ENDEXT® Technology

Plasmid preparation
(model case)

Ver.1.0 Mar 16, 2006

1 Important Information

1.1 pEU vector

pEU-E01 vector series is constructed based on pUC vector, and suitable for plant cell translation system. It has SP6 promoter, E01 translational enhancer, and ampicillin resistance gene. Blue-white color selection is not available because it doesn't have *LacZ* gene.

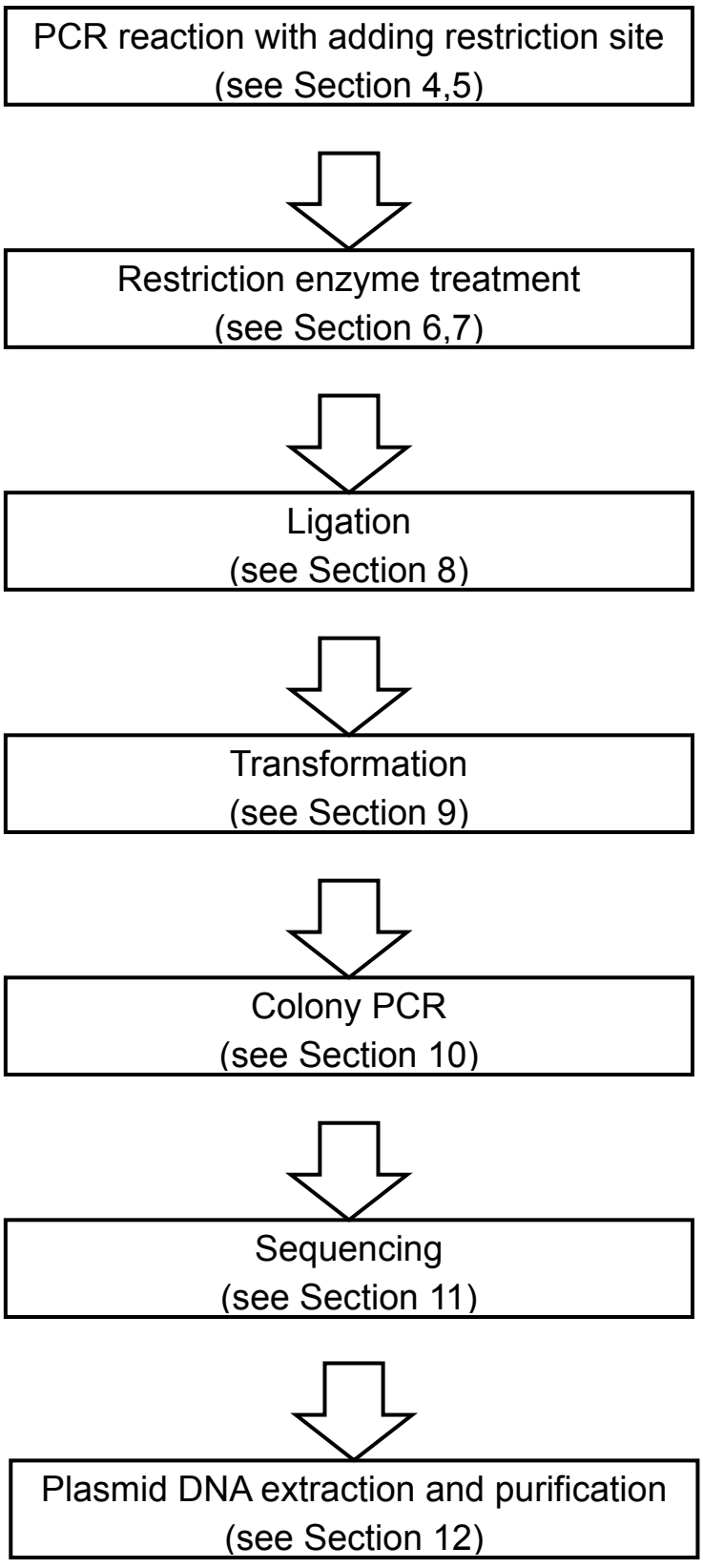
1.2 Selection of restriction enzyme site

In order to efficiently express the target protein, it is recommended to select a restriction enzyme site as near as possible to E01 translational enhancer, and not recommended to select Xho I site alone because of frequent self-ligation in case of pEU-E01-MCS.

1.3 Primer design

Restriction enzyme needs some nucleotides on both sides of restriction site to cut DNA. The least number of necessary nucleotides is depend on each enzyme.

2 Protocol Overview



3 Materials

Process	Item	Concentration
PCR reaction	Template DNA	10 ng /μl
	KOD -plus- (TOYOBO)	1U /μl
	10 x buffer for KOD-plus	10 x
	dNTPs mix	2 mM
	$MgSO_4$	25 mM
	5' Primer	20 μM
	3' Primer	20 μM
Restriction enzyme treatment	pEU-E01-MCS	1 μg /μl
	Buffer for enzyme	10 x
	Restriction enzyme	
	DNA extraction kit (ex. Gel-M™ Gel Extraction System, VIOGENE) (*1)	
Ligation	Ligation High (TOYOBO)	
Transformation	JM109 (competent cell)	
Colony PCR	Ex Taq (TaKaRa Bio)	5 U /μl
	Ex Taq Buffer	10 x
	dNTPs mix	2.5 mM
	SPU primer (5'- GCGTACGATTTAGGTG ACACT -3')	20 μM
	SP-A 1868 primer (5'- CCTGCGCTGGGAAGA TAAAC -3')	20 μM
Direct Sequencing	Exonuclease I	
	Shrimp Alkali Phosphatase	
	Big Dye Terminator V3.1 Cycle Sequencing Kit (Applied Biosystems)	

*1 : Gel-M™ is trademark of VIOGENE, Inc.

Process	Item	Concentration
Plasmid DNA extraction and purification	Plasmid DNA extraction Kit (ex. QIAGEN)	
	Phenol : Chloroform : Isoamyl alcohol (pH7.9)	Phenol : Chloroform : Isoamyl alcohol = 25 : 24 : 1
	Chloroform	
	Ethanol	100% and 70%
	3M Sodium acetate (pH5.2)	
	TE (pH8.0)	10mM Tris-HCl, 1mM EDTA

4 PCR reaction

4.1 Prepare following mixture.

Reagents	Volume (µl)	Final conc.
Template DNA(10 ng /µl)	2	1 ng /µl
10 x buffer for KOD-plus	2	1x
2 mM dNTP	2	0.2 mM
25 mM $MgSO_4$	0.8	1 mM
20 µM 5' Primer	0.3	0.3 µM
20 µM 3' Primer	0.3	0.3 µM
KOD -plus-	0.4	0.02 U /µl
DW	12.2	
Total	20	

4.2 Amplify DNA using following program.

Degrees C	Time	
98	4 min	
98	10 sec	35 cycles
55	1 min	
72	3 min	
72	5 min	
20	∞	

4.3 Apply 1µl of PCR product to agarose gel to check the product.

5 Purification of PCR product

5.1 Add an equal volume of phenol/chloroform (phenol:chloroform: isoamyl alcohol = 25:24:1, pH 7.9) to the PCR product and mix well.

5.2 Centrifuge the mixture at 15,000 rpm for 5 min.

5.3 Carefully transfer the upper aqueous phase to a new tube, add an equal volume of chloroform and mix well.

5.4 Centrifuge this mixture at 15,000 rpm for 5 min.

5.5 Carefully transfer the upper aqueous phase to another new tube. To this upper aqueous solution, add 100% ethanol, 2.5 times the volume, and 3M sodium acetate (pH 5.2), 1/10 of the volume, to precipitate the DNA.

5.6 Hold at -20 degrees C for 10 min, centrifuge at 15,000 rpm for 20 min at 4 degrees C.

5.7 Remove the supernatant. Add 800 µl of 70 % ethanol to wash the remaining DNA pellet in the tube.

5.8 Centrifuge the tube at 15,000 rpm for 10 min at 4 degrees C.

5.9 Remove the supernatant. Dry the DNA pellet for 10 to 20 min. Add an appropriate volume of TE buffer to resuspend the DNA pellet.

6 Restriction enzyme treatment

6.1 Prepare following mixture.

Vector			Insert		
Reagents	Volume (µl)	Final conc.	Reagents	Volume (µl)	Final conc.
pEU-E01-MCS (1 µg /µl)	2		PCR product	5	
10 x Buffer	2	1 x	10 x Buffer	2	1 x
10 x BSA	2	1 x	10 x BSA	2	1 x
Enzyme 1		0.5 U /µl	Enzyme 1		0.5 U /µl
Enzyme 2		0.5 U /µl	Enzyme 2		0.5 U /µl
DW	balance		DW	balance	
Total	20		Total	20	

6.2 Incubate at 37 degrees C.

6.3 To check the digested product, apply 1µl of them to agarose gel.

7 Purification of digested product

7.1 Apply 10 µl of the digested product to agarose gel, and electrophorese by the ordinary method.

7.2 Extract the product from gel with commercially available kit. (ex. Gel-M™ Gel Extraction System, VIOGENE) (*1)

*1 : Gel-M™ is trademark of VIOGENE, Inc.

8 Ligation

8.1 Prepare ligation mixture. The ideal ratio of insert to vector is variable. The reasonable starting point is [insert : vector = 2:1] as mole ratio.

(ex.)

	volume (µl)
Vector	1
Insert	2
Ligation High	3
Total	6

8.2 Incubate at 16 degrees C for 3 hrs.

9 Transformation

9.1 Transform the ligation mixture into the appropriate competent bacteria by ordinary method of transformation.

(ex.)

	volume (µl)
JM 109	20
Ligated product	2
Total	22

9.2 Plate on selective media (ex. LB with ampicillin).

9.3 Incubate at 37 degrees C over night.

10 Colony PCR

10.1 Prepare following PCR mixture.

Reagents	Volume (µl)	Final conc.
10 x Ex Taq buffer	2	1x
2.5 mM dNTP	1.6	0.2 mM
20 µM SPU	0.4	0.4 µM
20 µM SP-A 1868	0.4	0.4 µM
Ex Taq Polymerase (5U /µl)	0.05	0.0125 U /µl
DW	12.2	
Total	20	

10.2 Pick up one colony on plate by a toothpick, and then elute transformed competent cell into PCR mixture with stirring.

10.3 Amplify DNA using following program.

Degrees C	Time	
98	4 min	
98	10 sec	30 cycles
55	1 min	
72	3 min	
72	5 min	
20	∞	

10.4 Apply 2 µl of PCR product to agarose gel to check the product.

11 Direct Sequencing

11.1 Prepare following mixture.

	volume (µl)	Final conc.
PCR product (by colony PCR)	3	
Exonuclease I		0.6 U /µl
Shrimp Alkali Phosphatase		0.12 U/µl
DW	balance	
Total	10	

11.2 Incubate at 37 degrees C for 30 min, and then 80 degrees C for 15 min.

Note: This reaction product can be used to sequence reaction.

11.3 Do sequence analysis by the commercial cycle sequencing reaction kit and the sequencing equipment.

12 Plasmid DNA extraction

12.1 Cultivate competent cell containing pEU-E01-MCS with cDNA inserted.

12.2 Extract and purify the plasmid DNA from competent cell with commercial kit (ex. QIAGEN).

13 Plasmid DNA purification

A highly purified plasmid DNA is required for the transcription and subsequent translation. It is therefore mandatory to further purify the plasmid DNA that has been extracted from E. coli and purified with a commercially available kit. This additional purification is accomplished by extraction first with phenol/chloroform and then with chloroform, and by ethanol precipitation as described below:

13.1 Add an equal volume of phenol/chloroform (phenol: chloroform:isoamyl alcohol = 25:24:1, pH 7.9) to the plasmid DNA solution and mix well.

13.2 Centrifuge the mixture at 15,000 rpm for 5 min.

13.3 Carefully transfer the upper aqueous phase to a new tube, add an equal volume of chloroform and mix well.

13.4 Centrifuge this mixture at 15,000 rpm for 5 min.

13.5 Carefully transfer the upper aqueous phase to another new tube. To this upper aqueous solution, add 100% ethanol, 2.5 times the volume, and 3M sodium acetate (pH 5.2), 1/10 of the volume, to precipitate the DNA.

13.6 Hold at -20 degrees C for 10 min, centrifuge at 15,000 rpm for 20 min at 4 degrees C.

13.7 Remove the supernatant. Add 800 µl of 70 % ethanol to wash the remaining DNA pellet in the tube.

13.8 Centrifuge the tube at 15,000 rpm for 10 min at 4 degrees C.

13.9 Remove the supernatant. Dry the DNA pellet for 10 to 20 min. Add an appropriate volume of TE buffer to resuspend the DNA pellet.

14 Adjustment of the plasmid DNA concentration

14.1 Determine the concentration of the DNA with a spectrophotometer at the wavelengths of 260 nm and 280 nm. Absorbance at 260 nm represents the concentration of DNA. The ratio of absorbance at 260 nm to that at 280 nm represents the purity of the DNA

Note: Purity of plasmid DNA should be such that the A260/A280 ratio ranges between 1.70 and 1.85. Ratios outside the range indicate that the plasmid DNA has been contaminated. In that case, repeat Section 13 from the beginning.

14.2 Adjust the DNA concentration to 1.0 µg/µl by adding an appropriate volume of TE buffer

Note: Concentration of plasmid DNA should be within: [1.0 µg /µl +/- 0.05 µg /µl]