Immunoprecipitation using antibody immobilized FG beads

1. Materials

1) Antibody immobilized FG beads and protein solution

・Antibody immobilized beads:
Antibody immobilized beads 0.1 mg
(Antibody non-immobilized beads 0.1 mg)
When investigating some experimental conditions (protein concentration, salt concentration of binding and washing buffer, etc.), prepare 0.l mg of beads for each experimental conditions.

・Protein solution (typically cell extract)
Protein concentration 5 - 15 mg/ml
Dilute the above protein solution to 1 - 3 mg/ml with binding and washing buffer.
Prepare 200 µl of protein solution for each experimental conditions.

2) Reagents

2-[4-(2-Hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES)
Sodium hydroxide (NaOH)
Potassium chloride (KCl)
Magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_2O$)
Calcium chloride ($CaCl_2$)
Ethylenediaminetetraacetic acid (EDTA)
Glycerol
Nonidet P-40 (NP-40)
Phenylmethylsulfonyl fluoride (PMSF)
Glycine
Tris(hydroxymethyl)aminomethane (Tris)
Hydrochloric acid (HCl)
4×SDS-PAGE sample buffer (4×Dye)
Eectrophoresis (SDS-PAGE) gel
Eectrophoresis buffer
Silver stain kit

3) Apparatus

High-speed micro centrifuge (HITACHI CF15RX2)
Desktop centrifuge (for spin down)
Magnet stand (Tamagawa Seiki, TA4899N12)
Rotator (TAITEC BC-710)
Heat block (TAITEC DTU-1B)
Slab gel electrophoresis device

2. Method

1）Preparation of buffers

1. IP buffer (Binding and washing buffer):
   20 mM HEPES-NaOH (pH7.9)
   150 mM KCl
   1 mM $MgCl_2$
   0.2 mM $CaCl_2$
   0.2 mM EDTA
   10 % (v/v) Glycerol
   0.1 % NP-40
   0.2 mM PMSF

2. Acid elution buffer
   0.1 M Glycine-HCl（pH 2.5）

3. Neutralizing buffer
   1 M Tris-HCl（pH9.0）

4. 4×dye solution（Wako : 191-13272）
   0.25 M Tris-HCl（pH 6.8）
   0.02 % BPB
   8 % SDS
   40 % glycerol
   20 % 2-mercaptoethanol

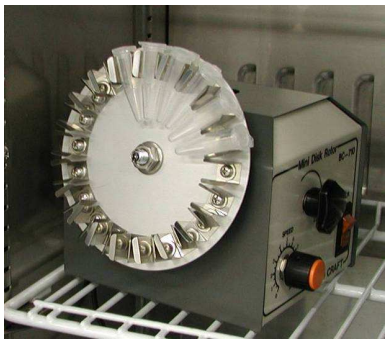
2）Buffer exchange of antibody immobilized beads and centrifugation of protein solution

1. Prepare an appropriate volume (typically 50 ml) of IP buffer on ice.
2. Using cold IP buffer, adjust protein concentration of the protein solution (usually cell extract) to appropriate concentration (the recommended concentration is 1 mg/ml or 3 mg/ml).
3. Transfer the adjusted protein solution into 1.5 ml microtubes and centrifuge at 15,000 rpm for more than 30 min at room temperature. After centrifugation, transfer the supernatant to fresh tubes (being careful not to recover precipitation)
4. While centrifugation (step 3), add 0.1 mg of antibody immobilized beads into 1.5 ml microtubes.
5. Add 200 µl of IP buffer into the tubes and disperse beads by brief sonication or manual dispersion method*.
   (*See supplementary instructions about dispersion method)
6. After brief spin down, perform a magnetic separation by using a magnet stand and remove the supernatant.
7. Repeat step 5 to 6 two times (totally three times).

3) Binding and washing

1. Add 200 µl of the centrifuged protein solution (step 2)-3) into each tube in which the beads have been collected, then disperse the beads.

2. Mix for 2 h at 4 °C by using Rotator (picture below).



3. After 2 h rotating, spin down briefly, perform a magnetic separation and remove the supernatant.
4. Add 200 µl of cold IP buffer into the tubes and disperse beads by manual dispersion method.
5. After brief spin down, perform a magnetic separation and remove the supernatant.
6. Repeat step 3 to 4 two times (totally three times).

4) Elution (choose acid elution or boil elution depending on your experimental system)

4-1) Acid elution

1. After removing the supernatant, add 28 µl of acid elution buffer and disperse beads by manual dispersion method.
2. After elution of bound proteins by incubating on ice for 5 min, spin down briefly and perform a magnetic separation.
3. Transfer the supernatant to fresh microtubes that contain 2 µl of neutralizing buffer.
4. Add 10 µl of 4×Dye solution to the acid elution sample and mix them.
5. Boil the sample for 5 min at 98 °C

4-2) Boil elution

1. After removing the supernatant, add 40 µl of 1×Dye solution and disperse beads.
2. Boil the sample for 5 min at 98 °C.
3. Spin down briefly and perform a magnetic separation at room temperature.
4. Transfer the supernatant (eluted protein solution) to fresh microtubes.

5) Analysis of eluted proteins

1. Apply an appropriate volume of the eluted sample (typically 10 μ l) to the gel of SDS-PAGE and perform electrophoresis.
2. After SDS-PAGE, perform an appropriate staining such as silver staining, fluorescent staining or CBB staining.

* If you need, perform further analysis such as western blotting.

3. Supplementary instructions

1) A method of beads dispersion

Two methods for dispersion of FG beads are used, sonication or manual agitation.

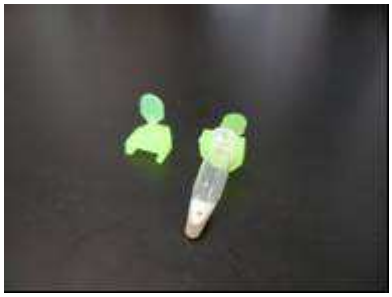
While sonication is effective, it is not recommended for steps involving protein affinity purification. In the manual dispersion method, the bottom of an micro-tube is glided over an uneven surface (side of plastic test tube rack in this case) creating turbulence through the collisions. (see left side picture below)

Please make sure to use well-constructed tubes with the caps tightly secured in order to prevent leakage/breakage. Use of cap lock is recommended in order to prevent leakage. (see right side picture below).

For more information, please visit FG beads web site and see the movie of the method.

http://www.magneticnanoparticle.jp/en/htdocs/af-notes.html

http://www.youtube.com/watch?v=6z6zyYww03Q&feature=player_embedded



2) Magnetic separation

Magnetic separation should be performed on ice.

3) Preparation of a protein solution

Dignam method is one of the method to prepare cell extract as a protein solution.

ference : J.D.Dignam, R.M.Lebovitz, and R.G.Roeder, *Nucleic Acids Res*. **11**, 1475（1983）

4. Notes

・Please confirm that no aggregates of beads are observed after dispersing the beads. An insufficient washing (dispersing) is likely to cause nonspecific protein bands to appear.

・Wear gloves to prevent keratin contamination.