Experiment Protocol 013

Drug elution in screening by using ligand immobilized beads (Affinity purification of ligand-binding proteins)

1. Materials

1.1 Ligand immobilized beads, Protein solution

- Ligand immobilized beads
  Beads with different amount of immobilized ligands: 0.5 mg for each type
  When investigating conditions other than the amount of ligand immobilization (e.g. protein concentration, salt concentration of binding/washing buffer, etc.): 0.5 mg 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
- Ligands (for drug elution)

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) ・ Dithiothreitol (DTT) ・ Phenyl fluoride methane sulfonyl (PMSF)
- Dimethylsulfoxide ・ Sample buffer (4×dye)
- Electrophoresis (SDS-PAGE) gel ・ Electrophoresis buffer ・ Silver staining reagent

1.3 Apparatus

- Micro centrifuge ・ Desktop centrifuge (for spin down)
- Magnetic stand (Tamagawa Seiki TA4899N12, etc.) ・ Rotator
- Heat block ・ Slab gel electrophoresis device

2. Method

2.1 Preparation for reagent solutions

1) 2×100 mM KCl buffer (500 mL): Mix 40 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) 100 mM KCl buffer: Mix 25 mL of ultrapure water and 25 mL of 2×100 mM KCl buffer. Add 50 µL of 1 M DTT solution and 10 µL of 1 M PMSF solution just before use.
3) 1 M DTT solution: Prepare 1 M DTT solution by dissolving DTT in ultrapure water. (Store this at -20℃)
4) 1 M PMSF solution: Prepare 1 M PMSF solution by dissolving PMSF in dimethylsulfoxide. (Store this at -20℃)
5) Solution for drug elution: Prepare 100 mM solution and 500 mM solution by dissolving ligands in dimethylsulfoxide. Dilute the solutions with binding/washing buffer until the concentration of DMSO becomes 1%, and prepare 0 mM, 1 mM, and 5 mM solutions. (30 µL of each solution is used.)

Composition of binding/washing buffer (100mM KCl buffer)

20 mM HEPES-NaOH(pH7.9), 100 mM KCl, 1 mM $MgCl_2$, 0.2 mM $CaCl_2$, 0.2 mM EDTA, 10%(v/v) glycerol, 0.1% NP-40, 1 mM DTT, and 0.2 mM PMSF

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

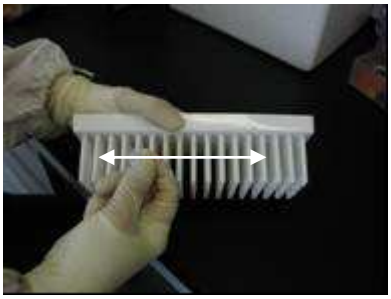
1) Prepare 100 mM KCl buffer, and place them on ice.
2) On the ice, adjust the concentration of the protein solution to a target concentration (1 mg/mL, 3 mg/mL, etc.)
3) Add the solution into 1.5 mL micro-tubes, and centrifuge at 15,000 rpm for 30 minutes or more at 4℃ to remove insoluble matter. (Transfer the supernatant to a fresh tube after the centrifugation.)
4) During the centrifugation, add 0.5 mg of ligand 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 100 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.)
8) Add 200 µL of centrifuged protein solution to each 1.5 mL micro-tube containing beads without the supernatant, and disperse the beads.
9) Perform binding reaction for four hours at 4℃ by agitating the beads with a rotator.
10) Four hours later, spin down, separate magnetically, and discard the supernatant.
11) Add 200 µL of 100 mM KCl buffer, and disperse the beads.
12) After spin down, separate magnetically, and discard the supernatant.
13) Repeat the above 11) to 12) two more times. (Wash the beads with buffer three times in total.)
14) Add 30 µL of each of the 0 mM, 1 mM, and 5 mM solutions for drug elution respectively to each tube containing the beads without supernatant, and disperse the beads.
15) Place the solution on the ice for an hour to allow bound proteins to elute. (by tapping at intervals). After spin down, separate magnetically.
16) Transfer the supernatant (drug elution sample) to a fresh 1.5 mL micro-tube.
17) Add 40 µL of 1×dye solution to the remaining beads, and disperse them.
18) Add 10 µL of 4×dye solution to the drug elution sample, and mix them.
19) Boil the beads dispersed solution and the drug elution sample for five minutes at 98℃. (using a heat block)
20) Spin down the beads dispersed solution, and separate magnetically at room temperature.
21) Transfer the supernatant (the boil elution sample) to a fresh 1.5 mL micro-tube. (Discard the beads.)
22) Proceed to the electrophoresis (SDS-PAGE) process. (Or store them in a freezer at -20℃.)
23) Apply the drug elution sample and the boil elution sample to SDS-PAGE. (e.g. 10 µL for each)
24) Silver-stain the electrophoresed gel, and analyze it.

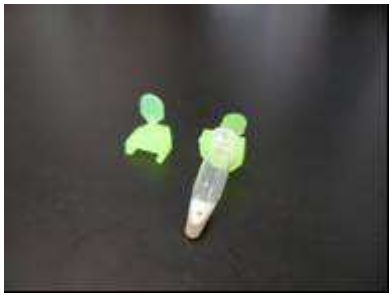
3. Supplements

・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 ligand concentration, 1 mM and 5 mM (the amount of ligands is 30 nmol and 150 nmol respectively) at the time of drug elution is set to 5 to 50 times of the estimated amount of immobilized ligands (3 nmol/0.5 mg). (The most suitable amount of immobilized ligands is generally 1 to 10 nmol per mg.)
The bound protein may be eluted even if the ligand concentration is set to (0.2 mM) about twofold the estimated amount of immobilized ligands.
Raise the concentration of ligands when you cannot confirm disappearance of a band, which is caused by competitive inhibition, and which appears to be a band of target proteins.
If you cannot raise the concentration because the ligands are slightly soluble in DMSO, increase the amount of the ligands by raising the concentration of DMSO up to 5% at the time of diluting them with buffer.

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.