ClonExpress MultiS One Step Cloning Kit

C113

Vazyme

Instruction for Use

Version 22.1

Contents

01/Product Description

The ClonExpress technology is simple, fast, and highly efficient DNA seamless cloning technology. It enables directional cloning of inserts into any site in any vector. Use any method to linearize the vector, and introduce the end sequence of the linearized vector at the 5' end of the insert forward/reverse amplification primer, so that the 5' and 3' ends of the PCR product have the same ends sequence (15 - 20 bp) as the linearized vector, respectively. The PCR product and the linearized vector are mixed in a certain proportion. Under the catalysis of recombinase, the transformation can be performed at 37℃ for 30 min to complete the directional cloning.

ClonExpress MultiS is a new generation of recombinant cloning kit. It is specifically optimized for multi-fragment recombination reactions, enabling up to five inserts to be cloned at one time. This kit has a unique ligase-independent system, which significantly reduces the self-ligation background of the vector, and does not need to consider the restriction endonuclease sites of inserts. Highly optimized reaction buffer and enhanced recombinase Exnase MultiS can significantly improve the recombination efficiency and the tolerance to impurities. These features makes it possible to use linearized vectors and inserts directly for recombinant cloning without purification, which significantly simplifies the experimental steps.

02/Components

Components	C113-01 (10 rxns)	C113-02 (25 rxns)
5 × CE MultiS Buffer	40 μl	100 μl
Exnase MultiS	20 μl	50 μl
pUC19 control vector, linearized (50 ng/μl, Amp^+)	5 μl	5 μl
Control insert Mix*	5 μl	5 μl

* 0.5 kb, 10 ng/μl; 1 kb, 20 ng/μl; 2 kb, 40 ng/μl.

03/Storage

Store at -30 ~ -15℃ and transport at ≤0℃.

▲Please avoid repeated freezing and thawing .

04/Applications

◇ Fast Cloning
◇ High-throughput Cloning
◇ Seamless Cloning
◇ DNA Site-directed Mutagenesis

05/Self-prepared Materials

PCR templates, primers, linearized vectors.

High-fidelity polymerase: Phanta Max Super-Fidelity DNA Polymerase (Vazyme #P505) or other equivalent products.

Competent cells: Chemically competent cells prepared by cloning strains;

DH5α Competent Cell (Vazyme #C502) for conventional cloning, applicable to plasmids <15 kb;

XL10 Competent Cell (Vazyme #C503) for long-fragment cloning, applicable to plasmids >10 kb.

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

06/Notes

For research use only. Not for use in diagnostic procedures.

1. Place the recombination products on ice and transform it to competent cells directly. It is recommended to use commercially competent cells (transformation efficiency >10^8 cfu/μg). The transformed volume of recombinant products should be ≤1/10 of the volume of competent cells.
2. The ClonExpress MultiS Kit can efficiently clone 50 bp - 10 kb fragments.
3. Preparation of inserts and vectors

◇Cloning of 2 - 3 inserts and total length of fragments <5 kb

▲ Linearized vectors, prepared by restriction endonucleases digestion, can be heated to inactivate the endonuclease (applicable to most endonucleases, please refer to the endonuclease instruction for the specific inactivation method) and then directly used in the recombination reaction.

▲ For linearized vectors prepared by Inverse PCR, if the amplification templates are pre-linearized and PCR products show single band, the PCR products can be used directly for recombination without purification.

▲ For inserts, if the yield and amplification specificity of the PCR products is confirmed by agarose gel electrophoresis and templates are not circular plasmids with the same antibiotic resistance as the cloning vector, high specific PCR products can be directly used in the recombination reaction without purification. Please refer to Table 1/Table 2 for the preparation of linearized vectors and inserts prepared in different situations.

Table 1. Preparation of Linearized Vectors

Method of Linearization		Template Type	Fast Protocol	Standard Protocol
Digestion		Circular plasmid	Use directly after inactivating restriction endonucleases	Gel extraction
PCR Amplification	Specific Amplification	Circular plasmid	Use directly after *Dpn* I digestion (degrade the PCR template)	Gel extraction or gel extraction after *Dpn* I digestion
		Pre-linearized plasmid, gDNA, cDNA	Use directly	Gel extraction
	Nonspecific Amplification	Gel extraction		

Table 2. Preparation of Amplified Inserts

PCR Amplification	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 (degrade the PCR template)	Gel extraction or gel extraction after *Dpn* I digestion
	Pre-linearized plasmid, gDNA, cDNA	Use directly	Gel extraction
Nonspecific Amplification	Gel extraction		

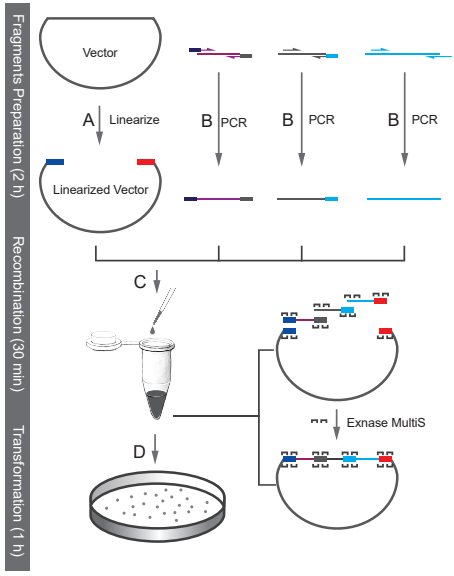
▲ When using enzyme cleavage products or amplification products directly for recombination, the volume should be ≤4 μl (1/5 of the total volume of recombination reaction system).

▲ After *Dpn* I digestion, the amplified inserts should be incubated at 85℃ for 20 min to inactivate *Dpn* I, thereby preventing the degradation of cloning vectors during recombination.

◇ Cloning of 4 - 5 inserts or total length of fragments >5 kb

It is recommended to purify the linearized vector and amplified inserts with the high-quality gel extraction kit, thereby improving the purity of the vector and insert.

07/Mechanism & Workflow



A. Vector linearization: The linearized vector can be obtained by digesting the circular vector with restriction endonucleases or by Inverse PCR.

B. Preparation of Inserts: Prepared by PCR. Add homologous sequences (marked in dark blue, dark grey, light blue and red in the figure) to the 5' end of the primers, so that there are 15 - 20 bp homologous sequences among all DNA fragments, respectively.

C. Recombination: Mix the linearized vectors and inserts at an appropriate ratio. Incubate with Exnase MultiS at 37℃ for 30 min to complete recombination reaction and realize the in vitro circularization of multiple linearized DNA.

D. Transformation: The recombination products can be used for transformation directly. The plate will form hundreds of single clones for later positive screening.

Fig 1. Mechanism of ClonExpress MultiS Multi-fragment Homologous Recombination

08/Experiment Process

08-1/Preparation of Linearized Vectors

1. Select appropriate cloning site to linearize the vector. Please ensure that there is no repetitive sequence near the cloning site, and the GC content in the upstream and downstream 20 bp region is between 40% - 60%.
2. Vector linearization: The linearized vector can be obtained by digesting the circular vector with restriction endonucleases or by Inverse PCR.

◇ When preparing the linearized vector by restriction endonuclease digestion, it is recommended to use double enzyme digestion method to make the vector linearized completely to reduce the transformation background (false positive clones); If single enzyme digestion is applied, please prolong the digestion time appropriately to reduce the cyclic plasmid residue.

▲ There is no DNA ligase in the recombination reaction system. It will not cause the self-ligation of vectors. Therefore, terminal dephosphorylation is not required even for linearized vectors prepared by single digestion. The false positive colonies (clones without inserts) are mainly from vectors that failed to be linearized. If the rate of false positive clones is high, it is recommended to reprepare the linearized vectors.

◇ When using Inverse PCR amplification to obtain linearized vectors, it is highly recommended to use a high-fidelity DNA polymerase (Phanta Max Super-Fidelity DNA Polymerase, Vazyme #P505) for vector amplification to reduce the introduction of amplification mutations. It is also recommended to use 0.1 - 1 ng circular plasmids or pre-linearized plasmids as PCR templates to reduce the influence of the residual circular plasmid template on the rate of positive clones in a 50 μl PCR reaction system.

08-2/Preparation of Inserts

1. The general principle of primer design: Introduce homologous sequences of linearized vector (15 - 20 bp, excludes restriction endonuclease sites) into 5' ends of both Forward & Reverse primers, respectively. Thereby, the ends of amplified inserts and linearized vectors are identical to each other.
   It is recommended to download the primer design software CE Design (http://www.vazyme.com) from the official website of Vazyme to automatically generate the amplification primers of inserts. For manual design, please refer to the following examples: Insert three gene fragments between the *Eco*R I and *Hin*d III sites of pUC19 vector. Take the primer design of inserts with lengths of 0.5 kb, 1 kb, and 2 kb as an example. The specific primer design protocol is as follows:

   Forward primer of the most upstream fragment:
   5' - homologous sequence of upstream vector end + restriction endonuclease site (optional) + sequence of gene specific forward amplification primer - 3'

Reverse primer of the most downstream fragment:

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

▲ Gene-specific forward/reverse amplification primer sequence refers to the forward/reverse amplification primer sequence of regular insert fragments. Tm value of 60 ~ 65℃ is recommended.

▲ Homologous sequences of vector upstream or downstream end refer to the terminal sequence of the linearized vector (for homologous recombination). GC content of 40% - 60% is recommended.

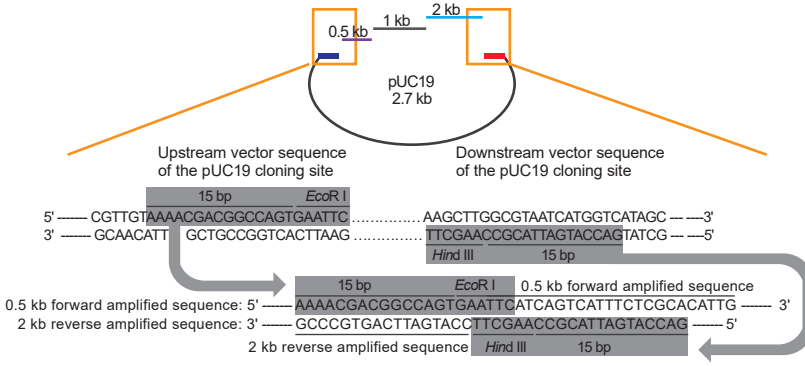


Fig 2. Primer Design for Multi-fragment Homologous Recombination

▲ If the length of primers exceed 40 bp, PAGE purification of synthetic primers is recommended, which will benefit the recombination efficiency. When calculating the Tm value of primers, the homologous sequence and restriction endonuclease site should be excluded, and only the gene-specific amplification sequence should be included.

◇Three ways to design primers between inserts in the middle are as follows:

a. Introduce homologous sequences (15 - 20 bp) from 3' end of previous fragment into 5' end of the latter fragment;

b. Introduce homologous sequences (15 - 20 bp) from 5' end of the latter fragment into 3' end of the previous fragment (as shown in Fig 3);

c. Take a part of each of the two inserts as a homologous sequence (15 - 20 bp) and add them to the end of the other fragment, respectively.

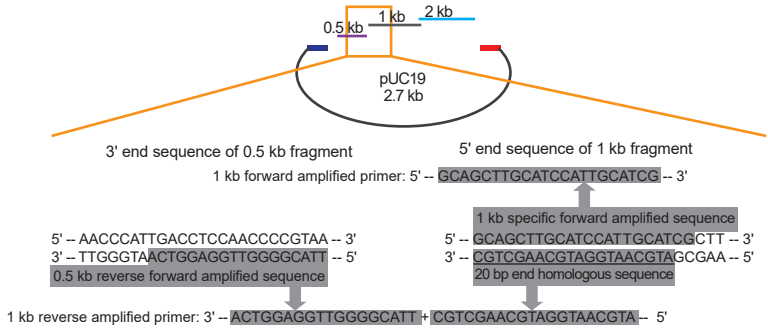


Fig 3. Primer Design for Multi-fragment Homologous Recombination

▲ If the length of primers exceed 40 bp, PAGE purification of synthetic primers is recommended, which will benefit the recombination efficiency. When calculating the Tm value of primers, the homologous sequence and restriction enzyme endonuclease site should be excluded, and only the gene-specific amplification sequence should be included.

2. PCR amplification of inserts

Inserts can be amplified by any polymerase (e.g. *Taq* DNA polymerase or high-fidelity polymerase). There is no need to consider whether there is an adenine at the 3' end of the product (It will be removed during the recombination process and not appear in the final vector). To reduce the introduction of amplification mutations, it is recommended to use the high-fidelity polymerase (Phanta Max Super-Fidelity DNA Polymerase, Vazyme #P505) for amplification.

08-3/The Amount of Linearized Vectors and Inserts

1. Determination of DNA concentration

The system of ClonExpress homologous recombination is recommended to quantify DNA by comparing the brightness of bands by electrophoresis.

◇ Suppose the high-quality gel DNA extraction kit has purified the linearized vectors and inserts, and there is no obvious nonspecific band or smear residue after gel electrophoresis. In that case, instruments based on absorbance, such as Onedrop, can be used to determine the DNA concentration, but the concentration value is only reliable when A260/A280 value is 1.8 - 2.0. When the sample concentration is lower than 10 ng/μl, the concentration values obtained by different models of instruments based on A260 may have significant differences. It is recommended to determine concentration by Qubit and PicoGreen, etc.

◇ If the linearized vector or insert is not purified, be sure to quantify DNA by comparing the brightness of bands by electrophoresis as shown in the figure below. After multiple serial dilutions of the linearized vector and inserts, load 1 μl of the stock solution and the

diluted solution for electrophoresis. Compare the band brightness of products with the standard DNA quantitative marker (the concentration of bands are uniform and determined) to determine its approximate concentration.

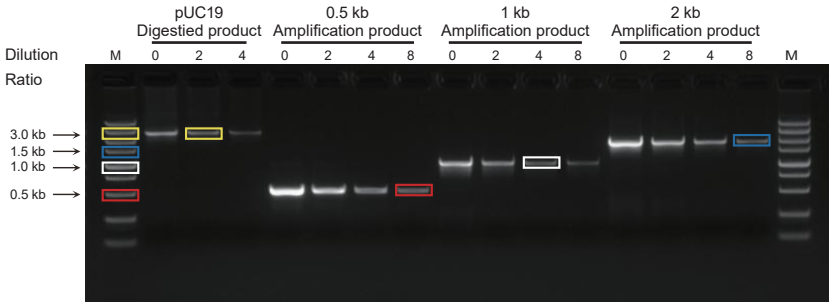


Fig 4. Concentration detection of linearized vectors and inserts by gel electrophoresis

▲ M: DL5000 Marker. Load 5 μl of solution, except for the 1.0 kb band, which is 100 ng, the DNA amount of the other bands is 50 ng. The yellow box/orange box/white box/blue box in the figure marks the linearized vector and the insert, respectively, and the brightness is similar to the marker band of similar size under a certain dilution gradient. Therefore:

The concentration of vectors is about: 50 ng × 2 = 100 ng/μl;

The concentration of 0.5 kb amplification products is about: 50 ng × 8 = 400 ng/μl;

The concentration of 1 kb amplification products is about: 100 ng × 4 = 400 ng/μl;

The concentration of 2 kb amplification products is about: 50 ng × 8 = 400 ng/μl.

2. The calculation of vectors and inserts input

For ClonExpress MultiS recombination reaction system, the optimal amount is 0.03 pmol per fragments (including linearized vector), These mass can be roughly calculated according to the following formula:

The optimal mass of vector required = [0.02 × number of base pairs] ng (0.03 pmol)

For example, when cloning inserts of 0.5 kb, 1 kb and 2 kb into the vector of 5 kb, the optimal mass of the vector and three inserts are as follows:

The optimal mass of linearized vector required: 0.02 × 5,000 = 100 ng;

The optimal mass of insert of 0.5 kb required: 0.02 × 500 = 10 ng;

The optimal mass of insert of 1 kb required: 0.02 × 1,000 = 20 ng;

The optimal mass of insert of 2 kb required: 0.02 × 2,000 = 40 ng;

a. The amount of linearized vectors should be between 50 - 200 ng. When the optimal amount calculated using the above formula is beyond these ranges, just choose the maximum or minimum amount for recombination.

b. The mass of each insert should be more than 10 ng. When the optimal mass calculated by the above formula is lower than 10 ng, just use 10 ng directly.

c. When the linearized cloning vector and the amplification product of inserts are used directly without DNA purification, the added volume should be ≤1/5 of the total volume of the reaction system, that is, 4 μl.

08-4/Recombination

1. The amount of DNA can be roughly calculated according to the above formula.

   Dilute the vector and insert at an appropriate ratio to ensure the accuracy of pipetting before preparing the recombination reaction system, and the amount of each component is not less than 1 μl.

2. Prepare the following reaction on ice:

Components	Recombination	Negative control-1[b]	Negative control-2[c]	Positive control[d]
Linearized Vector[a]	X μl	X μl	0 μl	1 μl
Insert[a]	Y_1 - Y_n μl	0 μl	Y_1 - Y_n μl	1 μl
5 × CE MultiS Buffer	4 μl	0 μl	0 μl	4 μl
Exnase MultiS	2 μl	0 μl	0 μl	2 μl
ddH_2O	to 20 μl	to 20 μl	to 20 μl	to 20 μl

   a. X/Y is the amount of vector/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 amplification template of inserts is circular plasmids with the same antibiotic resistance as the cloning vector. It is recommended that the circular plasmid residue detection of the linearized vector and inserts be performed independently.
   d. Positive controls can be used to exclude the influence of other materials and operation.

3. Gently pipette up and down for several times to mix thoroughly (DO NOT VORTEX!). Briefly centrifuge to collect the reaction solution to the bottom of the tube.

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

   ▲ It is recommended to perform the reaction on an instrument with precise temperature control such as a PCR machine. The recombination efficiency reached the highest at about 30 min of reaction. Insufficient or too long incubation time will reduce cloning efficiency.

   ▲ The recombinant product can be stored at -20℃ for one week. Thaw and transform when needed.

08-5/Transformation

1. Thaw the competent cells on ice (e.g., DH5α Competent Cell, Vazyme #C502).

2. Pipette 10 μl of the recombination products to 100 μl of competent cells, flick the tube wall to mix thoroughly (DO NOT VORTEX!), and then place the tube on ice for 30 min.

   ▲ The volume of recombination products should be ≤1/10 of the volume of competent cells.

3. Heat shock at 42℃ water bath for 45 sec and then immediately place on ice for 2 - 3 min.

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

5. Preheat the corresponding resistant LB solid medium plates in a 37℃ incubator.

6. Centrifuge the culture at 5,000 rpm (2,400 × g) for 5 min, discard 900 μl of supernatant. Then, use the remaining medium to suspend the bacteria and use a sterile bent glass rod to gently spread on the plate which contains the appropriate selection antibiotic.
7. Incubate at 37℃ for 12 - 16 h.

08-6/Recombinant Product Identification

◇ After overnight incubation, hundreds of single clones will form on the plate of recombination reaction, whereas fewer of those on the plate of negative control (Fig 5).

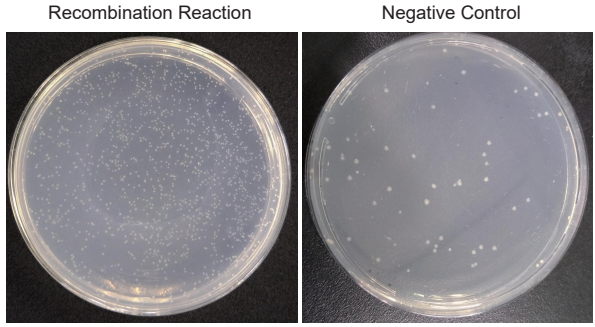


Fig 5. Plates Incubated Overnight

◇ Pick several clones from the plate of recombination reaction for colony PCR with at least one universal sequencing primer of the vector. If the colony is positive, a band slightly larger than the size of inserts should appear (Fig 6).

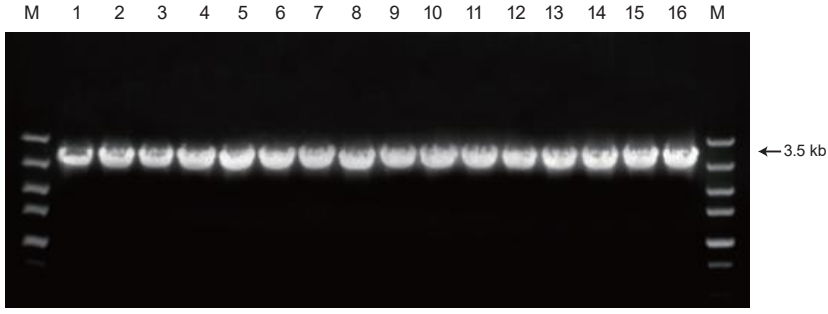


Fig 6. Agarose Gel Electrophoresis of Colony PCR Products

M: DL5000 Marker; 1 - 16: 16 Positive Colonies

◇ Inoculate the remaining bacterial solution of positive clones into the liquid LB medium (contains appropriate antibiotics) for overnight incubation. Then, extract the plasmid for restriction endonuclease digestion identification (Fig 7), or directly perform sequencing.

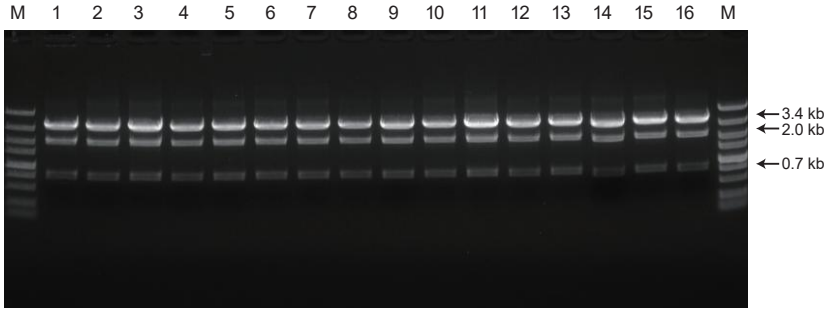


Fig 7. Agarose Gel Electrophoresis of Enzyme Digestion Products

M: DL5000 Marker; 1 - 16: 16 Positive Colonies

09/FAQ & Troubleshooting

◇ How to design primers?

① Primer design: It is recommended to use the primer design software CE Design, and select the corresponding module for design.

② Three ways to linearize the vector: Double digestion, Single digestion and Inverse PCR. Double digestion is preferred.

③ Three parts of primers: Homology arms (15 - 20 bp, exclude restriction sites and base residues, the content of GC is 40% - 60%) + restriction sites (optional) + specific primers (when calculating the Tm value of primers, the homology arms should be excluded)

◇ Few clones or no clone are formed on the plate.

① Incorrect primer design: The primer includes 15 - 20 bp homology arms (exclude restriction sites) and 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 is not appropriate. Please use the amount and ratio according to specification recommended.

③ Contamination in vector and insert inhibits the recombination: The total volume of unpurified DNA should be ≤4 μl (1/5 of the total volume of reaction system). It is recommended that the linearized vector and PCR product are purified by gel extraction. Then, dissolve the purified product in ddH_2O (pH 8.0).

④ The low efficiency of competent cells: Make sure the transformation efficiency of competent cells is >10^7 cfu/μg. The simple test can be performed. Transform the 1 ng of plasmids and take the 1/10 for spreading plates. If 1,000 clones are grown, the estimated transformation efficiency is 10^7 cfu/μg. The transformation volume of recombinant products should be ≤ 1/10 of the volume of competent cells, otherwise the transformation efficiency will be reduced. Select competent cells used for cloning (such as DH5α/XL10). Do not select competent cells used for expression.

◇ Most clones do not contain insert or contain incorrect insert.

① PCR products contain nonspecific amplification products: Optimize PCR system to improve the amplification specificity; purify PCR products by gel extraction; identify more clones.

② Incomplete linearization of the vector: The negative control can be used to detect whether the vector is completely linearized. Optimize the digestion system, increase the amount of restriction endonucleasess, prolong the time of digestion reaction, and purify the digestied products by gel extraction.

③ Plasmids with the same resistance mixed in reaction system: When the PCR amplification template is a circular plasmid, if the amplification product is directly used in the recombination reaction without purification, it is recommended to digest with *Dpn* I, or perform gel extraction to purify the amplification product.

◇ No target bands in Colony PCR.

① Incorrect primer: It is recommended to use the universal primer of the vector for colony detection, or use at least one universal primer.

② Inappropriate PCR system or program: It is recommended to optimize the PCR system and program; or extract plasmids as templates to perform PCR identification; or perform enzyme digestion identification.

③ Unsuccessful recombination: There is only the band of empty plasmids, indicating that the recombination is unsuccessful and the linearization of the vector is incomplete. It is recommended to optimize the enzyme digestion system.

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