LB medium and LB agar gel

Medium for cultivation of *E. coli*

◆ **LB medium (1 L)**

1; Add about 900 mL of distilled water to beaker.

2; Add reagents (Table) and stir.

3; Add distilled water up to 1 L and take LB medium to media bottle.

4; Autoclave for 20 min at 120℃

Table Composition of LB medium

Reagent	Volume
Bact Tryptone	10 g
Yeast Extract	5 g
NaCl	5 g
For 1 L LB medium	

◆ **LB agar gel (1 L)**

1; Prepare LB medium without autoclave (Steps 1-3 of 1L scale of LB medium).

2; Add 15 g of agar and stirrer bar.

3; Autoclave for 20 minutes at 120℃.

4; Stir and cool LB medium with agar, add appropriate antibiotic (50 μL).

5; Pour LB medium (Step 4) in plate and cool down in clean bench.

Transformation

Preparation of *E. coli* contain particular plasmid

1; Incubate frozen competent cell (DH5α) on the ice for a few minutes.

2; Add 1~5 μL of plasmid to competent cell (DH5α) on the ice.

3; Incubate for 20 – 30 minutes on the ice.

4; Incubate for 45 seconds at 42℃.

5; Add 1 mL LB medium and cultivate for 1 hour at 37℃.

6; Spread culture medium on LB agar plate with appropriate antibiotic.

Plasmid extraction

Preparation of plasmid extracted from *E. coli*

Solution	Solution composition	Attention
Solution 1	50 mM Tris-HCl (pH8.0), 5 mM EDTA, 20 mg/ml RNase A	Stare at 4℃
Solution 2	0.2 M NaOH,1% SDS	
Solution 3	3 M Potassium acetate (pH 5.5 adjusted by acetate)	
Solution 4	4 M NaClO4 (pH 6.0 adjusted by Tris)	Stare at 4℃
Bind mix	5 M GuSCN, 4%(w/v) Triton X-100, Silica (0.25 g/100 mL)	Shading

1; Pick up single colony from agar plate and cultivate it in 1.5 mL LB medium containing appropriate antibiotec (50 μg/mL) overnight at 37℃.

2; Move the culture medium to 1.5 mL tube.

3; Centrifuge for 5 seconds at 15,000×g and 4 °C and discard supernatant.

4; Add 100 µL Solution 1 to the pellet and resuspend and incubate for 3 minutes.

5; Add 100 µL Solution 2, invert tube gently 5 times and incubate for 3 minutes.

6; Add 100 µL Solution 3, invert tube 5 times and incubate for 3 minutes.

8; Add 200 µL Solution 4 and invert tube 5 times and centrifuge for 3 minutes at 15,000×g and 4 °C.

10; Take supernatant to new 1.5 mL tube and centrifuge for 3 minutes at 15,000×g and 4 °C.

11; Vortex Bind mix for 1 min and add 800 µL Bind mix to new 1.5 mL tube.

12; Add 400 µL supernatant after centrifugation (Step 10) to tube containing Bind mix (Step 11) and mix.

13; Incubate for 3 minutes, centrifuge for 3 seconds at 5,000×g and 4 °C, and discard supernatant.

14; Add 1 mL of 50% ethanol and resuspend.

15; Centrifuge for 3 seconds at 5,000×g and 4 °C and discard supernatant.

16; Repeat wash (Steps 14-15).

17; Dry pellet for a few minuet under a vacuum to remove residual ethanol.

18; Add 50 µL nuclease-free water or TE buffer and incubate for 3 minutes at 65℃.

19; Centrifuge for 3 minutes at 15,000×g and 4 °C.

19; 40 µL of supernatant into new 500 µL tube .

Restriction enzyme digestion of DNA

Cleavage of insert DNA from plasmid

1; Mix DNA and restriction enzyme (Table).

2; Incubate for 2 hours at 37℃.

3; Incubate for 10 minutes at 65℃.

4; Confirm the band of DNA by agar gel electrophoresis.

Table Composition of LB medium

Reagent	Volume
DNA plasmid	5 µL
10 × buffer	1 µL
Nuclease-free water	3 µL
*Eco*RI or *Xba*I	0.5 µL
*Spe*I or *Pst*I	0.5 µL
Total	10 µL

Agar gel electrophoresis

Confirmation and separation of digested DNA

◆ **Preparation of agar gel**

1; Add 1 g of agar to 100 mL of 1×TAE.

2; Boil and stir until solution is clear.

3; Cool down, pour to gel form and set gel corm.

4; Incubate until gel dry out.

5; Stare gel in 1×TAE.

Reagent	Volume	Composition
50 ×TAE	20 mL	2 M Tris, 2 M Acetate, 0.5 M EDTA (pH 8.0)
Distilled water	980 mL	
1×TAE	1,000 mL	

◆ **Agar gel electrophoresis**

1; Place agar gel and pour 1×TAE in electrophoresis chamber.

2; Load DNA ladder and DNA sample mixed with loading dye on agar gel.

3; Electrophorese for 20 minutes at 100 V.

4; Stain gel by Sybr Safe™ Gel Stain (Invitrogen).

5; Visualize the band of DNA using UV light.

6; Confirm the length of digested DNA

Gel purification

Purification of DNA from agar gel

GENECLEAN® II Kit(NaI、glass milk、NEW Wash)/Qbiogene

1; Cut the objective band in the agar gel after electrophoresis and stain with SYBR Safe.

2; Put the gel including objective DNA into 1.5 mL tube and measure the mass of that.

3; Add 2.5-3 fold volume NaI solution into the tube (Gel: NaI =1 mg : 1 µL).

4; Incubate the gel at 50℃ for 5 minute.

5; Add 10 µL of glass milk and vortex.

6; Incubate for 5 minutes and vortex per a minute.

7; Centrifuge for 5 seconds at 15,000×g and 4℃ and discard the supernatant.

8; Add the 500µL of New Wash and resuspend.

9; Centrifuge for 5 seconds at 15,000×g and 4℃.

10; Repeat wash (Steps 8-9).

11; Dry the pellet for 5-10 minutes under vacuum.

12. Add 20 µL of nuclease-free water and resuspend.

13. Centrifuge for 5 seconds at 15,000×g and 25℃.

14. Transfer supernatant including objective DNA into new tube.

Ligation

Ligation inset DNA and vector

DNA Ligation kit Ver 2.1(Solution Ⅰ)/Takara

1; Mix the insert DNA, vector and solution I (Table).

2; Incubation at 16℃ for 30 minute.

3; Transform *E. coli* with ligation sample.

Reagent	Volume
Solution 1	7 µL
Backbone plasmid	1 µL
Upstream insert DNA	4 µL
Downstream insert DNA	4 µL
Total	14 µL

Colony PCR

Confirm of insert DNA in plasmid, directly using *E. coli* at PCR

Table 1 Composition of reagent solution for colony PCR

Reagent	Volume	Final conc.
2 × Go Taq® Green Master Mix (Promega)	5 µL	1 ×
Forward primer (100 µM)	0.1 µL	1 µM
Reverse primer (100 µM)	0.1 µL	1 µM
Nuclease-free water	4.8 µL	
Total	10 µL	

Table 2 PCR program

Temp. (℃)	Time (min)	Cycle
95	2	1
95	0.5	30
55	0.5	
72	2*	
72	5	1

*: Adjust time to 1 min/kbp

1; Add 10 µL of reagent solution (Table 1) to PCR tube.

2; Pick up single colony from agar plate with tooth pick and sting replica plate (new LB agar plate).

3; Put and stir toothpick to reagent solution (Step 1).

4; Amplify insert DNA with PCR program (Table 2).

5; Electrophorese PCR sample with agar gel

6; Check the band and length of insert DNA and decide the colony with insert DNA

Sequence analysis

Identification of insert DNA

◆ **Preparation of PCR product**

Big Dye® Terminator Cycle Sequencing Kit Ver. 3.1 (Premix, Buffer) / Applied Biosystems

1; Add reagent solution (Table 1) to PCR tube and amplify insert DNA with PCR program (Table2).

Table 1 Composition of reagent solution for colony PCR

Reagent	Volume	Final conc.
Premix	1 µL	
5 × Buffer	4 µL	1 ×
Primer (1 µM)	4 µL	200 nM
Plasmid	1 µL	1 µM
Nuclease-free water	10 µL	
Total	20 µL	

Table 2 PCR program

Temp. (℃)	Time (min)	Cycle
95	5	1
96	0.5	25
50	0.25	
60	4	
4	∞	

◆ **Purification of PCR product and sequence analysis**

Agencourt CleanSEQ® and 96 R ring Super Magnetic Plate® / Beckman Coulter

1; Add 10 µL of Agencourt CleanSEQ® 10 µL to PCR product.

2; Add 62 µL of 85% ethanol, mix and incubate for 3 minutes.

3; Incubate for 3 minutes on 96 R ring Super Magnetic Plate® and discard supernatant.

4; Add 100 μL of 85% ethanol and mix.

5; Incubate for 3 minutes on 96 R ring Super Magnetic Plate® and discard supernatant.

6; Repeat wash (Steps 4-5).

7; Dry for 10 minutes.

8; Add 40 μL nuclease-free water and mix.

9; Transfer 30μL of clear sample into a new plate for loading on the detector.

10; Load sample on sequencer and analyze.