ClonExpress® MultiS One Step Cloning Kit

NB-54-0003-01
NB-54-0003-02

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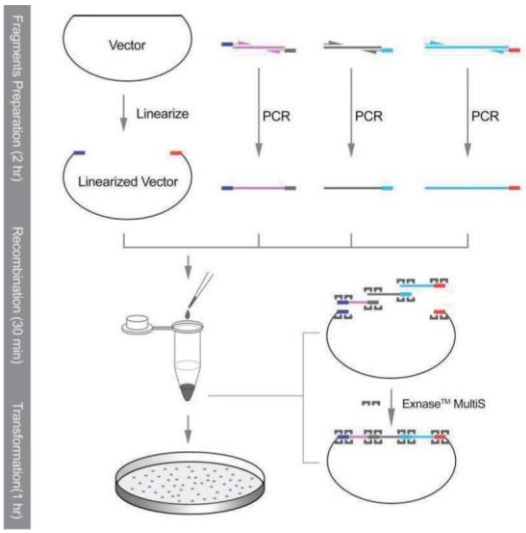
Cat# NB-54-0003-01

Introduction:

ClonExpress® MultiS One Step Cloning is a simple, fast, and high efficient cloning technology which is based on a homologous recombination technology. It 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 isadded onto the insert through PCR. The insert and the linearized vector, with over lapped sequences of 15 bp - 20 bp on both 5'- and 3' - end, respectively, are mixed and incubated with Exnase II at 37℃ for 30 min(Fig.1.). The cloning products can then be directly transformed to competent cells with apositive rate >95%.
ClonExpress® MultiS One Step Cloning Kit is a new version cloning kit based on ClonExpress One Step Cloning technology. Exnase MultiS and reaction buffer in this kit are especially optimized for the assembly of multiple fragments. With the help of this kit, up to five fragments can be orderly assembled in a single reaction. In addition, Exnase MultiS is compatible with the reaction systems of restriction enzyme digestion and PCR, which means that both the digestion products of vector and the PCR products of insert can be directly used for recombination without purification, significantly simplifying the procedures.

Advantages

- Easy, fast, and efficient.
- Directional cloning at any site on any vector.
- No need to consider the restriction enzyme cutting sites carried on inserts.
- Orderly assembly of up to five fragments simultaneously.
- Linearized vector and PCR products can be used directly without purification



Applications

- Multiple-fragment assembly
- Gene synthesis
- Site-specific mutagenesis

Components

Components	NB-54-0003-01 (10 rxn)	NB-54-0003-02 (25 rxn)
5 x MultiS Buffer	40 µl	100µl
Exnase MultiS	20 µl	50 µl
pUC19 control vector, linearized (50 nb/µl)	5µl	5 µl
Control Inser Mix	5µl	5 µl

(0.5 kb, 10 ng/µl ; 1 kb, 20 ng/µl ; 2 kb, 40 ng/µl)

Storage

All components can be stored at -20℃ for one year. Avoid repeated freezing and thawing.

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Protocol

1. Preparation of linearized cloning vectors

Select appropriate cloning sites on which the vector will be linearized. It is recommended to select cloning sites from regions with no repetitive sequence and even GC content. The maximum recombination efficiency can be achieved when the GC content is 40% - 60% both in the upstream and the downstream 20 bp regions flanking the cloning site.
The linearized vector can be obtained by digesting the circular vector with restriction enzymes (**1.A.**) or by reverse PCR (**1.B.**)

1.A. Linearizing vectors by restriction digestion

Double digestion: It is recommended to generate linearized vectors using double digestion due to its completeness of linearization and low false positive rate.

Single digestion: The linearization efficiency of single digestion is far lower than double digestion. A longer digestion time is helpful to reduce the false positive rate.

Note:There is no DNA ligase activity in the reaction system of ClonExpress MultiS, and no self-ligation of linearized vector will occur. Therefore, dephosphory- lation is unnecessary even when the linearized vectors are prepared by single digestion. The false positive clones (clones without inserts) are mainly from vectors that failed to be linearized. If the false positive rate is high,please redo the preparation of linearized vectors and try again.

Exnase MultiS is compatible with almost all reaction systems of digestion. Therefore, after inactivating the restriction enzymes (i.e.incu- bateat 65℃ for 20 min to inactivate Hind III), the linearized vectors can be directly used for recombination without purification.

1.B. Linearizing vectors by reverse PCR

It is highly recommended to use a high-fidelity DNA polymerase (i.e.Phanta Max Super-Fidelity DNA Polymerase, Vazyme, #P505) for vector amplification to reduce the amplification error rate. And it is also recommended to use pre-linearized plasmids as PCR templates to reduce the false positive rate in transformation caused by residual circular plasmids.
Exnase MultiS is compatible with almost all reaction systems of conventional PCR. Therefore, if the template is pre-linearized and the amplification is highly specific, the PCR products can be used directly for recombination without purification.
Low recombination efficiency and positive clone rate will be obtained if digestion or PCR products with low linearized vector concentration and with possible residual circular plasmids are used directly for recombination. Therefore,in case of large fragment (> 5 kb) cloning, it is recommended to purify the linearized vectors and amplified inserts with high quality gel recovery kit before recombination, so as to elevate the DNA purity and to eliminate residual circular vectors. Refer to **Table1** for the usages of linearized vectors 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		

Note: * In fast protocols, when using digesting products or PCR products directly for recombination, the volume should be ≤ 4μl (≤1/5 of the total volume of recombination reaction system).

2. Primer design for the inserts

The principle for the design of ClonExpress MultiS primers: introduce homologous sequences of linearized vector (15 bp-20 bp) into 5'-end of primers, aiming to making the ends of amplified inserts and linearized vectors identical to each other. An example of 3-fragment assem- bly (5' - 3': Insert 1#, Insert 2#, Insert 3#) is showed in **Fig.2**. Firstly, design the forward primer of insert 1# (the first insert) and the reverse primer of insert 3# (the last insert) (**Fig.2**.).

Forward primer of the first insert:

5'---homologous sequence of vector-upstream end+gene specific forward amplification sequence of insert---3'Reverse primer of the last insert:3'---gene specific reverse amplification sequence of insert + homologous sequence of vector - downstream end---5'

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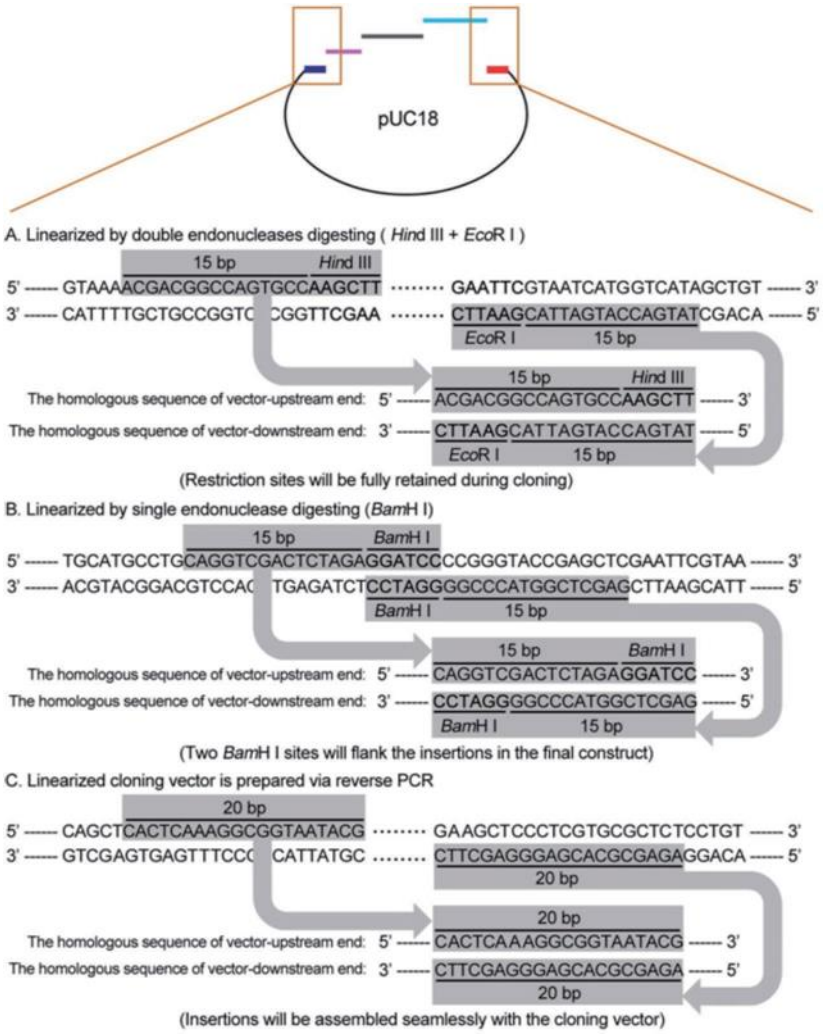
Note : Gene specific forward/reverse amplification sequence.

Homologous sequences of vector-upstream or-downstream end : the sequence at the end of the linearized vector(homologous reombination sites), which can be designed according to the following example:



Note: If the primer length 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 should be excluded and only gene specific amplification sequence should be used

Secondly, design the reverse primer of insert 1# (the first insert) and the forward primer of insert 2# (the second insert). Homologous sequence used for recombination between inserts can befully added to either the reverse primer of insert 1# or the forward primer of insert 2#, and also can be partially added to both of them.For example in Fig.3., the homologous sequence is added to the reverse primer of insert 1#.

Reverse primer of the first insert: 3'---gene specific reverse amplification sequence of insert 1# + homologous sequence of 5'-end of insert 2# ---5'Forward primer of the second insert: 5'---gene specific forward amplification sequence of insert 2#---3'

Note :Gene specific forward/reverse amplification sequence:the primer sequence to amlify the insert.

Homologous sequences of 5'-end of insert 2 is used for the recombination between insert 1 and insert 2, which can be designed according to the following example:

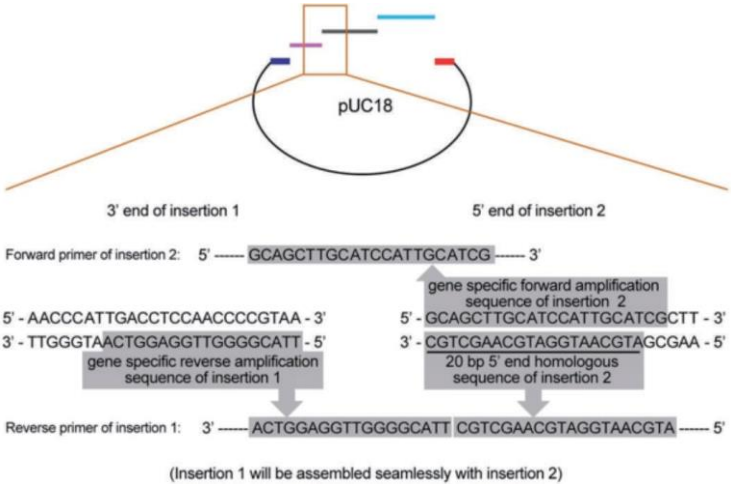


Fig. 3. Primer design for the first and second insert.

Note:If the primer length 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 should be excluded and only gene specific amplification sequence should be used.

Then, similarly, design the reverse primer of insert 2# and the forward primer of insert 3#.

3. PCR of the inserts

Inserts can be amplified by any polymerase (i.e. Taq DNA polymerase or high-fidelity polymerase). It will not interfere with the recombina- tion efficiency whether there is A-tail in the PCR products or not, which will be removed during recombination. To prevent possible mutations introduced during PCR, high-fidelity polymerases (i.e.Phanta Max Super-Fidelity DNA Polymerase, Vazyme, #P505) are highly recommended.

Confirm the yield and amplification specificity of the PCR products by agrose electrophoresis. ClonExpress MultiS is compatible with almost all reaction systems of conventional PCR. High specific PCR products can be directly used for recombination without further purification if the templates are not circular plasmids which share the same antibiotic resistance with the cloning vector.

Low recombination efficiency and positive clone rate will be obtained if PCR products with low insert concentration are used directly for recombination. Thus, in case of large fragment (>5 kb) cloning, it is recommended to purify the PCR products with high quality gel recovery kit before recombination. Amplified inserts can be used according to **Table2**.

Table2. Usages of Ampified 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		

Note: *In fast protocols, when using PCR products directly for recombination, the volume should be ≤ 4 μl(≤ 1/5 of the total volume of recombination reaction system).
**If linearized vectors are prepared by digesting circular plasmids, the amplified inserts should be incubated at 85℃ for 20 min after Dpn I digestion to deactivate Dpn I, so as to prevent linearized cloning vectors from degradation.

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4. Recombaination

Set up the following reaction on ice. Spin briefly to bring the sample to the bottom of the tube before reaction.

ddH_2O	Up to 20 µl
5× CE MultiS Buffer	4 µl
Linearized Vector	x ng
Amplified Insert	x ng
Exnase MultiS	2 µl

The optimal amount of each fragment (including vector) for recombination is 0.03 pmol. Their amount in molar can be roughly calculated according to the following formula:

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

For example, when orderly cloning inserts of 0.5 kb, 1 kb, and 2 kb to a vector of 5 kb, the optimal amounts are as follows: vector: 0.02 ×5000= 100 ng; insert (0.5 kb): 0.02 × 500=10 ng; insert (1 kb): 0.02 × 1000 = 20 ng; insert (2 kb): 0.02 × 2000=40 ng.

Note : 1. The amount of linearized vectors should be between 50 ng - 200 ng. When the optimal amount calculated using the above formula is beyond this range, just choose the maximum or minimum amount for recombination.
2. The amount of amplified inserts should be ≥ 10 ng. When the optimal amount calculated using the above formula is < 10 ng, just use 10 ng for recombination.
3. 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).

ClonExpress MultiS Cloning Kit provides pUC19 control vector(linearized, 5 µl, 50 ng/µl) and several control inserts (0.5 kb, 5 µl , 10 ng/µl ;1 kb, 5 µl , 20 ng/µl ; 2 kb, 5 µl , 40 ng/µl ;). Use 1 µl of each in one recombination reaction if positive control is needed.
After finishing setting up, gently pipette for several times to mix thoroughly and avoid bubbles. **DO NOT VOTEX!** Incubate at 37℃ for 30 min and immediately place the tube on ice for 5 min. The recombination products is now ready for transformation or storage at -20℃for future use.

Note : A PCR thermo-cyclers or water bath are recommended for the reaction. The recombination efficiency can reach its peak at 30 min. Longer or shorterreaction time will decrease on the cloning efficiency.

5. Transformation

Pipet 20 µl of the recombination products to 200 µl of competent cells, flip the tube for several times to mix thoroughly, and then place the tube on ice for 30 min. Heat-shock the tu be at 42℃ for 45 sec - 90 sec and then immediately place the tube on ice for 2 min. Add 900 µl of SOC or LB medium and incubateat 37℃ for 10 min to fully recover the competent cells. Then, shake the tube at 37℃ for 45 min. Pipet 100 µl of culture and plate evenly on a agar plate which contains appropriate selection antibiotic. Place the plate at 37℃ and incubate over night.

Note:Competent cells with transformation efficiency over 108 cfu/µg are highly recommended. If using competent cells with lower efficiency (i.e. competent cells prepared using CaCl2 have a efficiency of only 106 - 107 cfu/µg),before plating,it is necessary to centrifuge the cultureat 5,000 rpm for 3 min to collect the bacteria. Then, re-suspend the pellet with 100 µl of LB medium and plate.

6. Selection of positive colonies

Colony PCR is the most convenient selection method. Pick a single colony with tips to 20 µl - 50 µl of LB medium, mix thoroughly and use 1 µl as PCR template. To eliminate false positive PCR results, it is recommended to use at least one sequencing primer of the vector. Inoculate the remaining medium of positive clones into fresh LB medium and culture overnight. Then, extract the plasmids for further verification.

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Tips

Procedures	Dos	Don'ts
Selection of cloning site	Avoid GC-rich/poor regions or regions with repeated sequence. Recombination efficiency are maximum when GC content is within 40% - 60% both in the upstream and the downstream 20 bp regions flanking the cloning site.	Sequence flanking the cloning site is GC - rich / poor, or with repeated sequence.
Design of PCR primers	Design according to Fig.2. and Fig.3.	The recombination sequence is insufficient or improper.
Linearization of vector	Completely linearized.	Incompletely linearized and a trace of circular plasmids.
PCR of inserts	Highly-specific amplification.	Non-specific amplification.
Purification of the linearized vector and amplified inserts	PCR products should be purified by gel recovery if inserts are over 5 kb, circular plasmids are taken as PCR templates, or non-specific amplification exits.	PCR products are used directly without purification if inserts are over 5 kb, circular plasmids are taken as templates, or non-specific amplification exits.
Determination of DNA concentration	Determined by agarose electrophoresis.	Determined by absorbance.
Setting-up of the recombination reaction	Set up on ice with appropriate DNA amount and ratio.	Set up the reaction at room temperature; Use DNA freely without determination of its concentration.
Recombination temperature	Incubate at 37℃ for 30 min in a PCR thermo-cyclers or water bath.	The temperature is above or beneath 37℃; the time is longer or shorter than30 min.
Termination of recombination	Immediately place the reaction tube on ice for 5 min after reaction.	Place the reaction tube at room temperature after reaction.
Transformation	Transformation should be carried out within 1 hr after termination of the recombination and the reaction tube should stay on ice before transformation. Otherwise, store the recombination products at -20℃.	The reaction tube is left at either room temperature or 4℃ for a long time before transformation.
Colony PCR	Use at least one sequencing primer of the vector.	Use a pair of gene specific primers.

Trouble Shooting

- Few clones or no clone formed on the agar plate

1. The efficiency of the competent cell is very low: Make sure the transformation efficiency of competent cells is >107 cfu/μg.Set a group of plasmid transformation as control to detect the transformation efficiency of competent cells.
2. The amount of DNA is too low/high in the recombination reaction or the ratio of fragments is not appropriate. Please use the amount of DNA as 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 recommendedto purify the vector and insert. Avoid metal chelating agent (i.e.EDTA) in the recombination reaction. It is recommended to dissolve the purified DNA in ddH2O of pH8.0. Do not keep the DNA in the TE buffer.
4. Add too much DNA in the competent cell: DNA volume should be ≤1/10 volume of competent cells.
5. The transformation inhibitory effect occurs: High concentration of DNA inhibits the transformation.In this case, dilution of DNA (to 1/5) is suggested for transformation.

- The colony plasmids contain no insertion

1. Incomplete linearization of the vector : Even a trace amount of residual circular plasmids are sufficient to bring high false positive rate. Several approaches can be used to improve the digesting efficiency including elevating the amount of restriction endonuclease, prolonging the digesting time,and purifying the digesting products before the recombination reaction.
2. Plasmids with the same resistance mixed in reaction system: The PCR template for amplification of vectors or inserts is circular plasmid. When the amplification product is directly used for the recombination,the residual cyclic plasmid template will bring high false positive rate. Measures such as using a pre-linearized plasmid as the amplification template,digesting the amplification product with Dpn I, and gel recovery can effectively reduce or even eliminate the residue of cyclic plasmid template.

- Incorrect insert found in the colony plasmids

1. Non-specific amplification is mixed with target inserts: Optimize the PCR reaction system to elevate the amplification specificity. Purify the PCR products with a gel recovery kit. Select more colonies for verification.
2. Incomplete linearization of the vector: If the linearized vector is prepared by either PCR or restriction enzyme digestion from circular plasmids which already carry other inserts, a trace amount of residual template plasmids or incomplete digestion might result in high background with partial colonies containing the original plasmids rather than recombinants. Approaches to overcome such situation include evaluating the efficiency of restriction enzyme digestion, taking pre-linearized plasmids as PCR templates and purifying the DNA before recombination.

- Caution!

Selection of cloning cites should avoid regions with repetitive sequences flanking the cloning site (50 bp upstream and downstream of the cloning cites should be examined). GC content of 20 bp regions at both ends of linearized cloning vector has great impact on the recombi- nation efficiency. The maximum recombination efficiency can be realized when the GC content of these regions is within 40% - 60%. The recombination efficiency will be greatly reduced if the GC content of this region exceeds the range of 30% - 70%.

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