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Seamless DNA Assembly Plus Kit

1 Contents

Components	HY-K1041-50 rxns
Seamless Assembly Mix Plus	250 μL
pUC19 Control Plasmid, Linearized (Amp^r, 40 ng/μL)	5 μL
500 bp Control Fragment (20 ng/μL)	5 μL

2 Introduction

Based on gene recombination, seamless cloning is a simple, efficient, and fast DNA cloning technology. A 15-20 bp sequence homologous to the end of the linearized vector is added to the 5′ end of the insert fragment primers. In the presence of enzymes, recombination can be easily achieved.

MCE Seamless DNA Assembly Plus Kit contains an optimized mix of recombinase, reaction buffer, and additional cofactors that significantly improve the cloning efficiency and tolerance to impurities. This product can complete multiple DNA fragments recombination and takes only 5 minutes for single fragment, and the positive rate is more than 95%.

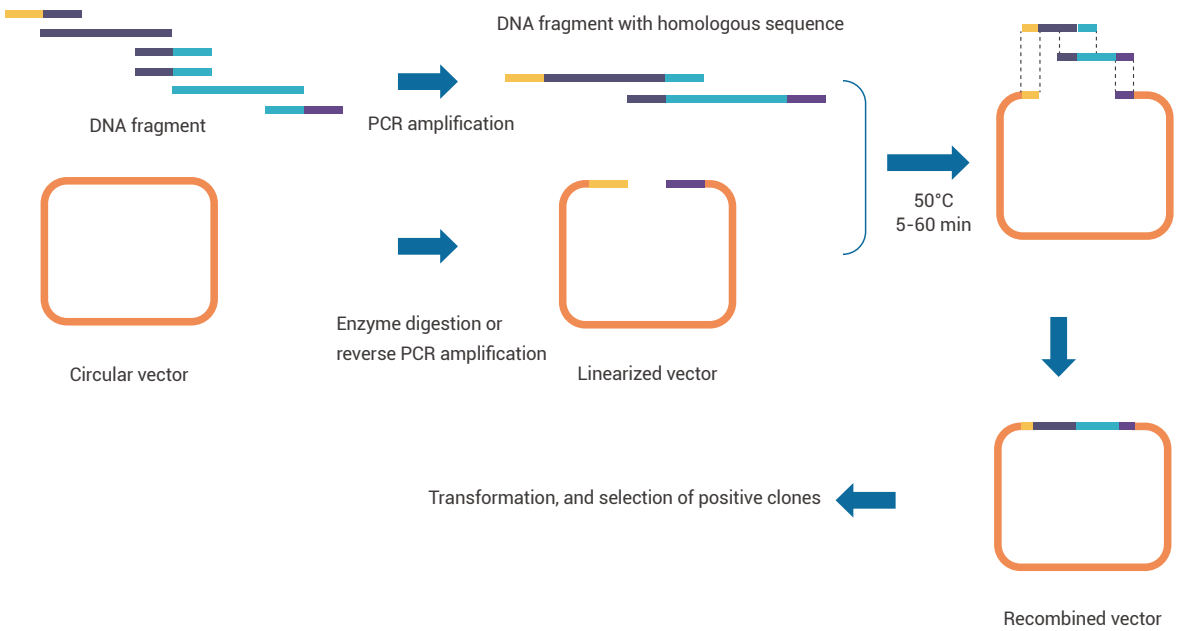


Figure 1 Seamless Cloning

3 General Protocol

1. Prepare the linearized cloning vector

Seamless cloning relies on homologous recombination, and the cloning vector must be linear. You can prepare the linearized vector using restriction enzymes (single or double digest) or using PCR amplification.

a. Enzyme digestion

When generating the linearized vector by restriction digest, we recommend that you digest the vector with two restriction enzymes rather than one single enzyme to reduce the amount of background. If single digestion is necessary, it is recommended to prolong the reaction time appropriately. Use agarose gel electrophoresis to verify that the digestion is complete, and then gel purify the linearized vector.

b. Reverse PCR amplification

It is recommended to use MCE 2× High-Fidelity PCR Master Mix (Cat. No.: HY-K0533) and use pre-linearized plasmid as a template for amplification.

2. Prepare the DNA fragment

a. Guidelines for designing PCR primers

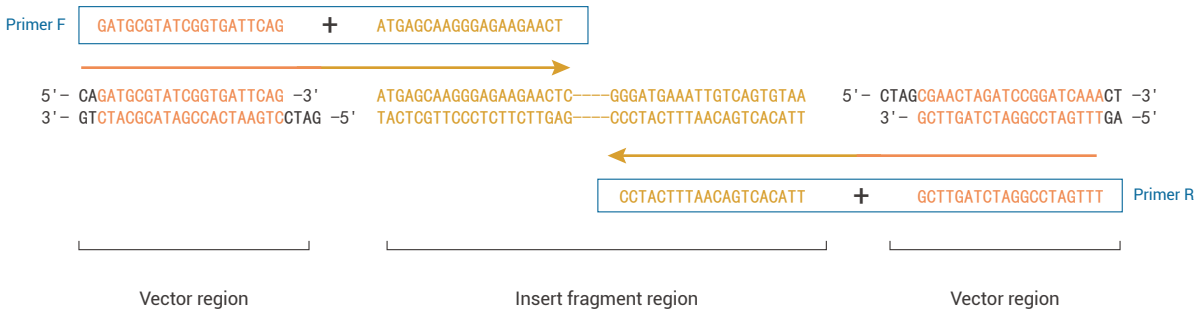
Seamless Cloning reaction requires that each DNA fragment share a 15-20 bp end-terminal homology with the adjacent fragment (including the cloning vector). Therefore, the 5′ ends of each primer pair (forward and reverse) must contain a 15- nucleotide overhang to provide this homology. The length of the primer should not exceed 40 bp (15 nucleotides to provide the requisite homology at the 5′ end and 18-25 nucleotides specific to your DNA fragment), Tm value is generally 55-65°C, and GC content is generally 40-60%.

Forward Primer (F):

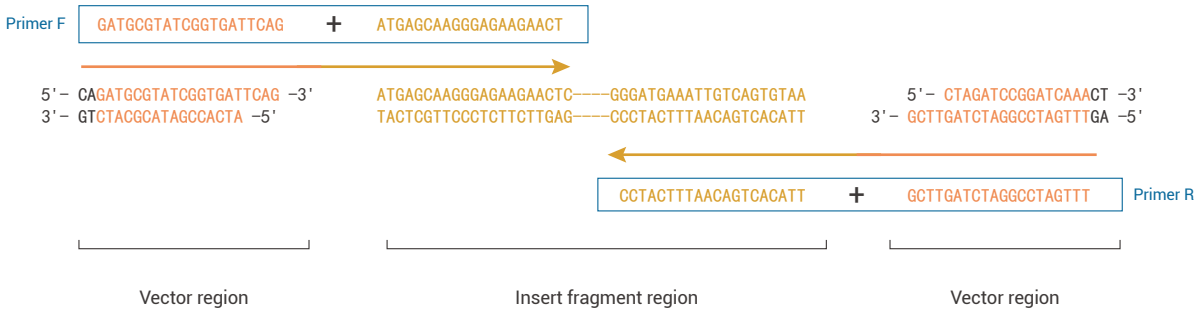
5′- upstream vector terminal homologous sequence + restriction site (optional) + gene specific forward sequence -3′

Reverse Primer (R):

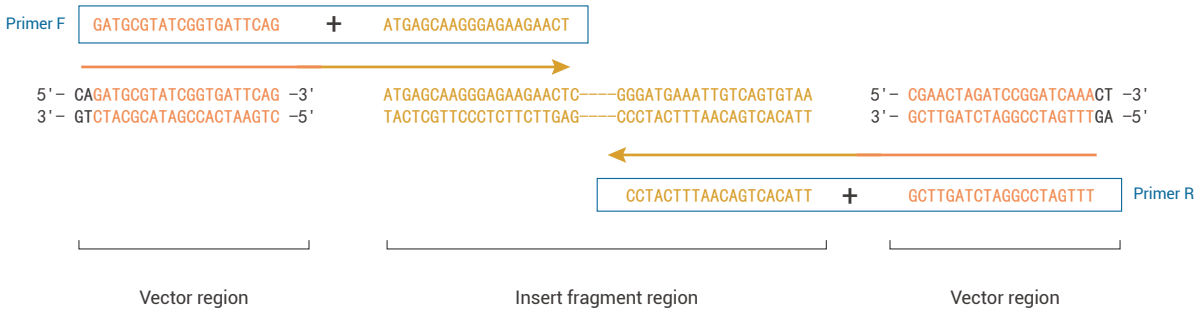
3′- gene specific reverse sequence + restriction site (optional) + downstream vector terminal homologous sequence -5′



a. Linearized vectors with 5' overhangs



b. Linearized vectors with 3' overhangs



C. Linearized vectors with blunt end

Figure 2 Primer design diagram

b. PCR amplification of the DNA fragment

We recommend MCE 2× High-Fidelity PCR Master Mix (Cat. No.: HY-K0533) for amplification. Afterward, the PCR products should be purified for subsequent seamless cloning reactions.

3. Seamless cloning reaction

a. Set up the following reaction on ice:

Components	Experimental group	Negative control	Positive control
Seamless Assembly Mix Plus	5 μL	5 μL	5 μL
Linearized vector	50-200 ng	50-100 ng	pUC19 Control Plasmid, Linearized, 1 μL
DNA fragment	10-200 ng	/	500 bp Control Fragment, 1 μL
ddH_2O	To 10 μL	To 10 μL	To 10 μL

For cloning a single fragment, we recommend adding 0.03 pmol of vector and 0.06 pmol of DNA fragment in an assembly reaction, that is to say, the optimal molar ratio of DNA fragment to vector is 2:1.

For cloning multiple fragments, the optimal molar ratio of each DNA fragment to vector is 1:1.

$$\text{Molar Ratio} = \frac{\text{Vector concentration (ng/μL)} \times \text{Vector volume (μL)} \times \text{Gene (bp)}}{\text{Gene concentration (ng/μL)} \times \text{Gene volume (μL)} \times \text{Vector (bp)}}$$

Note: 1) If the length of DNA is greater than the vector, the amount of the vector and DNA should be exchanged.

2) The amount of linearized vector should be between 50-200 ng, and the amount of DNA should be between 10-200 ng. If the usage calculated is beyond this range, it is recommended to use the minimum/maximum usage directly.

3) When the DNA fragment is < 200 bp, we recommend the molar ratio of DNA fragment to vector is 5:1.

4) When using unpurified vector or DNA, the volume should not exceed 20% of the total volume.

b. Mix the reactions gently, spin down and incubate at 50°C for 5-60 minutes.

Note: 1) It is recommended to use thermal cyclers.

2) The recommended reaction is 5-15 minutes for insertion of 1-2 fragments and 15-30 minutes for 3-5 fragments.

3) If the cloning vector is >10 kb or the DNA fragment is > 4kb, it is recommended to prolong the reaction time to 30-60 minutes.

c. After incubation, place the reaction mixture on ice and immediately proceed to the transformation step.

Note: The reaction mixture can be stored at -20°C for one week.

4. Transformation

a. Thaw 100 μL competent cells on ice.

b. Add 10 μL of the reaction mixture into 100 μL competent cells and mix gently. Do not mix by pipetting up and down or vortexing. Incubate the complex on ice for 30 minutes.

c. Heat-shock the reaction mixture for 45-60 seconds at 42°C without shaking. Immediately transfer the tube on ice and incubate for 5 minutes.

d. Add 500 μL LB or SOC medium (without antibiotics) to the reaction mixture and shake at 180 rpm at 37°C for 1 hour.

e. Concentrate the bacterial solution by centrifugation and spread 100 μL on a selective plate (with antibiotics). Incubate overnight at 37°C.

Note: 1) It is recommended to use competent cells with higher transformation efficiency.

2) Positive controls usually show a large number of colonies, while negative controls have almost no colonies.

5. Identification of positive clones

Pick several colonies and screen for positive clones containing your DNA fragment by isolating the plasmid DNA and performing enzyme digestion or by colony PCR.

Note: It is recommended to perform sequence analysis to rule out any errors.

a. Enzyme digestion

Inoculate several colonies in LB medium containing antibiotics, isolate plasmid DNA and perform enzyme digestion.

b. Colony PCR

Inoculate several colonies in 10 μL ddH_2O and lyse at 95°C for 10 minutes. Take 1 μL of lysate as a template for colony PCR. Use at least one universal primer for colony PCR to avoid false positives.

4 Storage condition

Store at -20°C for 2 years.

Avoid repeated freeze-thaw cycles.

5 Precautions

1. This product is for R&D use only, not for drug, household, or other uses.
2. For your safety and health, please wear a lab coat and disposable gloves to operate.

6 Troubleshooting

Problem	Possible cause	Solution
Low transformation efficiency	Low efficiency of competent cells.	Use freshly prepared competent cells. Thaw competent cells on ice.
	Incorrect amounts of DNA fragments or vector were used.	Prepare the reaction system according to the instructions.
	DNA fragments or vector were not pure.	Gel purify the DNA fragments and vector. Dissolve the purified product in ddH_2O. Do not use buffers such as Tris-EDTA.
Large numbers of the transformants contain no insert.	Cloning vector was incompletely linearized.	Increase the amount of endonuclease. Prolong the digestion reaction time. Gel purify the linearized vector.
	Insufficient antibiotic resistance.	Use correct antibiotics. Use freshly prepared plates containing the selection antibiotic.
Large numbers of the transformants contain incorrect insert.	PCR products were not pure enough.	Use MCE 2× High-Fidelity PCR Master Mix. Gel purify the PCR product. Screen for more clones.