffer at the same time. The optimal amount of antibody should be determined by the antibody titer.

②Add control IgG to lysate and incubation buffer as negative control, the species and added amount of control IgG are consistent with the specific antibody, and the volume of lysate and incubation buffer is consistent with step 4.1 (subsequent operations of the negative control are exactly same as the target sample).

③Incubate overnight or 2-4 h with rotation at 4 °C.

④Add 50 μL of resuspended rProtein A/G beads slurry into Spin columns to precipitate immune complexes, and incubate 1-4 h with rotation at 4 °C.

⑤Remove the End caps and discard the supernatant. If necessary, the flow rate of the supernatant can be increased by resuspending rProtein A/G beads.

⑥Wash the precipitate compound with 800 μL 1 × Washing buffer (take an appropriate amount of 20 × Washing buffer, dilute to 1× with pure water, and add Protease inhibitor to 1 × right before use) each time, and the Washing solution flows out naturally and discarded; Repeat washing 4-5 times. After washing, Spin columns were placed into Collection tubes at 4 °C and 500 × g, centrifuged for 30 s, and the Collection tubes and centrifuged products were discarded.

5. Elution

①Put the spin columns into a new 1.5 mL EP tube to collect the eluted product, use 40 μL Elution buffer to elute the precipitated complex, let it stand at room temperature for 5-10 min, and then centrifuge at 10,000 × g for 1 min at 4 °C to collect the product. Repeat the elution once with a new 40 μL Elution buffer (After 2 elutions, the products were pooled and collected).

②Add 10 μL Alkali neutralization buffer and 23 μL 5 × Sample Buffer to all eluted products, and heat it to 95 °C for 5 min.

6. Western blotting analysis

①Load 20-40 μL of IP samples into the corresponding gel lanes, and store the remaining IP samples at -80 °C for later use.

②After the IP samples were separated by SDS-PAGE, and the protein was then transferred to the PVDF membrane. Western blotting analysis was performed using detection antibody hybridization and HRP-conjugated protein A secondary antibody at 1:1500-1:3000 dilution.

7. Supplementary information

①If the operation time of the IP experiment needs to be shortened, the specific antibody corresponding to the target protein and the rProtein A/G beads slurry can also be added to the cell or tissue lysates for one-step incubation.

②If the antibody for IP and the primary antibody for WB are both mouse IgG1 or IgG3 subtype, the dilution of the HRP-conjugated protein A secondary antibody can be reduced during WB detection. If the signal of target is still weak, Peroxidase-conjugated Affinipure Goat anti-Mouse IgG(H+L) can be used.

③If the subtype of primary antibody is mouse IgM during WB detection, HRP-conjugated protein A secondary antibody cannot be used.

④If necessary, the kit can be scaled up for use.