1-Transformation and colony inoculation

Purpose:

Amplify target DNA sequence by transforming plasmid or ligation mixture into DH5α competent cell.

Materials:

- Plasmid or ligation product
- DH5α competent cell (GeneMark, 100 µl per tube)
- SOC solution or LB broth
- LB agar plate with antibiotic resistance such as Amp+ or CamR+
- LB broth with antibiotic resistance

Procedures:

[On ice]

- Thaw competent cell and pre-cool eppendorf tube.
- Inoculate 1 µl DNA into 30-50 µl competent cell and incubate on ice for 30 min. (*The volume of DNA added should not exceed over 1% volume of competent cell.)
- Heat shock at 42°C for 30 sec, and incubate on ice for 2 min.
- Add 250 µl SOC solution (or LB broth) and recover at 37°C with shaking 225rpm for 45 min.
- Spread all the mixture on the agar plate, and incubate at 37°C for 18~20 hr.

[The next day]

- Pick single colony and inoculate it into LB broth. Incubate at 37°C with shaking 225rpm for 18~20 hr.

2-Miniprep and Maxiprep

Purpose:

Purify plasmid and digest pBKS and endo-1,4-glucanase with EcoRI and PstI restriction enzyme for vector and insert.

Materials:

- Bacterial liquid
- 100% glycerol for stock
- Miniprep plus purification kit (BioTools)
- Maxiprep plus purification kit (GeneMark)

Procedures:

[Miniprep]

- Transfer 0.5 ml bacterial cells and add 0.5 ml 100% glycerol (1:1) as a stock. The rest 1.5 ml is for Miniprep.

- Harvest bacterial cells in an eppendorf tube by centrifugation at 13,400 rpm for 1 min.
- Resuspend the cell pellet with **200 µl P1 buffer** (RNase A added and stored at 4°C)
- Add **200 µl P2 buffer** and mix thoroughly by inverting the tube 10 times. (clarified)
- Add **300 µl P3 buffer** and mix thoroughly by inverting the tube 10 times. (cloudy)
- Centrifuge at 14,000 rpm for 10 min.
- Transfer supernatant to the spin column and centrifuge at 13,400 rpm for 1 min.
- Add **400 µl PD buffer** and centrifuge at 13,400 rpm for 1 min. Discard the flow-through.
- Wash by adding **600 µl PW buffer** and centrifuge at 13,400 rpm for 1 min. Discard the flow-through.
- Centrifuge at top speed for 3 min. Discard the flow-through, open the lid and stay for a while.
- Place the spin column to a new eppendorf tube. Add **50 µl elution buffer,** stay for a while and centrifuge at top speed for 2 min.
- Measure the concentration of purified plasmid. (ng/µl)

[Maxiprep]

✧ Pick Single Colony and incubation for Maxiprep.

Materials:

- Plate with pUC57-Gal-Ste12 to Kpn1 in DH5α
- Plate with pUC57-Light fusion to suffix in DH5α
- Plate with pUC57-Gluxyn to suffix in DH5α
- 500 ml flask *3

Procedures:

- **Pick single colony and stain it into LB broth with volume of 2-5 ml. Incubate at 37℃ with shaking 225 rpm for 8 hr.**
- Then prepare 250 ml of LB broth (with AmpR) in the flask.
- Transfer small volume of bacterial liquid to large volume of LB broth.

[Note]: Antibiotic need 1000x dilution, so as 400ml LB broth need 0.4 ml antibiotic.

1. Pellet down cells from 250 ml liquid culture, centrifugation at 14000g for 5 min and pour off the supernatant.
2. Completely resuspend the pellet in 10 ml Solution I by pipetting.
3. Add 10 ml Solution II and mix gently. Incubate at RT for 4 min.
4. Add 10 ml Solution III and mix gently. Incubate on ice for 10 min.
5. Centrifuge the lysate at 14000g for 20 min at 4℃. A compact white pellet will be formed at the bottom of the tube.

6. To remove the floating pellet, hold a Filter Net by hand above a sterile 50 ml centrifuge tube and gently pour the lysate through the net.
7. Open the cap of Maxi-Spin Column provided in 50 ml centrifuge tube and add about 12 ml of cleared lysate into column. Close the cap and wait for 2 min for equilibrium with the membrane. Centrifuge the column at 12000 g for 1 min.
8. Discard the filtrate, and add the remaining lysate into the same Maxi-Spin column. Repeat centrifugation and discard the filtrate.
9. Add 10 ml Endotoxin Removal Wash Solution to the Maxi-Spin Column, wait for 2 min for equilibration with the membrane and centrifuge at 12000g for 1 min. Discard the filtrate.
10. Add 10ml Wash Solution to the column, and wait for 2 min for equilibration with the membrane and centrifuge at 12000g for 1 min and discard the filtrate. Repeat this step once more.
11. Reassemble the column to the tube and centrifuge at 12000 g for 10 min to dry the column.
12. For complete evaporation of ethanol, incubate the column at 60℃ for 15 min.
13. To elute the DNA, place the Maxi-Spin Column into a sterile 50 ml centrifuge tube and add 2 ml preheated Elution Solution to the membrane. Let stand for 3 min and centrifuge at 12000g for 5 min to elute DNA. Withdraw eluent from 50 ml centrifuge tube and add to the Maxi-Spin Column, repeat centrifuge from this step to increase DNA yield.
14.Collect the DNA solution into four 1.5 ml Eppendorf and store at -30℃.

3-Restriction enzyme analysis and digestion

Purpose:

Digest DNA with restriction enzyme to analyze the plasmid mapping or cut the restriction site to make vector and insert.

Materials:

- DNA plasmid
- NEB restriction enzyme
- NEB restriction enzyme buffer
- ddH2O
- Gel electrophoresis

Procedures:

	Vector	**Insert**	**Plasmid mapping**
DNA	2000 ng	2000 ng	**1000 ng**
10X buffer (cutsmart)	5	5	**2**
EcoRI-HF	0.5	0.5	**0.2**
PstI-HF	0.5	0.5	**0.2**

ddH20	Up to total	Up to total	**Up to total**
Total volume	**50**	**50**	**20**

- Incubate at 37 °C water bath for 1-2 hr.

4.1-Gel extraction

Purpose:

Extract DNA fragments from enzymatic reaction or PCR amplification.

Materials:

- Electrophoresis with 1% agarose gel
- DNA extraction kit (GeneMark)

Procedures:

- After electrophoresis, cut out the target DNA band from agarose gel.
- Transfer gel slice into an eppendorf tube and add 2 volumes of binding buffer. Incubate at 60°C until the gel dissolved.
- Transfer the solution into the spin column, and centrifuge at 15,000 g for 1 min. Discard the filtrate.
- Add 500 µl of Binding solution and centrifuge at top speed for 1 min. Discard the filtrate.
- Add 700 µl of Wash solution and wait for 1 min. Centrifuge at 15,000 g for 1 min and discard the filtrate. Repeat one more time.
- Centrifuge at top speed for additional 5 min to remove residual ethanol.
- Transfer the spin column into a new Eppendorf tube, add 30 µl of Elution buffer and wait for 2 min.
- Centrifuge at top speed for 2 min to elute DNA.

4.2-PCR/DNA clean-up

Purpose:

Extract DNA fragments from enzymatic reaction or PCR amplification without running electrophoresis on agarose gel.

Materials:

- DNA extraction kit (GeneMark)

Procedures:

- Transfer PCR product or other enzymatic reaction mixture into an eppendorf tube and add 3 volumes of binding buffer. Vortex briefly to mix.

- Transfer the solution into the spin column, and centrifuge at 15,000 g for 1 min. Discard the filtrate.
- Add 700 µl of Wash solution to the spin column and wait for 1 min. Centrifuge at 15,000 g for 1 min and discard the filtrate. Repeat one more time.
- Centrifuge at top speed for additional 5 min to remove residual ethanol.
- Transfer the spin column into a new Eppendorf tube, add 30 µl of Elution buffer and wait for 2 min.
- Centrifuge at top speed for 2 min to elute DNA.

5-T4 DNA Ligation

Purpose:

Ligate target insert into vector which both digested with the same cutting site and make a recombinant plasmid.

Materials:

- Digest vector and target insert
- T4 ligase and 10X T4 ligation buffer (NEB)
- ddH2O
- Gel electrophoresis equipment for ligation check

Procedures:

[Verification of digested vector and insert before ligation]

- Load: marker 5, uncut 5+1, digest vector 5+2, uncut5+1, digest insert5+2
- According to the brightness of the band on the gel, estimate the ratio of concentration between vector and insert. [Calculation]-vector: insert=the ratio of brightness/ basepairs = estimated ratio of concentration.

- Insert : vector= 3 : 1

	µl
Vector	1.0
Insert	1.5
T4 ligase	1.0
10X ligation buffer	2.0
ddH2O	14.5
	20.0

Calculation: according to the band brightness of digested vector and insert, 3/1 multiply 1441/2961 = 1.46/1 (insert: vector)

- Incubate at RT for 30-60 min. (More incubation time (2 hr) for blunt end)

[Note]:

Ligation information (insert → vector)

1. Light fusion to suffix (4323 bp, 529ng/µl) →pRS423 (HIS3) (change the plasmid to pRS112)
→Restriction enzyme: Kpn1 & Pst1
2. Gal-Ste12 to Kpn1 (3082 bp, 302 ng/µl) →YEplac195 (URA3. 5233+8 bp)
→Restriction enzyme: EcoR1 & Kpn1
3. Gluxyn to suffix (2887 bp, 50 ng/µl-condense to 80 ng/µl) →YEplac181 (LEU2. 5214+27 bp)
→Restriction enzyme: Kpn1 & Pst1