Experiment Protocol 101

Immobilization of antibodies or proteins on COOH beads

1. Materials

1.1 Beads and Ligands (Antibodies)

- COOH beads (TAS8848N1140):10 mg (Functional groups : Approx. 200 nmol/mg)
- Antibodies or Proteins: Approx.50 µg

1.2 Reagents

- 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid(HEPES)
- Sodium hydroxide ・ Potassium chloride ・ Ethylenediamine tetra acetic acid (EDTA)
- Glycerol ・ Morpholinoethane sulfonic acid (MES)
- Methanol ・ N,N-Dimethylformamide（DMF）
- 1-etyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride（EDC・HCl） M.W. 191.70
- Amino ethanol M.W. 61.08 ・ Hydrochloric acid ・ Isopropyl alcohol (IPA)

Composition of protein immobilization buffer

25mM MES-NaOH（pH6.0）・・・・・Antibodies
25mM HEPES-NaOH（pH7.0）・・・・・Other proteins

Composition of protein immobilized beads wash/storage buffer

10mM HEPES-NaOH（pH7.9）
50mM KCl
1mM EDTA
10% glycerol

1.3 Apparatus

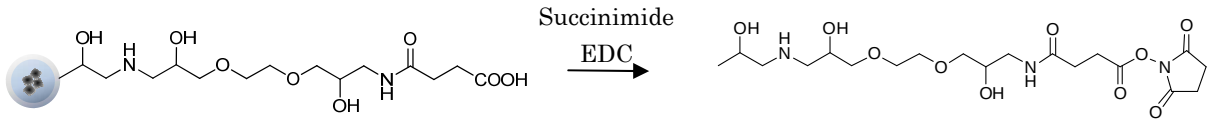
- Micro centrifuge ・ Micro tube mixer（TOMY MT-360, etc.）
- Ultrasonic dispersing device
  We have performed operation checks with an ultrasonic homogenizer:VP-15S with a cup horn (TAITEC), or an ultrasonic dispersing device:TA4905（Tamagawa Seiki）.

2. Method

2.1 Outline

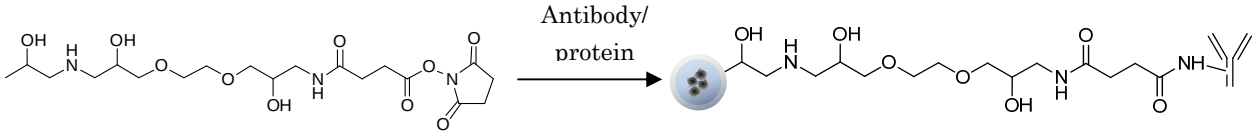
The following is a schematic view of ligand immobilization. Refer to the next section 2.2 “Procedures” for details.

1) NHS activation



COOH beads　　NHS beads

2) Antibody/protein immobilization



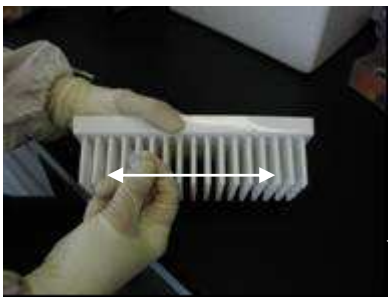
NHS beads　　Antibody (protein) immobilized beads

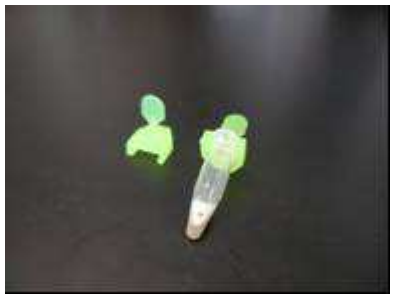
2.2 Procedures

1) Prepare protein immobilization buffer and washing/storing buffer.
2) Dissolve succinimide in DMF, and prepare 250 µL of 1M succinimide solution.
3) Dilute antibodies or proteins with protein immobilization buffer, and prepare 50 µg/50 µL antibody solution by 50 µL or more. (Prepare the solution slightly more than 50 µL so that you can fully secure 50 µL.)
4) Dissolve amino ethanol in ultrapure water, and prepare 1 mL of 1 M amino ethanol solution (pH8.0). (Adjust the pH with hydrochloric acid.)
5) Add 1 mg of COOH beads (TAS8848N1140) into a 1.5 mL micro-tube.
6) Centrifuge at 15,000 rpm for five minutes at room temperature, and discard the supernatant.
7) Add 100 µL of DMF, and disperse the COOH beads with an ultrasonic device.
8) Centrifuge at 15,000 rpm for five minutes at room temperature, and discard the supernatant.
9) Repeat the above 7) to 8) two more times. (Wash the beads three times in total.)
10) Add 7.68 mg (40 µmol) of EDC・ HCl into another 1.5 mL micro-tube.
11) Add 160 µL of DMF to COOH beads, and disperse the beads with an ultrasonic device.
12) Add 40 µL of 1M succinimide solution, and mix them.
13) Add 200 µL of COOH beads - succinimide solution to EDC measured in the above 10), and disperse them with an ultrasonic device.
14) React them for 2 hours at room temperature by using Microtube Mixer.
15) Centrifuge at 15,000 rpm for five minutes at room temperature, and discard the supernatant.
16) Add 100 µL of DMF, and disperse the beads (NHS beads) with an ultrasonic device.
17) Centrifuge at 15,000 rpm for five minutes at room temperature, and discard the supernatant.
18) Repeat the above 16) to 17) four more times. (Wash the beads five times in total.)
19) Add 100 µL of DMF, and disperse the beads (NHS beads) with an ultrasonic device. (Concentration of NHS beads: 1 mg/100 µL (When storing them in a state of NHS beads, store them at -30℃ after completely replacing them to IPA.)
20) Add 1 mg of NHS beads into a 1.5 mL micro-tube.
21) Centrifuge at 15,000 rpm for five minutes at 4℃, and discard the supernatant.
22) Add 50 µL of methanol, and disperse the NHS beads with an ultrasonic device.
23) Centrifuge at 15,000 rpm for five minutes at 4℃, and discard the supernatant.
24) Add 50 µL of protein immobilization buffer, and disperse the beads with an ultrasonic device.
25) Add 50 µL of antibody or protein solution.
26) React them for 30 minutes at 4℃ by using Microtube Mixer.
27) Centrifuge at 15,000 rpm for five minutes at 4℃, and transfer the supernatant to a fresh micro-tube. (For protein quantification)
28) Add 250 µL of 1 M amino ethanol solution, and disperse the beads with an ultrasonic device.
29) React them for 16 to 20 hours at 4℃ by using Micro-tube Mixer.
30) Centrifuge at 15,000 rpm for five minutes at 4℃, and discard the supernatant.
31) Add 200 µL of protein immobilization beads washing/storing buffer, and disperse the beads.
32) Centrifuge at 15,000 rpm for five minutes at 4℃, and discard the supernatant.
33) Repeat the above 31) to 32) two more times. (Wash the beads three times in total.)
34) Disperse the beads in 200 µL of protein immobilized beads wash/storage buffer, and store them at 4℃. (Concentration of antibody immobilization beads : 0.1 mg/20 µL)

3. Supplements

・ Beads are easily dispersed by using an ultrasonic dispersing device. But if you do not have such a device, they are dispersed by using an ultrasonic washer, or 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.)





・ When dispersing the beads after immobilizing antibodies or proteins, disperse them by the manual agitation. When you cannot disperse the beads easily, disperse them in a short time by using an ice-cold ultrasonic homogenizer or ultrasonic washer.
・ Recover the beads not by magnetic separation but by centrifugation.
・ The amount of antibodies and proteins immobilized on beads can be calculated from protein quantification (Bladford method or SDS-PAGE) of the transferred supernatant. It can also be calculated directly from protein immobilized beads by BCA method.
・ When you want to increase the volume of antibodies and proteins immobilized on beads, increase the volume of the proteins to be added.
・ Because EDC・HCl are prone to absorb moisture, be careful not to contaminate them with moisture.
・ Use DMF which is hydrated with a molecular sieve, or a low-moisture solvent. If the solvent contains moisture, succinimide may be liberated from beads, and ligands are not properly immobilized on the beads.
・ Because NHS (succinimide) is liberated quickly into water, perform the work as promptly as possible under a condition of 4℃.
・ If the protein immobilization solution contains Tris or BSA, remove it because it is immobilized on the beads.