Experiment Protocol 011

Immunoprecipitation by using antibody immobilized beads

1. Materials

1.1 Antibody Immobilized beads, Protein solution

・ Antibody immobilized beads

Antibody (-) beads and antibody (+) beads: 0.1 mg for each type

When investigating conditions (e.g. protein concentration, salt concentration of binding / washing buffer, etc.): 0.1mg for each condition

・ Protein solution

Protein concentration: 5 to 15 mg/mL (Not applicable if the original concentration is less than 5 mg/mL.)

Dilute the solution with binding/washing buffer (usually protein concentration should be 1 mg/mL). Required volume : 200 µl for each condition

1.2 Reagents

・ 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES)
・ Sodium hydroxide (NaOH) ・ Potassium chloride (KCl) ・ Magnesium chloride ($MgCl_2$)
・ Calcium chloride ($CaCl_2$) ・ Ethylenediamine tetraacetic acid (EDTA) ・ Glycerol (Glycerin)
・ Nonidet P-40 (NP-40) ・ Phenyl fluoride methane sulfonyl (PMSF)
・ Dimethylsulfoxide ・ Sample buffer (4×dye)
・ Glycine ・ Tris (hydroxymethyl) amino methane (Tris) ・ Hydrochloric acid (HCl)
・ Electrophoresis (SDS-PAGE) gel ・ Electrophoresis buffer ・ Silver staining reagent

1.3 Apparatus

・ Micro high-speed cold centrifuge ・ Desktop centrifuge (for spin down)
・ Magnetic stand (Tamagawa Seiki TA4899N1, etc.) ・ Rotator
・ Heat block ・ Slab gel electrophoresis device

2. Method

2.1 Preparation for reagent solutions

1) 2×150 mM KCl buffer (500 mL): Mix 60 mL of 2.5 M KCl, 126 g of glycerol, 20 mL of 1 M HEPES-NaOH solution (pH 7.9), 1 mL of 1 M $MgCl_2$ solution, 200 µL of 1 M $CaCl_2$ solution, 400 µL of 0.5 M EDTA solution(pH 8.0), and 10 mL of 10% NP-40 solution.Dilute this with ultrapure water in a measuring cylinder to 500 mL total. (Store this at room temperature after filtration.)
2) 150 mM KCl buffer: Mix 25 mL of ultrapure water and 25 mL of 2×150 mM KCl buffer. 10 µL of 1 M PMSF solution just before use.
3) 1 M PMSF solution: Prepare 1 M PMSF solution by dissolving PMSF in dimethylsulfoxide. (Store this at -20℃)

Composition of binding/washing buffer (150mM KCl 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, and 0.2 mM PMSF

Composition of acid elution buffer

0.1 M Glycine (pH 2.5)

Composition of neutralizing buffer

1 M Tris-HCl (pH 9.0)

Composition of 4×dye solution (Wako Pure Chemical Industries Ltd.: 191-13272)
0.25 M Tris-HCl(pH 6.8), 0.02% BPB, 8% SDS, 40% glycerol, and 20% 2-mercaptoethanol

2.2 Procedures

2.2.1 Preparation

1) Prepare 150 mM KCl buffer, 1 M KCl buffer and acid elution buffer, and place them on ice.
2) On the ice, adjust the concentration of the protein solution to a target concentration (1 mg/mL, 3 mg/mL, etc.)
3) Add the solution into 1.5 mL micro-tubes, and centrifuge at 15,000 rpm for 30 minutes or more at 4℃ to remove insoluble matter. (Transfer the supernatant to a fresh tube after the centrifugation.)
4) During the centrifugation, add 0.1 mg of antibody immobilized beads into each 1.5 mL micro-tube. (Beads must be fully dispersed in advance to make a uniform suspension)
5) Add 200 µL of 150 mM KCl buffer to the suspension and disperse the beads.
6) After spin down, separate magnetically, and discard the supernatant.
7) Repeat the above 5) to 6) two more times. (Wash the beads with buffer three times in total.)

2.2.2 Binding and washing

1) Add 200 µL of protein solution obtained after the centrifugation to each 1.5 mL micro-tube containing beads without the supernatant, and disperse the beads.
2) Perform binding reaction for two hours at 4℃ by agitating the beads with a rotator.
3) Two hours later, spin down, separate magnetically, and discard the supernatant.
4) Add 200 µl of 150 mM KCl buffer, and disperse the beads.
5) After spin down, separate magnetically, and discard the supernatant.
6) Repeat the above 3) to 4) two more times. (Wash the beads with buffer three times in total.)

2.2.3 Elution

2.2.3.1 In case of acid elution

1) Add 28 µL of acid elution buffer to the beads without supernatant, and disperse the beads.
2) Place the solution on the ice for five minutes to elute acid. After spin down, separate magnetically.
3) Transfer the supernatant (acid elution sample) to a fresh 1.5 mL micro-tube. (Discard the beads.)
4) Add 2µL of neutralizing buffer to the acid elution sample to neutralize the sample.
5) Add 10 µL of 4×dye solution to the sample, and mix them.
6) Boil them for five minutes at 4℃. (using a heat block)

2.2.3.2 In case of boil elution

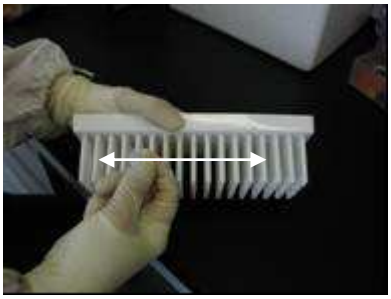
1) Add 40 µL of 1×dye solution to the beads without supernatant, and disperse the beads.
2) Boil them for five minutes at 98℃. (using a heat block)
3) Spin down the beads dispersed solution, and separate magnetically at room temperature.
4) Transfer the supernatant (the boil elution sample) to a fresh 1.5 mL micro-tube. (Discard the beads.)

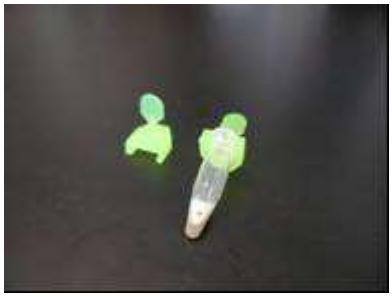
2.2.4 Analysis of proteins

1) Proceed to the electrophoresis (SDS-PAGE) process. (Or store them in a freezer at -20℃.)
2) Apply the acid elution sample and the boil elution sample to SDS-PAGE. (e.g. 10 µl for each)
3) Silver-stain the electrophoresed gel, and analyze it.

3. Supplements

・ Beads are easily dispersed by the manual agitation. In the manual dispersion method, the bottom of a 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.
(Please click: http://www.magneticnanoparticle.jp/en/htdocs/technique/affinity.html for moving pictures.)





・ Perform magnetic separation by placing a magnetic stand on ice.

Magnetic stand

Before separation

After separation

- For protein solution, we recommend using plasmatic compartments (or nuclear fractions or membrane fractions) prepared by Dignam method.
  A reference book：J.D.Dignam, R.M.Lebovitz, and R.G.Roeder, *Nucleic Acids Res.* **11**, 1475(1983)
  The Dignam method, however, requires numerous cells (>10^9 Cells). Therefore, when conducting the experiment on a small scale, the use of NP-40 lysis method or marketed cell extract reagents are also allowable.
- The salt elution is used to isolate weak affinity proteins. When the affinity is disregarded because all types of proteins can be eluted by boil elution, the salt elution should be excluded. Conversely, when you need to recover strong affinity proteins only, strong affinity proteins alone can be purified by increasing the volume of salt elution buffer, and increasing the number of the elution in a washing process.
- Salt concentration of the salt elution sample is so high that it may yield a white turbidity or precipitation. And as it does not affect the results of the experiment, proceed directly to the electrophoresis.

4. Notes

- Be sure to centrifuge the cell extract before mixing with beads. Otherwise, insoluble fractions caused by freezing and thawing remain in the extract, which can cause a background.
- Recover beads not by centrifugation but by magnetic separation. If centrifuged, insoluble fractions of proteins yielded during the reaction are recovered along with the beads, which can cause a background.
- When dispersing beads in washing and elution process, ensure that the beads are fully dispersed. Otherwise, non-specific bands are likely to appear.
- Wear gloves during the experiment to prevent keratin contamination.