Experiment Protocol 301

Immobilization of double strand DNA on Plain beads

This experiment protocol describes the immobilization of double strand DNA to purify DNA-bound proteins such as transcription factors. This protocol includes annealing of complementary single strand DNA, 5' end phosphorylation, ligation, and immobilization on beads. Double strand DNA is immobilized on beads by reacting guanine amino groups of DNA protruding ends with epoxy groups on beads surfaces.

1. Materials

1.1 Beads and Ligands (DNA)

- Plain beads (TAS8848N1010): 20mg (Functional groups: Approx. 1μmol/mg)
- Complementary single strand DNA: approximately 150μg for each annealing
  1. Target sequence (Example)
     5'-AGGGTATGCAAATTAAGAAG-3'
     3'-TCCCATACGTTTAATTCTTC-5'
  2. Synthetic oligonucleotide for immobilization
     Sense: 5'-GGGGAGGGTATGCAAATTAAGAAG-3'
     Antisense: 3'-TCCCATACGTTTAATTCTTCCCCC-5'

1.2 Reagents

- T4 Polynucleotide Kinase (Takara Bio 2021S, etc.)
- T4 DNA Ligase (Takara Bio 2011A, etc.)
- Phenol/Chloroform 2mL ・ Sodium chloride ・ Ethanol 10mL
- NICK columns (GE Healthcare 17-0855-02, etc.)
- 3M Sodium acetate (pH5.3) ・ 2.5M Potassium chloride ・ Agarose gel
- TES buffer
  10 mM Tris-HCl (pH 8.0)
  0.3 M KCl
  1 mM EDTA
  0.02 % NaN_3

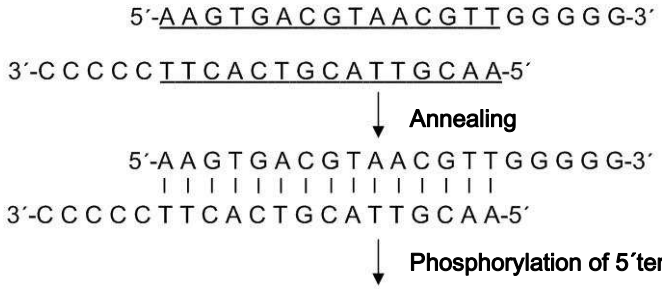
1.3 Apparatus

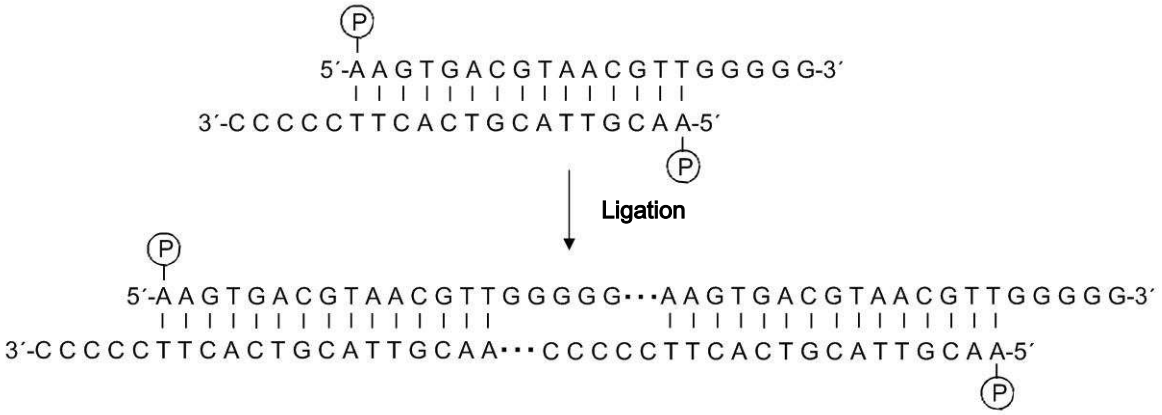
- Micro high-speed cold centrifuge ・ Thermostat bath ・ Electrophoresis unit
- Spectrophotometer ・ UV lamp or imager ・ Vortex mixer
- Ultrasonic dispersing device
  We have performed operation checks with an ultrasonic homogenizer: VP-15S with a cup horn(TAITEC), and an ultrasonic dispersing device: TA4905 (Tamagawa Seiki).

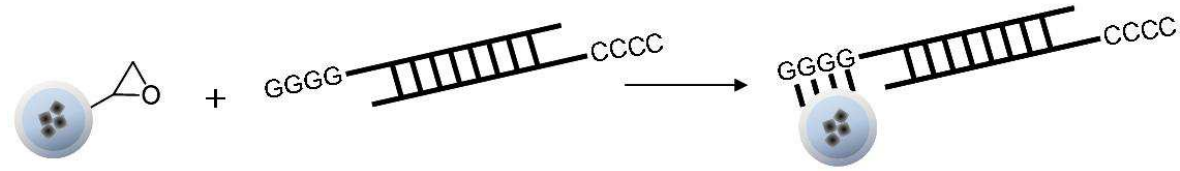
2. Method

2.1 Outline

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







Plain beads DNA immobilized beads

2.2 Procedures

2.2.1 Annealing

1) Dilute each single strand DNA with ultrapure water to adjust its concentration to 1μg/μL.
2) Add 150μg of sense oligonucleotide and 150μg of antisense oligonucleotide into a 1.5mL micro-tube as below, and mix them.

Sense oligonucleotide	150μL	(150μg)
Antisense oligonucleotide	150μL	(150μg)
	300μL	(300μg)

3) Heat the solution for ten minutes at 98℃, float it in 200mL of water at 98℃, and leave it until it cools down to room temperature (overnight).

2.2.2 PNK (polynucleotide kinase) treatment of DNA ends

Note: When using 5' end phosphorylated synthetic oligonucleotide, skip this process.

1) Divide the annealed DNA into eight tubes, mix each of them with PNK as below, and stand them still for 60 minutes at 37℃ to react them.

DNA	35μL	(35μg)
10× Kinase buffer	10μL	
10 mM ATP	10μL	
H_2O	43.2μL	
T4 Polynucleotide Kinase	1.8μL	
	100μL	

2) Add 100μL of phenol/chloroform, and mix them for 30 seconds. (Vortex mixer)

3) Centrifuge at 15,000 rpm for five minutes at room temperature, and transfer a water layer to a fresh 1.5mL micro-tube.
4) Add 10μL of 3M sodium acetate (pH5.3) and 250μL of ethanol, and mix them.
5) Stand the solution still for one hour at −30℃ to precipitate the ethanol.
6) Centrifuge at 15,000 rpm for 30 minutes at 4℃, and discard the supernatant.
7) Add 500μL of 70% ethanol gently, and rinse them.
8) Centrifuge at 15,000 rpm for five minutes at 4℃, and discard the supernatant.
9) Dry them. (Vacuum drying, etc.)
10) Add 10μL of ultrapure water, and dissolve them.
11) Collect the DNA solutions into one tube. (80μL in total)
12) Quantify the volume of DNA, and adjust its concentration to 1μg/μL. (280μg /280μL)

2.2.3 Ligation

1) Divide the annealed DNA into eight tubes, and mix each of them with DNA Ligase as below.

DNA	25μL	(25μg)
10× Ligation buffer	10μL	
H_2O	63μL	
T4 DNA ligase	2μL	
	100μL	

2) After spin down, stand them still at 4℃ to react them overnight.
3) Measure the length of DNA with agarose gel. (Target length：200 to 400bp)。
4) Add 100μL of ultrapure water. (Fluid volume: 200μL)
5) Add 200μL of phenol/chloroform, and mix them for 30 seconds. (Vortex mixer)
6) Centrifuge at 15,000 rpm for five minutes at room temperature, and transfer a water layer to a fresh 1.5mL micro-tube.
7) Add 20μL of 3M sodium acetate (pH5.3) and 500μL of ethanol, and mix them.
8) Stand the solution still for one hour at −30℃ to precipitate the ethanol.
9) Centrifuge at 15,000 rpm for 30 minutes at 4℃, and discard the supernatant.
10) Add 1mL of 70% ethanol gently, and rinse them.
11) Centrifuge at 15,000 rpm for five minutes at 4℃, and discard the supernatant.
12) Dry them. (Vacuum drying, etc.)
13) Add 10μL of ultrapure water, and dissolve them.
14) Collect the eight DNA solutions into one tube. (80μL in total)
15) Load the solution onto NICK columns. (Equilibrate the NICK columns with ultrapure water prior to loading.)
16) Elute them four times with 400μL of ultrapure water.
17) Perform quantification of nucleic acids on each recovered fraction, and store a sample (2 fractions) which has the highest concentration. (The DNA concentration: approximately 250μg/mL)

2.2.4 Immobilization of double strand DNA on beads

1) Add 10mg of Plain beads into each of two 1.5 mL micro-tubes.
2) Centrifuge at 15,000 rpm for five minutes at room temperature, and discard the supernatant.
3) Add 500μL of ultrapure water, and disperse the beads.
4) Centrifuge at 15,000 rpm for five minutes at room temperature, and discard the supernatant.

5) Repeat the above 3) to 4) two more times. (Wash the beads twice in total.)
6) Add 400µL of ultrapure water into one of the two micro-tubes to control the solution, and add 400µL of the ligated DNA solution (250µg/mL) into the other micro-tube as below, and disperse the beads. (When you cannot disperse the beads easily, disperse them in a short time by using an ice-cold ultrasonic homogenizer.)

	−	+	
DNA	0µL	400µL	(100µg)
H_2O	400µL	0µL	

7) Stand the solutions still for 24 hours at 50℃ to react them.
8) Centrifuge at 15,000 rpm for five minutes at room temperature, and transfer the supernatant to another tube.
9) Add 400µL of 2.5M potassium chloride, and disperse the beads.
10) Centrifuge at 15,000 rpm for five minutes at room temperature, and transfer the supernatant to another tube.
12) Repeat the above 9) to 10) one more time. (Wash the beads twice in total.)
13) Add 400µL of ultrapure water, and disperse the beads.
14) Centrifuge at 15,000 rpm for five minutes at room temperature, and transfer the supernatant to another tube.
15) Repeat the above 13) to 14) one more time. (Wash the beads twice in total.)
16) Add 400µL of TES buffer, and disperse the beads.
17) Centrifuge at 15,000 rpm for five minutes at room temperature, and discard the supernatant.
18) Repeat the above 16) to 17) two more times. (Wash the beads three times in total.)
19) Disperse them into 400µL of TES buffer, and store them at 4℃. (The concentration of double strand DNA immobilized beads：25mg/mL)

Note: The volume of DNA immobilization is approximately 2µg/mg beads (10pmol/mg).
(Calculate the volume by subtracting the volume of DNA in the washing supernatant and the volume of DNA in the reaction supernatant from the volume of the input DNA.)

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.)