ClonExpress Ultra One Step Cloning Kit

C115

Vazyme

Instruction for Use

Version 22.3

Vazyme

Contents

01/Quick Workflow

01-1/The Amount of Linearized Vectors and Inserts

1. Entry clone and single-fragment homologous recombination:

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

2. Multi-fragment (2 - 5) homologous recombination:

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

01-2/Recombination

1. The amount of DNA required for recombination reaction can be calculated according to the formula.
   Dilute linearized vectors and inserts before recombination to assure the loading accuracy. The volume of each component loaded should be no less than 1 μl.
2. Prepare the following reaction system on ice:

Components	Recombination	Negative control-1	Negative control-2	Positive control
Linearized Vector	X μl	X μl	0 μl	1 μl
n Inserts (n≤5)	$Y_1+Y_2+...+Y_n$ μl	0 μl	$Y_1+Y_2+...+Y_n$ μl	1 μl
2 × ClonExpress Mix	5 μl	0 μl	0 μl	5 μl
ddH_2O	to 10 μl	to 10 μl	to 10 μl	to 10 μl

3. Gently pipette up and down for several times to mix thoroughly (**DO NOT VOTEX!**). Centrifuge briefly to collect the reaction solution to the bottom of the tube.
4. **Single-fragment homologous recombination:** Incubate at 50℃ for 5 min and chill the tube immediately at 4℃ or on ice.
   Multi-fragment homologous recombination: Incubate at 50℃ for 15 min and chill the tube immediately at 4℃ or on ice.
   ▲ Increase the volume of reaction system to 20 μl if the total volume of vector and insert is more than 5 μl. For single-fragment homologous recombination, increasing the recombination time to 15 min may be helpful to improve the recombination efficiency when the amount of DNA is 300 - 400 ng. For 4 - 5 multi-fragment homologous recombination, increasing the recombination time to 30 min but no more than 1 h can improve the recombination efficiency.

01-3/Transformation

1. Thaw the competent cells on ice (e.g., DH5α Competent Cell, Vazyme #C502).
2. Pipette 5 - 10 μl of the recombination products to 100 μl competent cells, flick the tube wall to mix thoroughly (DO NOT VOTEX!), 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°C 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 LB plate which contains appropriate selection antibiotic at 37℃.
6. Centrifuge at 5,000 rpm (2,500 × g) for 5 min, discard 900 μl of supernatant. Then, use the remaining medium to suspend the bacteria and use a sterile spreading rod to gently spread on an agar plate which contains appropriate selection antibiotic.
7. Incubate at 37℃ for 12 - 16 h.

02/Product Description

The ClonExpress technology is simple, fast, and highly efficient DNA seamless cloning technology. It enables rapid 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 with the same sequence as the end of the vector and the linearized vector are mixed in a certain proportion. Under the catalysis of recombinase, the transformation can be performed at 50℃ for 5 - 15 min to complete the directional cloning.

ClonExpress Ultra One Step Cloning Kit is a new generation of recombinant cloning kits, compatible with 1 - 5 fragments homologous recombination. The unique ligase-independent system of this kit significantly reduces the self-ligation background of the vector. Restriction endonuclease sites do not need to be considered. Highly optimized 2 × ClonExpress Mix (It contains enhanced recombinase Exnase) can significantly improve the recombination efficiency and the tolerance to impurities. pCE-Zero vector is compatible with most PCR products, enabling the specific PCR products to be used directly for recombination without any treatments, which significantly simplify the procedure.

03/Components

Components	C115-01 (25 rxns)	C115-02 (50 rxns)
2 × ClonExpress Mix	125 μl	2 × 125 μl
pCE-Zero vector, linearized (50 ng/μl)*	25 μl	50 μl
500bp control insert (20 ng/μl)	5 μl	5 μl

* Double-resistance vector: Amp^+, Kan^+

04/Storage

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

05/Applications

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

06/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.

07/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 the recombinant product should be ≤1/10 of the volume of competent cells.
2. ClonExpress Ultra One Step Cloning Kit is applicable for efficient clone of 50 bp - 10 kb fragments.
3. Usages of inserts and vectors

◇ Cloning fewer or short fragments (<5 kb)

▲ Linearized vectors, prepared by restriction 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 electrophoresis and the 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 usages of linearized vectors and inserts prepared in different situations.

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
PCR Amplification	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, gDNA, cDNA	Use directly	Gel recovery
	Non-specific Amplification	Gel recovery		

Table 2. Usages of Ampified 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 recovery or gel recovery after *Dpn* I digestion
	Pre-linearized plasmid, gDNA, cDNA	Use directly	
Non-specific Amplification	Gel recovery		

▲ When using enzyme cleavage products or amplified products directly for recombination, the volume should be ≤2 μl (1/5 of the total volume of recombination reaction system).

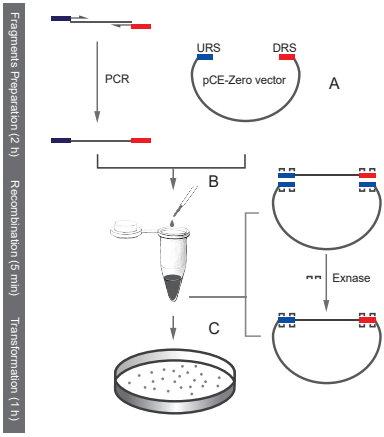
▲ After *Dpn* I digestion, the amplified inserts should be incubated at 85℃ for 20 min to deactivate *Dpn* I, so as to prevent cloning vectors from degradation when recombination.

◇Cloning multi-fragment (4 - 5) or long fragment (>5 kb)

It is recommended to purify the linearized vector and amplified inserts with the high-quality gel recovery kit, so as to improve the purity of the vector and insert.

08/Entry Clone Homologous Recombination

08-1/Workflow



A. Acquisition of Inserts: Introduce URS/DRS sequences (marked in blue and red) into 5'-end of Forward (F) & Reverse (R) primers, aiming to make the ends of amplified inserts and pCE-Zero vector identical to each other.

B. Recombination: Mix pCE-Zero vectors and inserts at an appropriate ratio and incubate with Exnase at 50℃ for 5 min to make two linearized DNA cyclized in vitro.

C. Transformation: The recombination products can be used for transformation directly, and hundreds of single clones will be formed on the plate for later positive screening.

Fig 1. Mechanism of ClonExpress Ultra Entry Clone Homologous Recombination

08-2/Preparation of Inserts

1. Primer design principles for entry clone homologous recombination: Introduce URS and DRS sequence into 5'-end of Forward (F) & Reverse (R) primers, respectively, aiming to make the ends of amplified inserts and pCE-Zero vectors identical to each other (15 bp).

   It is recommended to use Vazyme's software, CE Design (available on https://www.vazymebiotech.com), to design primers. The CE Design automatically generates amplification primers of insert. For manually design, please refer to the principle below:

   Forward primer of insert fragments:

   5' - URS sequence + restriction enzyme cutting site (optional) + gene specific forward amplification sequence of insert - 3'

   Reverse primer of insert fragments:

   5' - DRS sequence + restriction enzyme cutting site (optional) + gene specific reverse amplification sequence of insert - 3'

a. URS is the abbreviation of Upstream Recombination Sequence; DRS is the abbreviation of Downstream Recombination Sequence. The specific sequences are as follows:

   URS: 5' - GGATCTTCCAGAGAT - 3', DRS: 5' - CTGCCGTTCGACGAT - 3'.

b. pCE-Zero vector contains two *Eco*R I cutting sites, which can be used for verifying the inserts by enzyme *Eco*R I digestion analysis. Other suitable enzyme cutting site can also be added between UDS/DRS sequence and gene specific forward/reverse amplification sequence.

c. Gene specific forward/reverse amplification sequence refers to the sequence to amplify the insertion. The Tm value of 60 ~ 65°C is recommended.

d. 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 value of primers, the URS/DRS sequence and additional restriction enzyme cutting site should be excluded, and only gene specific amplification sequence should be included.

2. Design the insert forward amplification primer and reverse amplification primer as follows:

   ClonExpress Ultra entry clone, forward primer of insert:

5' - GGATCTTCCAGAGAT + ATCTCGCACATTGCC - 3'
URS Gene specific forward amplification sequence of insert

   ClonExpress Ultra entry clone, reverse primer of insert:

5' - CTGCCGTTCGACGAT + GTTGCCGCATTATCG - 3'
DRS Gene specific reverse amplification sequence of insert

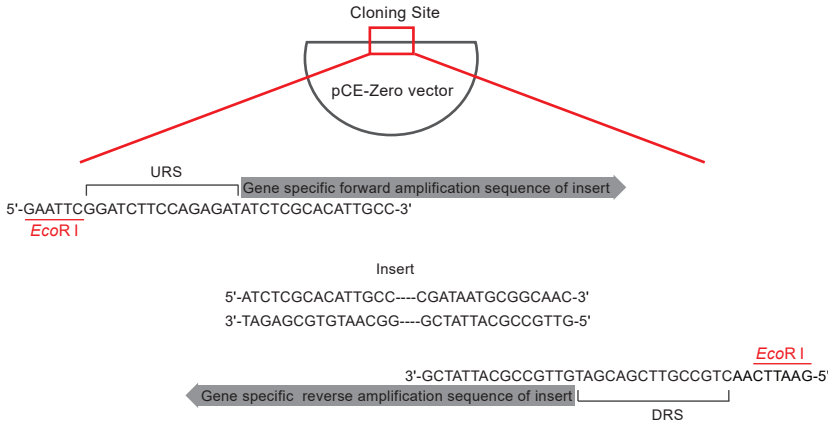
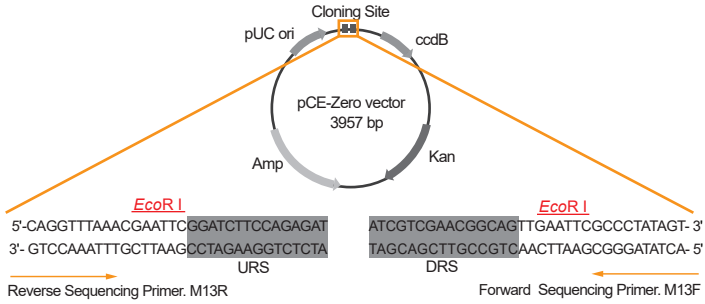


Fig 2. Primer Design for ClonExpress Ultra Entry Clone Homologous Recombination

3. PCR Amplification of Inserts

Inserts can be amplified by any polymerase (*Taq* DNA polymerase or high-fidelity polymerase). There is no need to consider whether there is an A tail at the end of the product (it will be removed during the recombination process and will not appear in the final vector). To prevent possible mutations introduced during PCR, high-fidelity polymerases (Phanta Max Super-Fidelity DNA Polymerase, Vazyme #P505) are recommended.

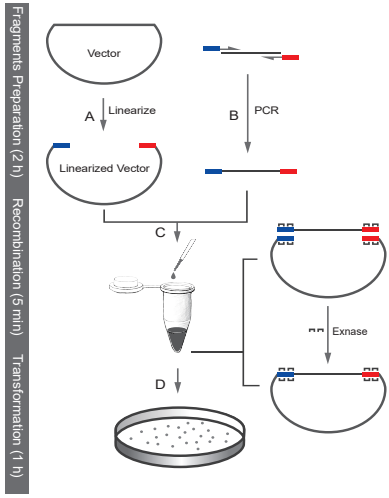
08-3/The Sequence Information of the Vector



General primer (M13) can be used for pCE-Zero vector sequencing. Inquire the complete sequences of pCE-Zero vector, please log in https://www.vazymebiotech.com

09/Single-fragment Homologous Recombination

09-1/ Workflow



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

B. Acquisition of Inserts: Introducing homologous sequences of linearized vector ends about 15 - 20 bp (marked in blue and red) 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 at 50℃ for 5 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, and hundreds of single clones will be formed on the plate for later positive screening.

Fig 3. Mechanism of ClonExpress Ultra Single-fragment Homologous Recombination

09-2/Preparation of Linearized Vectors

1. Select appropriate cloning site to linearize the vector. It is recommended to select the cloning site from regions with no repetitive sequence and the GC content of the certain region is 40% - 60% both in the upstream and the downstream 20 bp regions flanking the cloning site.
2. Vector linearization: The linearized vector can be obtained by digesting the circular vector with restriction enzymes or by reverse PCR amplification.

◇ When preparing the linearized vector by enzyme digestion, it is recommended to use double enzyme digestion method to make the vector linearized completely, while single enzyme digestion linearization is not the first choice. Restriction endonuclease digestion, please prolong the digestion time appropriately, such as overnight digestion, to reduce the cyclic plasmid residue, reduce the transformation background (false positive clone).

▲ There is no DNA ligase activity in the reaction system, so the vector will not self-ligation. Therefore, dephosphorylation is unnecessary even if the linearized vectors are prepared by single digestion. The false positive colonies (clones without inserts) are from vectors that failed to be linearized. If the false positive rate is high, it is recommended to prepare linearization again.

◇ When using Inverse PCR amplification to obtain the linearized vector, 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 amplified 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 positive rate of clones in a 50 μl PCR reaction system.

▲ When the PCR templates are circular plasmids, digesting the amplification products with *Dpn* I to reduce the influence of the residual circular plasmid template on the positive rate of clones in a 50 μl PCR reaction system.

09-3/Acquisition of Inserts

1. The primer design principles for single-fragment homologous recombination: Introduce homologous sequences of linearized vector (15 - 20 bp, excludes restriction enzyme cutting sites) into 5' end of both Forward (F) & Reverse (R) primer, respectively, aiming to make the ends of amplified inserts and linearized vectors identical to each other.

   Forward primer of insert fragments:

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

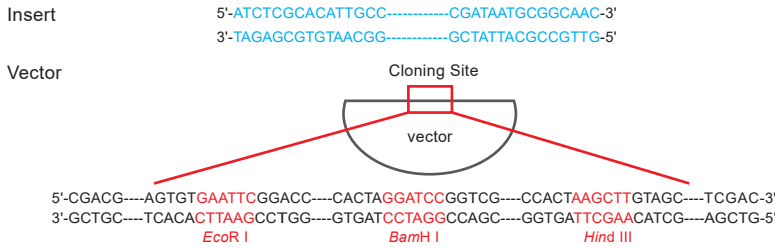
   Reverse primer of insert fragments:

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

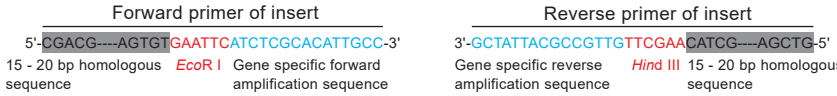
   ▲ Gene-specific forward/reverse amplification primer sequence refers to the forward/reverse amplification primer sequence of regular insert fragments. Tm value of 60 ~ 65℃ is recommended.

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

It is recommended to use Vazyme's software, CE Design (available on https://www.vazymebiotech.com), to design primers. The CE Design automatically generates amplification primers of insert. For manually design, please refer to the principle below:

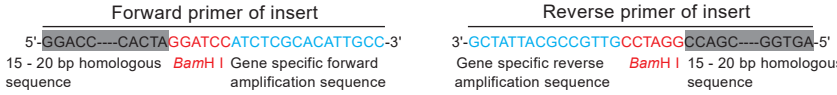


A: Linearized by double endonucleases digesting (e.g., *Hin*d III + *Eco*R I)



▲ The restriction sites of *Eco*R I and *Hin*d II keep intact after cloning.

B: Linearized by single endonucleases digesting (e.g., *Bam*H I):



▲ Both ends of the insert keep intact *Bam*H I restriction sites after cloning.

C: Linearized by Inverse PCR

Fig 4. Primer Design for Single-fragment Homologous Recombination

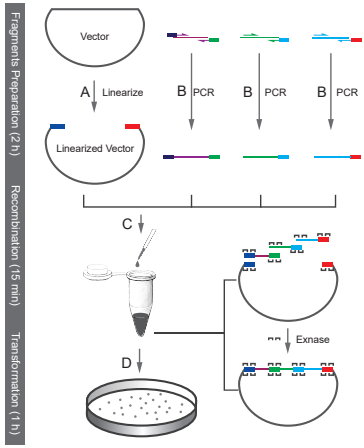
▲ If the length of primer exceeds 40 bp, PAGE purification of synthetized primers is recommended, which will benefit the recombination efficiency.

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 a poly(A) tail at the end of the product (it will be removed during the recombination process and will not appear in the final vector). To prevent possible mutations introduced during PCR, high-fidelity polymerases (Phanta Max Super-Fidelity DNA Polymerase, Vazyme #P505) are recommended.

10/Multi-fragment Homologous Recombination

10-1/ Workflow



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

B. Acquisition of Inserts: Prepared by PCR. The amplification primers used need to add homologous sequences (marked in dark blue, green, light blue and red in the figure) at the 5' end of the primers, so that there are 15 - 20 bp homologous sequences between the amplification products and between the amplification products and the linearized vector, respectively.

C. Recombination: Mix the linearized vectors and all inserts at an appropriate ratio and incubate with Exnase at 50℃ for 15 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，and hundreds of single clones will be formed on the plate for later positive screening.

Fig 5. Mechanism of ClonExpress Ultra Multi-fragment Homologous Recombination

10-2/Preparation of Linearized Vectors

1. Select appropriate cloning site to linearize the vector. It is recommended to select the cloning site from regions with no repetitive sequence and the GC content of the certain region is 40% - 60% both in the upstream and the downstream 20 bp regions flanking the cloning site.
2. Vector linearization: The linearized vector can be obtained by digesting the circular vector with restriction enzymes or by Inverse PCR.

◇ When preparing the linearized vector by enzyme digestion, it is recommended to use double enzyme digestion method to make the vector linearized completely, while single enzyme digestion linearization is not the first choice. Restriction endonuclease digestion, please prolong the digestion time appropriately, such as overnight digestion, to reduce the cyclic plasmid residue, reduce the transformation background (false positive clone).

▲ There is no DNA ligase activity in the reaction system, so the vector will not self-ligation. 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 Inverse PCR amplification to obtain linearized vector, 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 positive rate of clones in a 50 μl PCR reaction system.

▲ When the PCR templates are circular plasmids, digesting the amplification products with *Dpn* I to reduce the influence of the residual circular plasmid template on the positive rate of clones in a 50 μl PCR reaction system.

10-3/Acquisition of Inserts

1. The primer design principles for multi-fragment homologous recombination: By introducing homologous sequences at the 5' ends of the primers, the amplification products, as well as between the amplification products and the linearized cloning vector, have completely identical sequences capable of homologous recombination with each other.

   It is recommended to use Vazyme's software, CE Design (available on https://www.vazymebiotech.com), to design primers. The CE Design automatically generates amplification primers of insert. For manually design, example of three fragments, A, B, C (0.5 kb, 1 kb and 2 kb) inserted into pCE-Zero vector is shown as follows:

   ◇ The design of the primers between fragments on both sides and the recombination end of the vector:

   Forward primer of the most upstream fragment:

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

   Reverse primer of the most downstream fragment:

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

   ▲ Gene specific forward/reverse amplification sequence refers to the sequence to amplify the insertion. Tm value 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.

◇ 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 6) ;**

   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.**

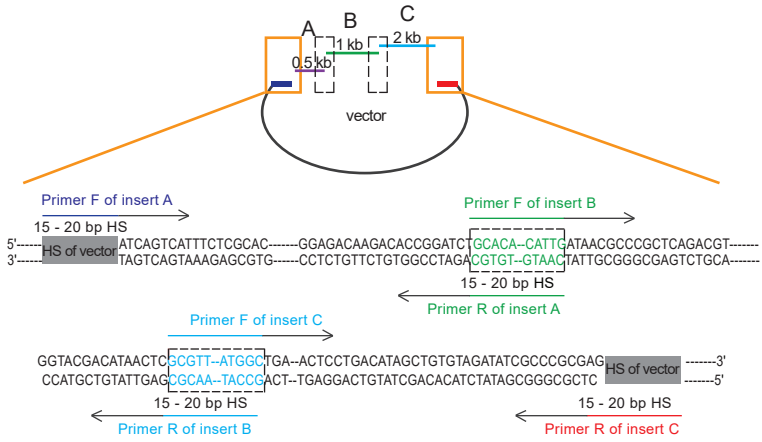


Fig 6. Primer Design for Multi-fragment Homologous Recombination

HS: Homologous Sequences Primer F: Forward primer Primer R: Reverse primer

▲ Primer design of both side fragments and the recombination end of the vector (Refer to Fig 4. Primer design for single-fragment homologous recombination).

▲ If the length of primer exceeds 40 bp, PAGE purification of synthetized primers is recommended, which will benefit the recombination efficiency.

2. PCR amplification of inserts

Inserts can be amplified by any PCR polymerase (e.g. conventional *Taq* DNA polymerase or high-fidelity DNA polymerase). There is no need to consider whether there is an A tail at the end of the product (it will be removed during the recombination process and will not appear in the final vector). To prevent possible mutations introduced during PCR, amplification with a high-fidelity polymerases (Phanta Max Super-Fidelity DNA Polymerase Vazyme #P505) are recommended.

11/Experiment Process

11-1/The Amount of Linearized Vectors and Inserts

1. Determination of DNA concentration

◇ Suppose the high-quality gel DNA recovery 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 One drop, can be used to determine the DNA concentration, but the results of concentration are only reliable when A260/A280 value is 1.8 - 2.0. It is recommended to determine concentration by Nanodrop, Onedrop, Qubit, PicoGreen, etc. When the sample concentration is lower than 10 ng/μl, the concentration values obtained by different models of instruments based on A260 may have large differences.

2. The calculation of vector and inserts usage

ClonExpress Ultra Kit is compatible with 1 - 5 fragments for homologous recombination. The pCE-Zero vector included in the kit can be used as a recombinant vector for recombination reaction with the insert. For single-fragment homologous recombination, the optimal amount of vector required is 0.03 pmol, the optimal amount of insert required is 0.06 pmol (the molar radio of vector to insert is 1:2). For multi-fragment homologous recombination, the optimal amount of inserts and linearized vectors are both 0.03 pmol (the molar radio of vector to insert is 1:1). These mass can be roughly calculated according to the following formula:

◇ Entry clone homologous recombination

The optimal mass of insert required = [0.04 × number of base pairs] ng (0.06 pmol)

◇ Single-fragment homologous recombination

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

The optimal mass of insert required = [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 × 5,000 =100 ng, and that of insert is 0.04 × 2,000 = 80 ng.

◇ Multi-fragment homologous recombination

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

The optimal mass of each insert 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 to a vector of 5 kb, the optimal mass of 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. For single-fragment homologous recombination: The mass of amplified insert should be more than 20 ng. When the length of the insert is larger than that of the vector, the calculation method of the optimal mass of vector and insert should be inverted.

b. For multi-fragment homologous recombination: The mass of each insert should be more than 10 ng. When the optimal mass calculated by the above formula is under 10 ng, just use 10 ng directly.

c. 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.

d. For entry clone or single-fragment homologous recombination: If there are no obvious non-specific bands or smear shows in gel electrophoresis, the DNA can be directly used without purification and the total volume of vectors and inserts should be ≤2 μl (1/5 of the total volume of recombination reaction system), which will reduce the recombination efficacy (Purification is recommended before recombination).

11-2/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] (n≤5)	$Y_1+Y_2+...+Y_n$ μl	0 μl	$Y_1+Y_2+...+Y_n$ μl	1 μl
2 × ClonExpress Mix	5 μl	0 μl	0 μl	5 μl
ddH_2O	to 10 μl	to 10 μl	to 10 μl	to 10 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 templates are circular plasmids which share the same antibiotic resistance with the cloning vector.

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 VOTEX!**). Briefly centrifuge to collect the reaction solution to the bottom of the tube.

4. **Single-fragment homologous recombination:** Incubate **at 50℃ for 5 min** and immediately chill the tube at 4℃ or on ice.

   Multi-fragment homologous recombination: Incubate **at 50℃ for 15 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.

   ▲ If the total volume of vector and insert is more than 5 μl, the volume of reaction system can be increased to 20 μl. For single-fragment homologous recombination, if the amount of DNA is 300 - 400 ng, the time of recombination can be prolonged to 15 min to improve the recombination efficiency. For multi-fragment of 4 - 5 homologous recombination, the time of recombination can be prolonged to 30 min to improve the recombination efficiency, but not more than 1 h.

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

11-3/Transformation

1. Thaw the competent cells on ice (e.g., DH5α Competent Cell, Vazyme #C502).
2. Pipette 5 - 10 μl of the recombination products to 100 μl of competent cells, flick the tube wall to mix thoroughly (**DO NOT VOTEX!**), and then place the tube still 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°C 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°C incubator.
6. Centrifuge the culture at 5,000 rpm (2,500 × g) for 5 min, discard 900 μl of supernatant. Then, use the remaining medium to suspend the bacteria and use a sterile spreading rod to gently spread on an agar plate which contains appropriate selection antibiotic.
7. Incubate at 37℃ for 12 - 16 h.

11-4/Recombinant Product Identification

◇ After overnight culture, hundreds of single clones will form on the transformation plate of recombination reaction, whereas fewer of those on the transformation plate of negative control (Fig 7).

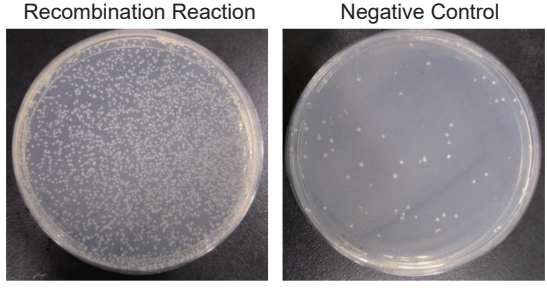


Fig 7. Plate Incubated Overnight

◇ 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, there should be a band whose length is slightly bigger than that of insert (Fig 8).

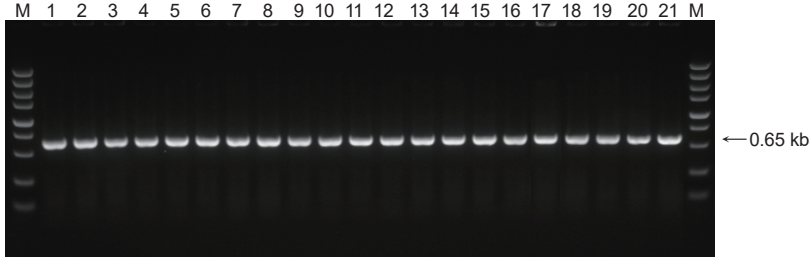


Fig 8. Agarose Gel Electrophoresis of Colony PCR Products
M: DL5,000 Marker; 1 - 21:21 Positive Colonies

◇ Inoculate the remaining medium of positive clones into fresh LB medium and culture overnight. Then, extract the plasmids for further enzyme digestion analysis (Fig 9) or DNA sequencing.

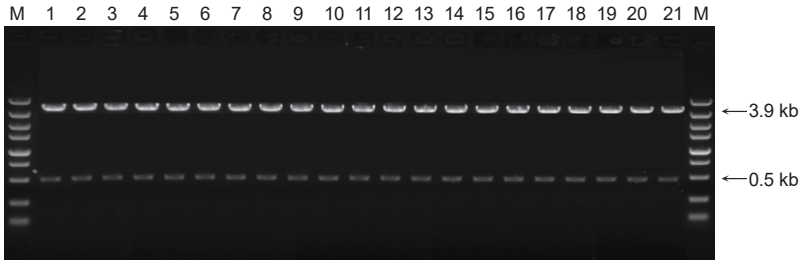


Fig 9. Agarose Gel Electrophoresis of Enzyme Digestion Products
M: DL5,000 Marker; 1 - 21:21 Positive Colonies

12/FAQ & Troubleshooting

◇ How to design primers?

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

② Three ways to linearize the vector: Double restriction digestion, single restriction digestion, Inverse PCR, and double restriction 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 ≤2 μl (1/5 of the total volume of reaction system). It is recommended that the linearized vector and PCR product be recovered and purified by gel, and the purified product is dissolved in ddH_2O.

④ The low efficiency of the competent cells: Make sure the transformation efficiency of competent cells is >10^8 cfu/μg. The simple test can be performed. If 0.1 ng of the plasmid is transformed, 1/10 of the plasmid is used for plating, and 1,000 plaques are grown, and the estimated transformation efficiency is 10^8 cfu/μg. The transformation volume of the recombinant product should be ≤ 1/10 of the volume of competent cells, otherwise the transformation efficiency will be reduced. Choose competent cells used for cloning (such as DH5α/XL10) not those for expressing.

◇ Most clones do not contain inserts or contain incorrect inserts

① Non-specific amplification is mixed with PCR products: Optimize the PCR reaction system to improve the amplification specificity; purify the PCR products with a gel recovery kit; select more clones for verification.

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 endonucleases used, prolong the time of digestion reaction, and recover and purify the digestion products by gel.

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 recovery and purification on the amplification product.

◇ Colony PCR without bands

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: No bands of target or empty plasmid. It is recommended to optimize the PCR reaction system or program; or extract plasmids as PCR templates or use enzyme digestion for confirmation.

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

Nanjing Vazyme Biotech Co.,Ltd.
Tel: +86 25-83772625
Email: info.biotech@vazyme.com
Web: www.vazyme.com
Loc: Red Maple Hi-tech Industry Park, Nanjing, PRC
Follow Us