ClonExpress II One Step Cloning Kit

NB-54-0002-01
NB-54-0002-02

Sommaire

ClonExpress II One Step Cloning Kit

#Cat: NB-54-0002-01 Size: 25rxns
#Cat: NB-54-0002-02 Size: 50rxns

01/Product Description

The ClonExpress technology is a 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 II is a new generation of recombinant cloning kit. It has a unique ligase-independent system, which significantly reduces the self-ligation background of the vector, and the positive rate can reach more than 95%. Highly optimized reaction buffer and enhanced recombinase Exnase II can significantly improve the recombination efficiency and the tolerance to impurities. These features make it possible to use linearized vectors and inserts directly for recombinant cloning without purification, which significantly simplifies the experimental steps.

02/Components

Components	NB-54-0002-01 (25 rxns)	NB-54-0002-02 (50 rxns)
5 x 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

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 or other equivalent products.

Competent cells: Chemically competent cells prepared by cloning strains;

DH5α Competent Cell for conventional cloning, applicable to plasmids <15 kb;

XL10 Competent Cell 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. The transformed volume of recombinant products should be ≤1/10 of the volume of competent cells.
2. The ClonExpress kit can efficiently clone 50 bp - 10 kb fragments.
3. Preparation of inserts and vectors

◇ Cloning short fragments (<5 kb)

▲ Linearized vectors, prepared by restriction endonucleases digestion, can be heated to inactivate the endonucleases (applicable to most endonucleasess, please refer to the endonucleases 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

<table>
<tr><th colspan="2">Method of Linearization</th><th>Template Type</th><th>Fast Protocol</th><th>Standard Protocol</th></tr>
<tr><td colspan="2">Digestion</td><td>Circular plasmid</td><td>Use directly after inactivating restriction endonucleases</td><td>Gel extraction</td></tr>
<tr><td rowspan="3">PCR Amplification</td><td rowspan="2">Specific Amplification</td><td>Circular plasmid</td><td>Use directly after Dpn I digestion (de grade the PCR template)</td><td>Gel extraction or gel extraction after Dpn I digestion</td></tr>
<tr><td>Pre-linearized plasmid, gDNA, cDNA</td><td>Use directly</td><td>Gel extraction</td></tr>
<tr><td>Nonspecific Amplification</td><td colspan="3">Gel extraction</td></tr>
</table>

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		

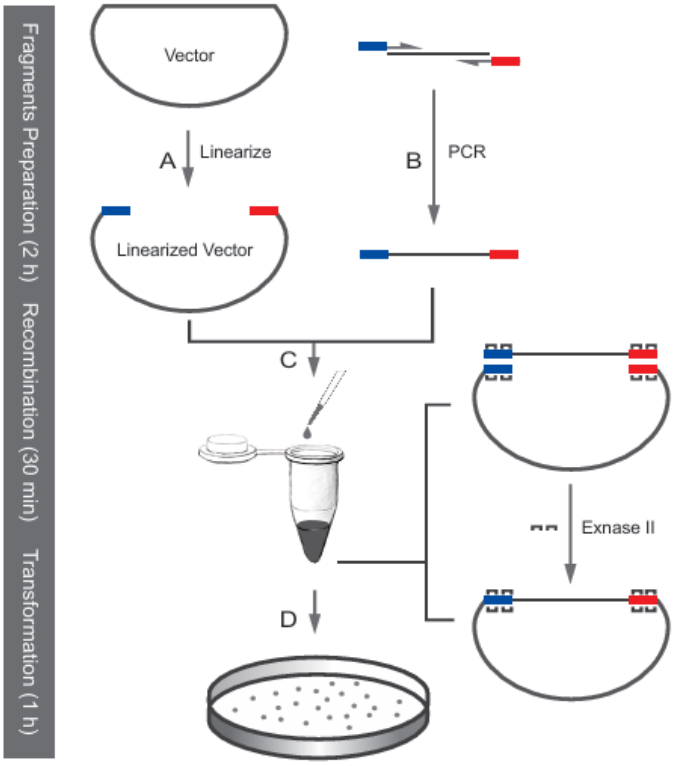
▲ When using enzyme cleavage products or amplification products directly for recombination, the volume should be ≤4 μl (1/5 of the total volume of recombination reaction system).

▲ 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 long 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: Introducing homologous sequences of linearized vector ends about 15 - 20 bp (marked in blue and red) into 5' ends 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. Incubate with Exnase II at 37℃ for 30 min to complete recombination reaction and realize the in vitro circularization of double 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 rapid cloning technology

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.

◇ 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) 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.

   Forward primer of insert fragments:

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

   Reverse primer of insert fragments:

   5'- homologous sequence of downstream vector end + restriction endonuclease 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 terminal sequence of the linearized vector (for homologous recombination). GC content of 40% - 60% is recommended.

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

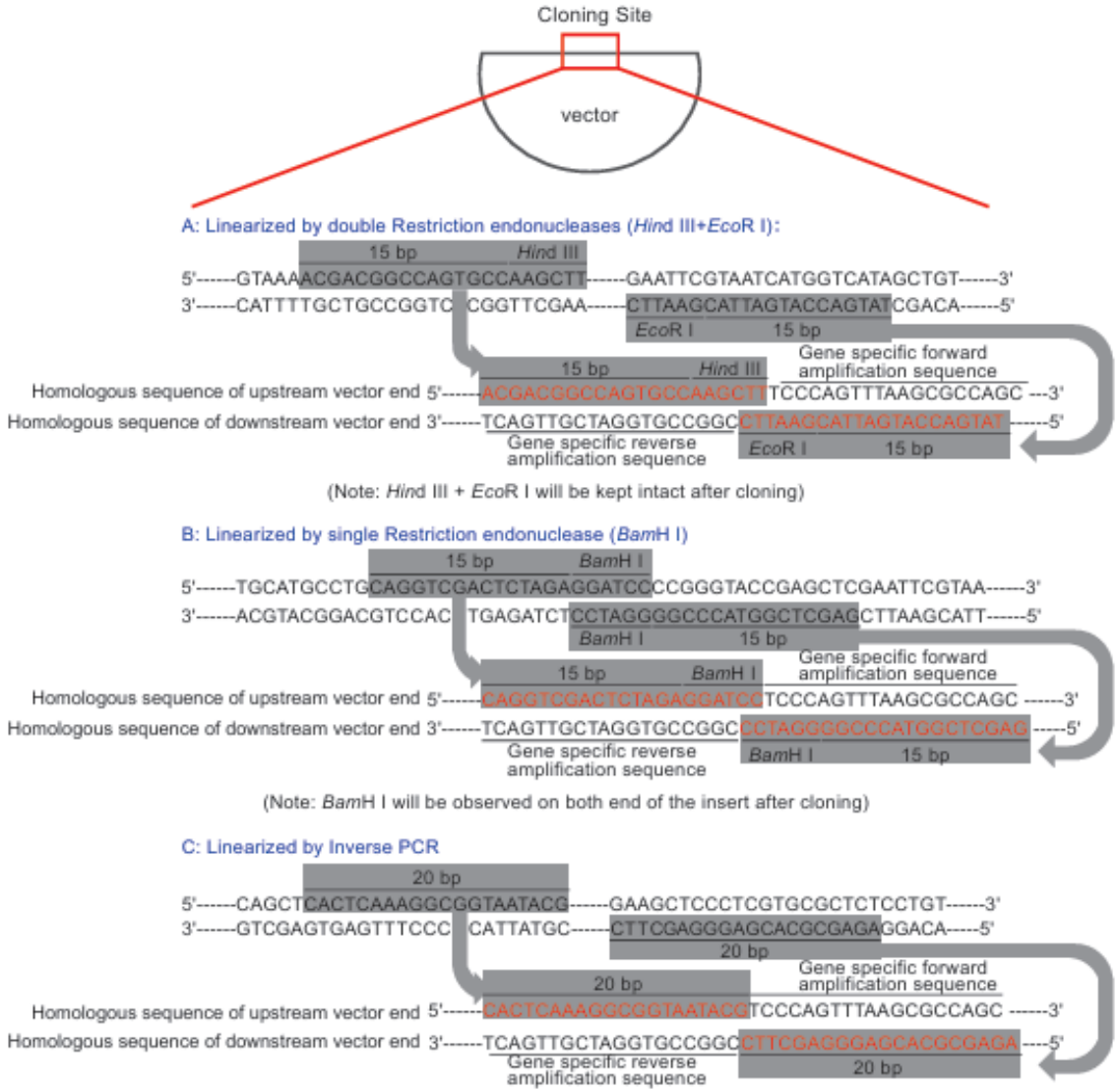


Fig 2. Primer Design for Single-fragment Homologous Recombination

▲ 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.

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 Polymeras) for amplification.

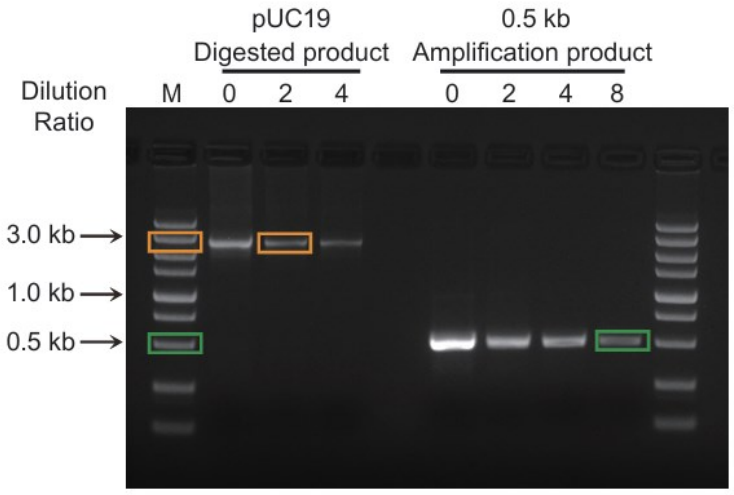
08-3/The Amount of Linearized Vectors and Inserts

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 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 bright ness 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.



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 orange box/green 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.

Fig 3. Concentration detection of linearized vectors and inserts by gel electrophoresis

2. The calculation of vectors and inserts input

   For ClonExpress II recombination reaction system, 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).

   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)

   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 into a vector of 5 kb, the optimal mass of vector is 0.02 × 5,000 =100 ng; the optimal mass of insert is 0.04 × 2,000 = 80 ng.

a. When the length of the insert is larger than that of the vector, the calculation method of the optimal mass of vector and inserts should be inverted. That is, the insert is regarded as a cloning vector, and the cloning vector is regarded as an insert for calculation.
b. The amount of linearized vectors should be between 50 - 200 ng; the amount of the insert should be between 10 - 200 ng. When the optimal amount calculated using the above formula is beyond these ranges, just choose the maximum or minimum amount for recombination.
c. When the linearized cloning vector and the amplification product of the insert 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 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/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.
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°C 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).
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°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 (2,400 × 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 4).

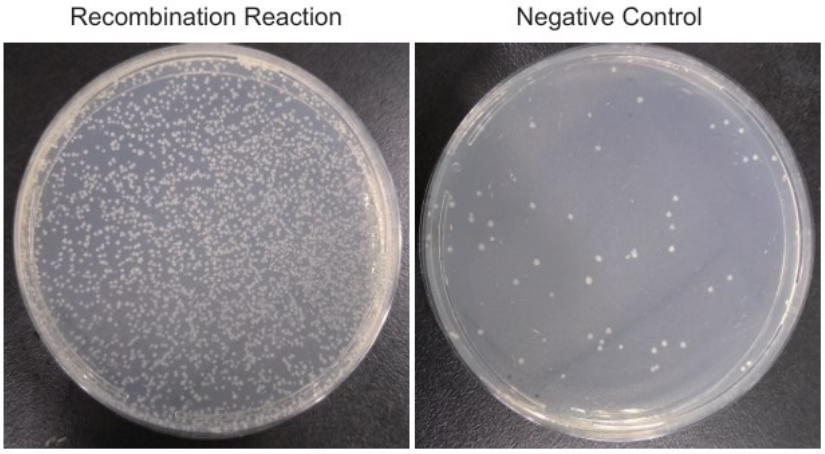


Fig 4. Plates Incubated Overnight

◇ 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 the insert should appear (Fig 5).

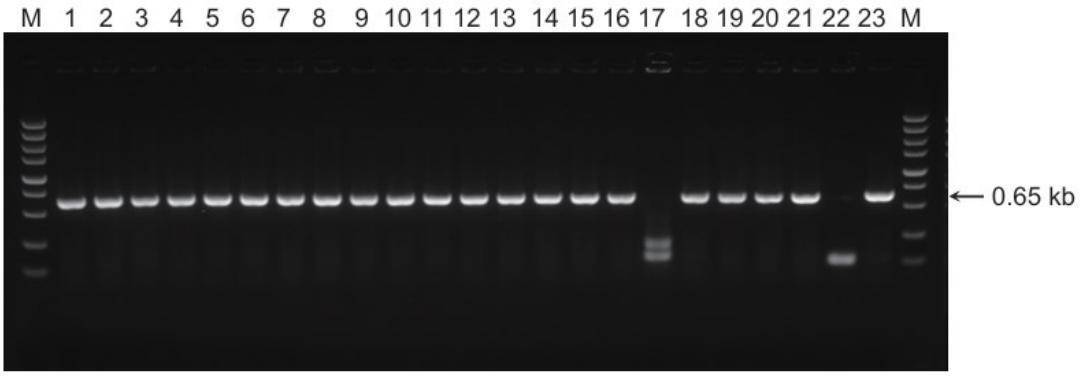


Fig 5. Agarose Gel Electrophoresis of Colony PCR Products

M: DL5000 Marker; 1 - 23: 21 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 6), or directly perform sequencing.

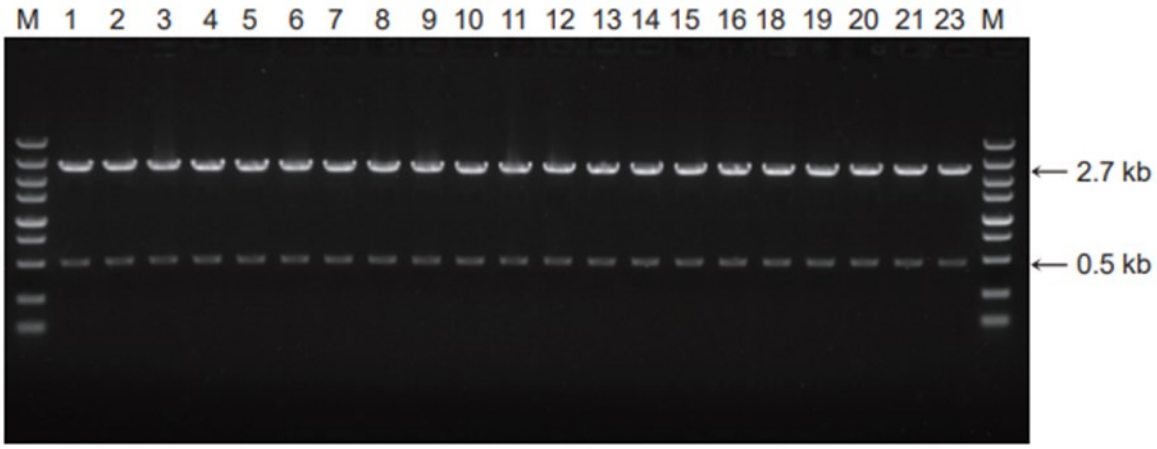


Figure 6. Agarose Gel Electrophoresis of Enzyme Digestion Products

M: DL5000 Marker; 1 - 23: 21 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 re combination is unsuccessful and the linearization of the vector is incomplete. It is recommended to optimize the enzyme digestion system.