ClonExpress II One Step Cloning Kit

NB-54-0002-01 (25 rxns)
NB-54-0002-02 (50 rxns)

1 Introduction

Neo BiotechClonExpress II One Step Cloning is a simple, fast, and high efficient cloning technology and enables directional insertion of any amplified DNA product into any linearized vector at any site. Firstly, the vector is linearized at the cloning site. A small sequence overlapped with each end of the cloning site is added onto the insert through PCR. The insert and the linearized vector, with overlapped sequences of 15 bp - 20 bp on both 5'- and 3'-end, respectively, are mixed in an appropriate ratio and incubated with recombinase Exnase II at 37℃ for 30 min. The ClonExpress II is a novel cloning Kit, independent of DNA ligase, significantly reducing the vself-ligated colonies and bringing a true positive rate > 95%. The enhanced Exnase II and highly optimized buffer significantly improve the recombination efficiency and the tolerance to impurities.Both the linearized vector and the PCR products of insert can be directly used for recombination without purification, significantly simplifying the procedures.

2 Package Information

Components	NB-54-0002-01 (25 rxn)	NB-54-0002-02 (50 rxn)
5× CE II Buffer	100 µl	200 µl
Exnase II	50 µl	100 µl
500 bp Control Insert (20 ng/µl)	5 µl	5 µl
pUC19 Control Vector, Linearized (50 ng/µl, Amp+)	5 µl	5 µl

3 Storage

All components should be stored at -20°C
▲avoid repeated freezing and thawing.

4 Applications

◇Fast Cloning

◇High-throughput Cloning

◇Seamless Assembly

◇Site-specific Mutagenesis

5 Additional Materials Required

PCR templates, primers, linearized vectors.

High-fidelity polymerase: Phanta Max Super-Fidelity DNA polymerase (Neo Biotech #NB-54-0153-01 / -02 / -03) or other equivalent products.

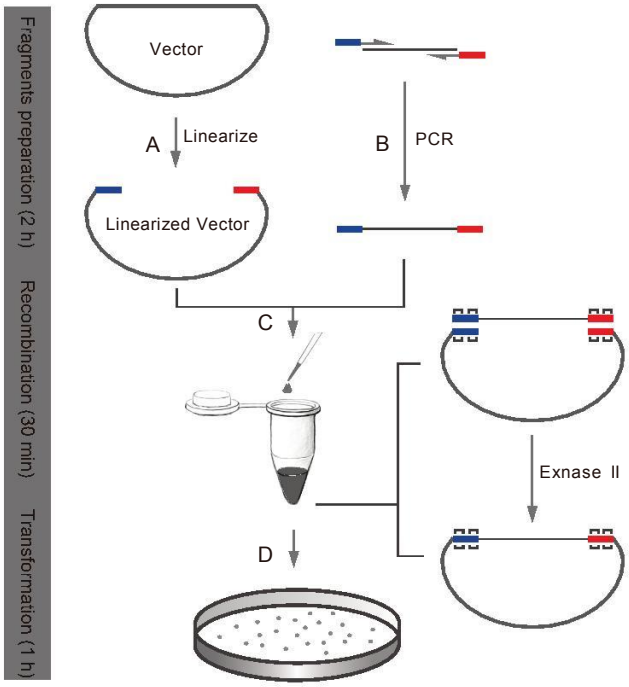
Competent cells: chemically competent cells by cloning strains:

DH5α competent E.Coli strain for conventional cloning, applicable to plasmids < 15 kb;

XL10 competent E.Coli strain or long-fragment cloning, applicable to plasmids > 10 kb.

Other materials: ddH_2O, PCR tubes, PCR instrument, etc.

6 Workflow



A: Preparation of Linearized Vectors: The linearized vector can be obtained by digesting the circular vector with restriction enzymes or by reverse PCR.

B: Acquisition of Inserts: Introducing homologous sequences of linearized vector ends about 15 bp - 20 bp (highlighted as red and blue) into 5'-end of Forward (F) & Reverse (R) primer, respectively, aiming to make the ends of amplified inserts and linearized vectors identical to each other.

C: Recombination: Mix the linearized vectors and inserts at an appropriate ratio and incubate with Exnase Ⅱ at 37℃ for 30min for recombination reaction and to make two linearized DNA cyclization in vitro.

D: Transformation: The recombination products can be used for transformation directly.

Fig 1. Mechanism of ClonExpress

7 Protocol

7.1 Preparation of Linearized Vectors

1.Select an appropriate cloning site on the vector that will be linearized. It is recommended to select the cloning site from regions with no repetitive sequence and the GC content of the certain region (within 20 bp up and downstream of the site) stays between 40% and 60%.

2.Vector linearization: the linearized vector can be obtained by digesting the circular vector with restriction enzymes or by reverse PCR.

◇Double digestion is recommended because it brings complete linearization and low false positive rate. If single digestion is adopted, a longer digestion time is necessary to reduce intact plasmid residues and decrease the false positive rate.

▲There is no DNA ligase activity in the reaction system of ClonExpress II, and no occurrence of self-ligation of linearized vector. Therefore, dephosphorylation is unnecessary even if the linearized vectors are prepared by single digestion. The false positive colonies (clones without inserts) are mainly from vectors that failed to be linearized.

◇When using reverse PCR amplification to obtain linearized vector, it is highly recommended to use a high-fidelity DNA polymerase (i.e. Phanta Max Super-Fidelity DNA Polymerase, Neo Biotech, # NB-54-0153-01 /-02 /-03) for vector amplification to reduce the PCR error rate. It is also recommended to use o.1 ng –1 ng circular plasmids or pre-linearized plasmids as PCR templates to reduce the false positive rate caused by residual circular plasmids in a 50 μl PCR reaction system.

7.2 Acquisition of Inserts

1.The principle for the design of ClonExpress II primers: add homologous sequences of linearized vector (15 bp – 20 bp) to the 5'-ends of both Forward (F) and Reverse (R) primers. This is to make the ends of amplified inserts and linearized vectors identical to each other (15 bp - 20 bp, excludes restriction enzyme cutting sites).

Forward primer of insert:

5'- homologous sequence of vector-upstream end + restriction enzyme cutting site (optional) + gene specific forward amplification sequence of insert -3'

Reverse primer of insert:

3'- gene specific reverse amplification sequence of insert + restriction enzyme cutting site (optional) + homologous sequence of vector-downstream end -5'

▲Gene specific forward/reverse amplification sequence refers to the sequence to amplify the insertion. Tm of 60℃ ~ 65℃ is recommended.

▲The homologous sequences (for homologous recombination) of vector-upstream or -downstream end are the sequences at the ends of the linearized vector. GC content of 40% - 60% is recommended.

It is recommended to use software, CE Design, to design primers. The CE Design automatically generates amplification primers of insert. You can aslo design the primers manually. An example is showed in Fig 2.

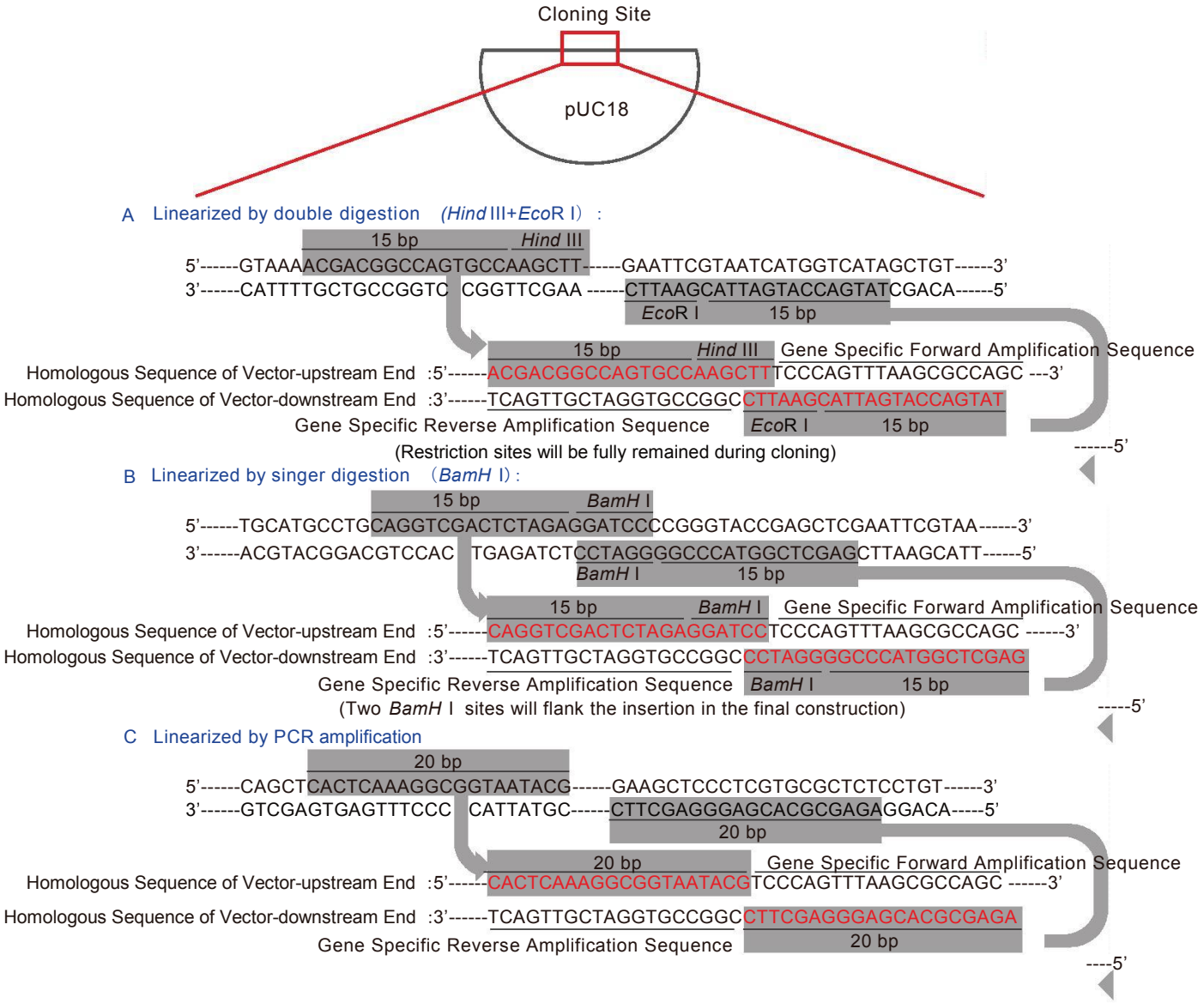


Fig 2. Primer Design for ClonExpress II

▲If the length of primer exceeds 40 bp, PAGE purification of synthetized primers is recommended, which will benefit the recombination efficiency. When calculating the Tm of primers, the homologous sequence of vector ends and restriction enzyme cutting site should be excluded and only gene specific amplification sequence should be used.

2.PCR of the inserts

Inserts can be amplified by any PCR polymerase (i.e. conventional Taq DNA polymerase or high-fidelity DNA polymerase). It will not interfere with the recombination efficiency whether there is A-tail in the PCR products or not. To prevent possible mutations introduced during PCR, amplification with a high-fidelity polymerases (i.e. Phanta Max Super-Fidelity DNA Polymerase, Neo Biotech, # NB-54-0153-01 /-02 /-03) are highly recommended.

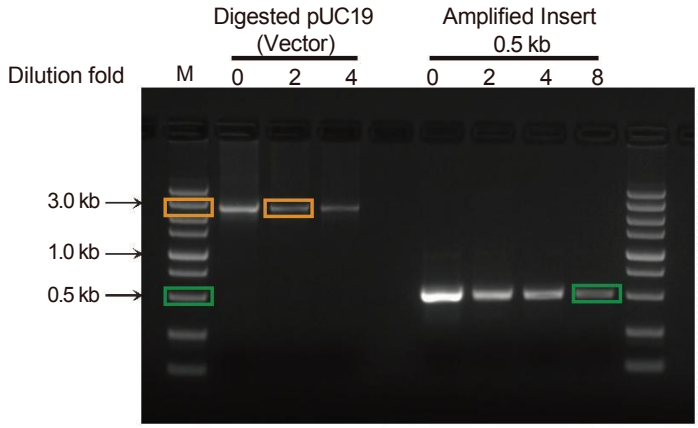
7.3 Amount and Ratio of Linearized Vectors and Inserts

1. Determination of DNA concentration

For a rough determination of DNA concentration, it is recommended to compare the intensity of bands by DNA gel electrophoresis, which is rapid and convenient.

◇If the linearized vectors and inserts have been purified by a high quality gel DNA recovery kit and no obvious nonspecific bands or smear shows in gel electrophoresis, the DNA concentrations can also be determined using instruments based on absorbance, i.e. Onedrop. But the results of concentration are only reliable when the A260/A280 value is between 1.8 and 2.0. For samples with DNA concentration lower than 10 ng/μl, the detection results may differ significantly between instruments based on the A260. In that case, instruments like Qubit ® and PicoGreen are recommended.

◇If the linearized vectors and inserts have not been purified, it is necessary to compare the intensity of bands after gel electrophoresis for the determination of DNA concentration (as showed in the picture below). Make serial dilutions of the linearized cloning vectors and amplified insertions, respectively, and then take 1 μl of each for gel electrophoresis. The approximate concentration of vector and insert can be determined by comparingthe intensity of their bands with DNA marker bands, because most DNA maker give determined mass for each band when a certain volume is loaded.



M: DL5, 000 Marker.

When 5 μl is loaded, the DNA mass of 1.0 kb band is 50 ng, while other bands are all 100 ng.

The boxes indicate the bands of linearized vectors (highlighted in orange) or inserts (highlighted in green), which shares the similar band intensity with corresponding band of DNA marker under a certain dilution ratio.
Therefore, it can be roughly inferred that:

Vectors concentration: 50 ng x 2 = 100 ng/μl;
Insets concentration: 50 ng x 8 = 400 ng/μl.

Fig3. Determination of the Concentration of Linearized Vect˙ors and Inserts via Gel Electrophoresis

2.Calculation of the amount of vectors

The optimal amount of vector for the recombination with ClonExpress II is 0.03 pmol, while the optimal amount of insert is 0.06 pmol (molar ratio of vector to insertion is 1:2), as roughly calculated as follows:

The optimal mass of **vector** = **[0.02 × number of base pairs] ng (0.03 pmol)**
The optimal mass of **insert** = **[0.04 × number of base pairs] ng (0.06 pmol)**

For example, when cloning an insert of 2 kb to a vector of 5 kb, the optimal mass of vector is 0.02 × 5000 = 100 ng, and the optimal mass of insert is 0.04 × 2000 = 80 ng.

a.When the length of the insert is larger than that of the vector, the calculation method of the optimal amount of vector and insert should be inverted.

b.The amount of linearized vector should be between 50 ng - 200 ng. The amount of amplified insert should be 20 ng - 200 ng. When the optimal amount calculated by above formula is beyond those ranges, choose the maximum or minimum amount for recombination.

c.When using digested vectors and amplified inserts directly for recombination (without purification), the total volume of vectors and inserts should be ≤ 4 μl (1/5 of the total volume of recombination reaction system).

7.4 Recombination

1. Calculate the amount of DNA for recombination by formula.

Dilute linearized vectors and inserts before recombination to make sure the loading accuracy. The volume of each component loaded should be no less than 1 μl.

2.Set up the following reaction on ice:

components, recombination reaction, negative control-1 [b], negative control-2 [c], positive control [d].

Components	Recombination	Negative Control-1 [b]	Negative Control-2 [c]	Positive Control [d]
Linearized Vectors [a]	X μl	X μl	0 μl	1 μl
Inserts [a]	Y μl	0 μl	Y μl	1 μl
5 × CE II Buffer	4 μl	0 μl	0 μl	4 μl
Exnase II	2 μl	0 μl	0 μl	2 μl
ddH_2O	to 20 μl	to 20 μl	to 20 μl	to 20 μl

a. X or Y indicates the amount of vector or insert calculated by formula.

b. It is recommended to use negative control-1 which can confirm the residue of cyclic plasmid template.

c. It is recommended to use negative control-2 when the amplified inserts templates are circular plasmids which share the same antibiotic resistance with the cloning vector.

d. Positive controls are used to exclude other materials and operational factors.

3.Gently pipette up and down for several times to mix thoroughly (**DO NOT VOTEX!**). Spin briefly to bring the sample to the bottom of the tube before reaction.

4.Incubate at 37℃ for 30 min and immediately place the tube at 4℃ or on ice.

▲It is recommended to use an instrument with high accurate temperature controlling system (i.e. a PCR instrument) for the reaction. The recombination efficiency can reach its peak at 30 min. Longer or shorter reaction time will decrease on the cloning efficiency.

▲The recombination product can be stored at -20℃ for one week. Thaw the product before transformation.

7.5 Transformation

1.Place the competent cells on ice (i.e. DH5α competent E.coli strain)

2.Pipet 10 µl of the recombination products to 100 µl of the competent cells, flip the tube several times to mix thoroughly (**DO NOT VOTEX!**), and then place the tube still on ice for 30 min.

▲The volume of transformation products should not be more than 1/6 of the volume of competent cells.

3. Heat-shock the tube at 42℃ for 45 sec and then immediately chill on ice for 2 - 3 min.

4. Add 900 µl of SOC or LB medium (without antibiotics) to the tube. Then, shake at 37℃ for 1 hour at 200 - 250 rpm.

5. Preheat the LB plate which contains appropriate selection antibiotic at 37℃.

6. Centrifuge the culture at 5,000 rpm for 5 min, discard 900 µl of supernatant. Then, re-suspend the pellet with 100 µl of remaining medium and plate it on an agar plate which contains appropriate selection antibiotic.

7. Incubate at 37℃ for 12 -16 hours.

7.6 Selection of Positive Colonies

◇After overnight culture, hundreds of mono-colonies will form on the transformation plate of recombination reaction, whereas fewer of those on the transformation plate of negative control.

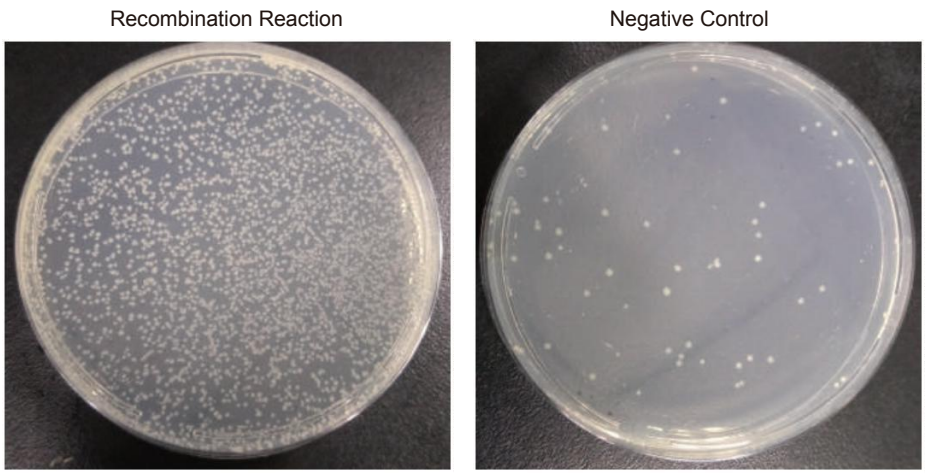


Fig4. Plate Incubated Overnight

◇Pick several mono-colonies from the plate of recombination reaction for colony PCR with at least one common sequencing primer of the vector. If the colony is positive, there should be a band which length is slightly bigger than that of insert (Fig 5).

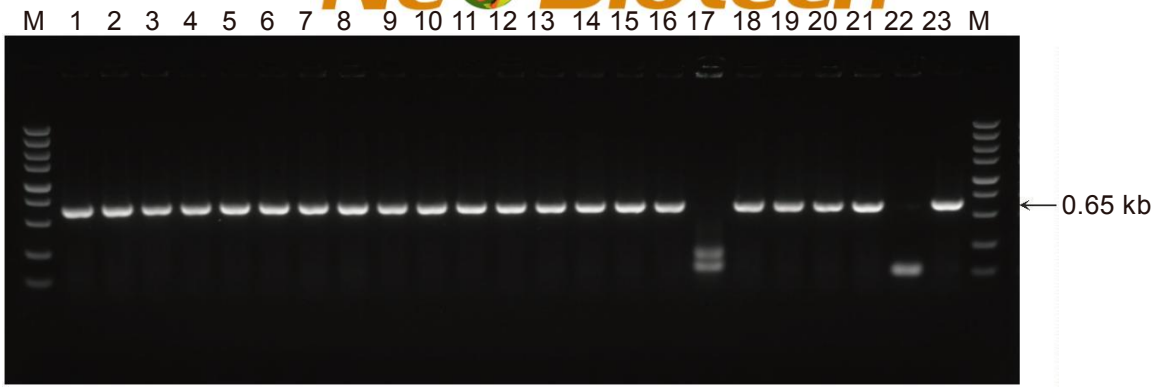


Fig 5. Agarose Gel Electrophoresis of Colony PCR Products
M: DL5, 000 Marker; 1 - 23: 21 positive colonies

▲Inoculate the remaining medium of positive clones into fresh LB medium and culture overnight. Then, extract the plasmids for further enzyme digestion analysis (Fig 6) or DNA sequencing.

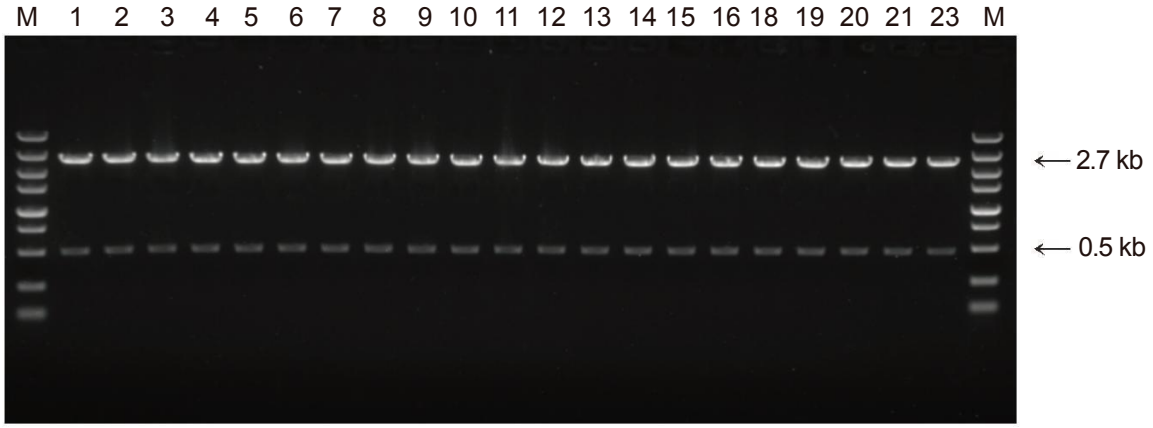


Fig 6. Agarose Gel Electrophoresis of Enzyme Digestion Products
M: DL5, 000 Marker; 1 - 23: 21 Positive Colonies

8 Tips

1.Place the recombination products on ice and transform it to competent cells directly. When transforming, the volume of recombination products should not be more than 1/6 of the volume of competent cells.
2.The ClonExpress Kit is applicable for efficient clone of DNA fragments of 50 bp - 10 kb.
3.Usages of inserts and vectors
◇Cloning short fragments (< 5 kb)
▲Linearized vectors, prepared by restriction digestion, can be directly used for recombination after inactivating the restriction enzymes. Inactivation is available for most restriction enzymes, please refer to instruction of specific inactivation methods.
▲For linearized vectors prepared by reverse PCR, the PCR products can be used directly for recombination without purification if the amplification templates are pre-linearized and PCR products show singer band.
▲For inserts, if the yield and amplification specificity of the PCR products is confirmed by agarose electrophoresis and the templates are not circular plasmids which share the same antibiotic resistance with the cloning vector, high specific PCR products can be directly used. For recombination without further purification, please refer to Table 1 / Table 2 for the usages of linearized vectors and inserts prepared in different ways.

Table 1. Usages of Linearized Vectors

Method of Linearization		Template Type	Fast Protocol*	Standard Protocol
Digestion		Circular Plasmid	Use directly after inactivating restriction enzymes	Gel Recovery
Reverse PCR	Specific Amplification	Circular Plasmid	Use directly after *Dpn I* digestion (degrade the PCR template)	Gel recovery or gel recovery after *Dpn I* digestion
		Pre-linearized Plasmid, Genomic DNA, cDNA	Use directly	Gel recovery
	Non-specific Amplification	Gel recovery		

Table 2. Usages of Amplified Inserts

Amplification Specificity	Template Type	Fast Protocol*	Standard Protocol
Specific Amplification	Circular plasmids sharing the same antibiotic resistance with the cloning vector	Use directly after *Dpn I* digestion**	Gel recovery or gel recovery after *Dpn I* digestion
Non-specific Amplification	Pre-linearized plasmid, genomic DNA, cDNA	Use directly	Gel recovery
	Gel recovery		

▲When using enzyme digestion products or amplified products directly for recombination, the volume should be ≤ 4μl (1/5 of the total volume of recombination reaction system).

▲After Dpn I digestion, the amplified inserts should be incubated at 85℃ for 20 min to deactivate Dpn I , so as to prevent cloning vectors from degradation when recombination.

◇ Cloning long fragment (> 5 kb)

It is recommended to purify the linearized vectors and amplified inserts with high quality gel recovery kit before recombination, so as to improve the DNA purity and to eliminate residual circular vectors.

9 Troubleshooting

◇ **Primers design notes**

1. It is recommended to use the software CE Design for primer design.
2. The linearized vector can be obtained by double digestion, single digestion and reverse PCR, among which, double digestion is recommended.
3. Three parts of primers: homologous sequences (15 bp – 20 bp, exclude restriction sites and base residues, the content of GC is 40% - 60%)+ restriction sites (include or exclude according to experiment need) + specific primers (when calculating the Tm of primers, the homologous sequence of vector ends should be excluded)

◇ **Few clones or no clones formed on the plate**

1. Improper primer design: primer includes 15 bp-20 bp homologous sequences (exclude restriction sites), the content of GC is 40% - 60%.
2. The amount of linearized vectors and amplified inserts are too low/high in the recombination reaction or the ratio of fragments is not appropriate. Please use the amount and radio as specification recommended.
3. Contamination in vector and insert inhibits the recombination: The total volume of unpurified vector and insert digested should be ≤ 4 μl (1/5 of the total volume of recombination reaction system). Gel extraction purification is recommended to purify the vector and insert. It is recommended to dissolve the purified DNA in ddH2O of pH 8.0.
4. The low efficiency of the competent cells: Make sure the transformation efficiency of competent cells is $>10^{7}$ cfu/μg. Transform 1 ng of vector and pick 1/10 of that to transform, and there are 1000 colonies growing on the plate. Then, the transformation efficiency of competent cells can be estimated as 10^{7}cfu/μg. The volume of transformation products should not be more than 1/6 of the volume of competent cells. Choose competent cells used for cloning (such as DH5α/XL10) not those for expressing.

◇ **Incorrect/none inserts found in the colony plasmids**

1. Non-specific amplification is mixed with target inserts: Optimize the PCR reaction system to improve the amplification specificity; purify the PCR products with a gel recovery kit; select more colonies for verification.
2. Incomplete linearization of the vector: Approaches to overcome such situation include using negative controls to confirm the complete linearization of vectors, elevating the amount of restriction endonuclease, prolonging the digesting time, and purifying the digesting products before the recombination reaction.
3. Plasmids with the same resistance with vectors mixed in reaction system: When the PCR templates for amplification of vectors or inserts are circular plasmids, digesting the amplification products with Dpn I or purifying them by gel recovery can both effectively reduce or even eliminate the residues of cyclic plasmid templates.

◇ **No electrophoretic bands in colony PCR**

1. Improper primer: it is recommended to use at least one common sequencing primer of the vector.
2. Inappropriate PCR system or program: No bands of target or empty- plasmid. It is recommended to optimize the PCR reaction system or program; extract plasmids as PCR templates or use enzyme digestion for confirmation.
3. Unsuccessful recombination: There is only the band of empty plasmid after colony PCR, which indicates the unsuccessful recombination and incomplete linearization of the vector. One of the approaches to overcome such situation is to optimize the enzyme digestion system.