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Peachtree Corners, GA 30092
www.RayBiotech.com | info@raybiotech.com
770-729-2992 | 1-888-494-8555
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RayBio® Seamless Gene Cloning Kit

Catalog #: 230-K6004

User Manual
Last Revised: May 28, 2024

Introduction

The Raybio® Seamless Gene Cloning Kit is designed for fast and direct cloning of one or multiple DNA fragments into any linearized vector without introducing any extra amino acid sequences (seamless cloning) using homologous recombination (Figure 1). The procedure includes the following steps:

1. Linearize the vector through restriction enzyme digestion or reverse PCR.
2. Introduce 15-25 nt homologous sequences to the 5’ ends of PCR products using PCR primers.
3. Mix the PCR products and vector in a specific ratio, then add 2× Raybio Assembly Premix. Incubate at 37℃ for 15-25 minutes.
4. Transfer the reaction mixture into competent bacterial cells. Screen the positive colonies by PCR or DNA sequencing.

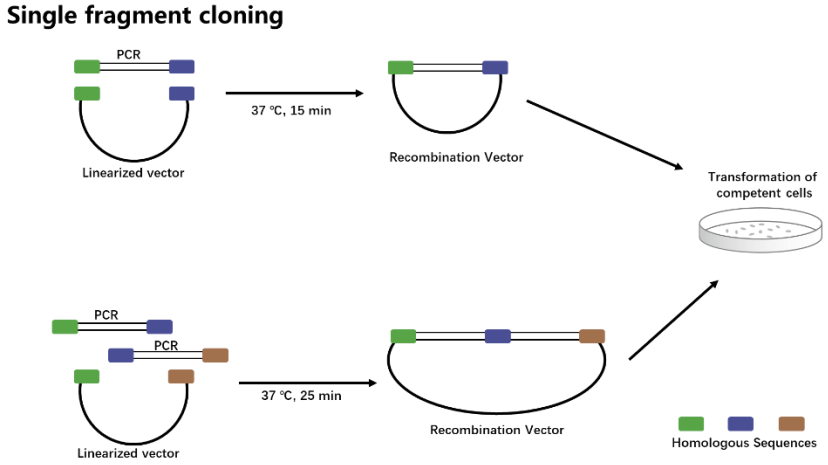

Figure 1. Schematic Diagram of the Raybio® Seamless Gene Cloning Kit Workflow

The schematic diagram illustrates the operation of the Raybio® Seamless Gene Cloning Kit. The cloning vector is first linearized by restriction enzyme digestion. PCR primers are designed to include overlapping sequences at the 5' ends, covering both the overlapping and gene-specific sequences. The 2× Raybio Assembly Premix directs precise homologous recombination. This kit allows for the quick cloning of single DNA fragments (top panel) as well as multiple DNA fragments (bottom panel) into the linearized vector.

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This product is suitable for:

- Seamless and rapid gene cloning
- Site-directed DNA mutagenesis
- Cloning of multiple DNA fragments
- High-throughput gene cloning

Storage / Stability

The product is shipped in ice packs. The entire kit may be stored at -20°C. Avoid repeated freeze-thaw cycles.

Kit Components

Name	Volume
2X Raybio® Assembly Premix	125 µL
Positive control vector, linearized pUC19 (2.6 kb, 20 ng/µL)	5 µL
Positive control PCR fragment (0.5 kb, 20 ng/µL)	5 µL
Sterile DNase and RNase Water	200 µL

Additional Materials Required

- PCR Primers
- Commercial PCR Gel Purification Kits
- Linearized Vector
- High-Fidelity Taq DNA Polymerase
- High-Efficiency Competent Bacterial Cells

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Assay Procedure

1. Preparation of linearized vector

The linearized vector can be generated using restriction enzymes or reverse PCR.

1.1 Restriction Enzyme Digestion: Use one or two restriction enzymes to linearize the vector. Ensure that the enzymatic digestion is complete to avoid high background in bacterial transformation.

1.2 Reverse PCR: Use Fidelity Taq polymerase to avoid any mutations during PCR amplification. To prevent false positive colonies caused by residual cyclic vectors, treat the amplified linearized vectors with Dpn I enzyme digestion.

1.3 Gel Purification: After restriction enzyme digestion or reverse PCR, purify the linearized vector using agarose gel electrophoresis and commercial gel extraction kits.

1.4 DNA Concentration Measurement: Measure the DNA concentration using a Nanodrop or other suitable methods.

Note:

- Homologous recombination does not require T4 DNA ligase; the vector will not self-ligate, thus preventing false transformants.
- Even with single enzyme digestion, dephosphorylation treatment of the linearized vector is unnecessary.
- Always check for background caused by non-linearized vector. Transform competent cells with 5-10 ng of the purified linearized vector without adding the purified PCR fragments. If the background is high, increase the restriction enzyme digestion time to 2 hours or even overnight.

2. Preparation of PCR fragment

2.1 PCR primer design

PCR primers used in this kit consist of two parts: the overlapping regions and the gene-specific regions. Among all primers, the 5’ ends contain 16-25 nucleotides (nt) of homologous sequence to the vector or DNA fragments, while the 3’ ends contain 15-25 nt of gene-specific sequence (refer to Figures 2 & 3 below). For example, the primer pair for cloning a single fragment is shown below:

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Forward primer (5'-3'):

Vector homologous sequence (16-25 nt), including the optional restriction enzyme site, + Gene-specific sequence (18-25 nt)

Reverse primers (5'-3'):

Vector homologous sequence (16-25 nt), including the optional restriction enzyme site, + Gene-specific sequence (18-25 nt).

The homologous region typically consists of more than 16 nucleotides (nt) with a melting temperature (Tm) value between 60-65 °C (with 40%-60% GC content, where A/T pairs contribute 2 ℃ and G/C pairs contribute 4 ℃ to the Tm value). It's advisable to maintain consistent Tm values across all overlapping regions. If not possible, extend the nucleotide length to meet these requirements. Additionally, the last 5 nucleotides at the 3' end of each primer should contain no more than two guanines (G) or cytosines (C) to avoid hairpin structures or primer-dimer formations. When calculating primer Tm values, exclude sequences from overlapping regions.

In Figure 2, an example demonstrates the design of PCR primers for cloning a single fragment. Both the F1 forward primer (5'-GAAGGAGATATACCCATATG/CTGGTCGTCACACCC-3') and the R1 reverse primer (5'-TGGTGGTGGTGGTGGTGCTCGAG/CAGGAAGCTATCCTC-3') feature homologous sequences with the vector (marked in red) to facilitate the recombination reaction.

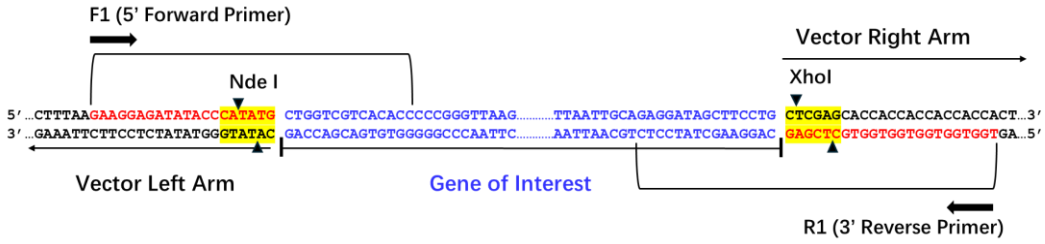

Figure 2. The schematic diagram shows PCR primer design for single fragment cloning.

F1 forward primer includes left arm vector part (20 nt, red) and 5' end of target gene sequence (15 nt, blue). R1 reverse primer includes 3' end of target gene sequence (15 nt, blue) and right arm vector part (23 nt, red). Two restriction enzymes (NdeI, XhoI) used to linearize the vector. PCR products don't need any restriction enzyme digestion.

Figure 3 illustrates an example of PCR primer design for multiple fragment recombinant cloning. In addition to the F1/R1 primers mentioned previously (Figure 1), the F2 forward primer (5'-ACTCCTTGCCCTTTAAGGTG/AGCTTTGGCAGAAGAAG-3') and R2 reverse primer (5'-CACCTTAAAGGGCAAGGAGT/GTGGCACCACGATAT-3') have

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been designed to include overlapping sequences for homologous recombination. Similarly, F3/R3 primers, F4/R4 primers, etc., can be generated accordingly.

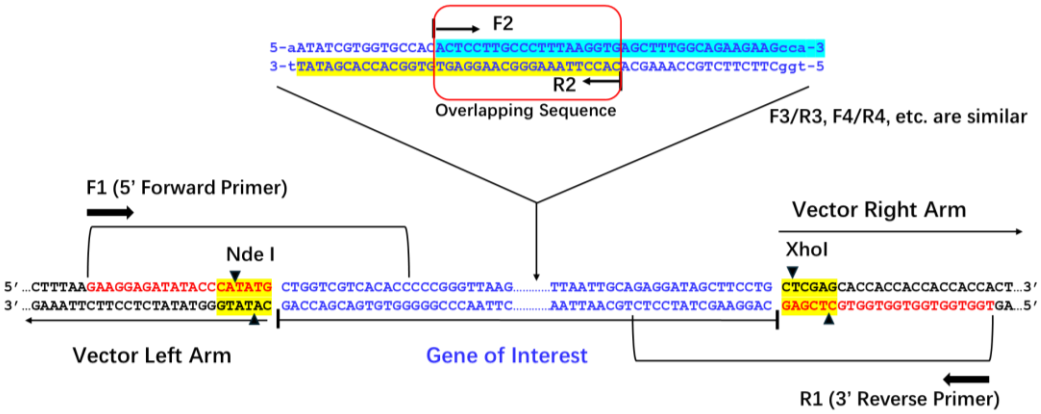

Figure 3. PCR primer design for multiple fragment cloning.

The F1 forward primer and R1 reverse primer remain the same as in Figure 2. The F2 forward primer includes a 20 nt overlapping sequence at the 5' end and a 17 nt sequence at the 3' end. Likewise, the R2 reverse primer comprises a 20 nt overlapping sequence at the 5' end and a 15 nt sequence at the 3' end. Similarly, F3/R3 primers, F4/R4 primers, etc., are generated accordingly. The red box highlights the overlapping sequence.

2.2 PCR amplification of target fragments

We recommend using high-fidelity Taq DNA polymerase for PCR amplification to minimize mutations. Please consult the product instructions for the specific DNA polymerase used for amplification. Following PCR amplification, purify the PCR products using a commercial agarose gel extraction kit. Measure the DNA concentration using a Nanodrop or other suitable methods.

3. Setting up the enzymatic reaction

3.1 Calculation of linearized vector and PCR fragment usage:

Typically, the optimal cloning efficiency is attained by using 50 ng of vector and 200 ng of insert, irrespective of their lengths. In a 10 µl reaction system, it is advisable to add a total amount of vector and PCR fragments ranging between 0.01 and 0.3 pmol. The recommended molar ratio between PCR fragments and the linearized vector is between 2:1 and 10:1. When conducting a cloning reaction involving more than two fragments (including the linearized vector), the molar ratio between inserts should be 1:1, while the ratio of insert to vector should always be 2:1. In summary, the molar ratio of two inserts to vector should be 2:2:1.

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Note: The recommended amount of cloning vector for the reaction is 0.03 pmol, and for insertion fragments, it is 0.06 pmol. The pmol quantities for PCR fragments and vectors can be calculated using the following formula:

pmols = (Weight, ng) / [(Length, nt) × 0.65 kDa]

For instance, in an enzyme reaction setup as follows, the molar ratio of PCR fragment to the linearized vector was approximately 5:1 in pmols.

- 20 ng of a 2,600-bp linearized vector corresponds to approximately 0.012 pmols
- 20 ng of a 500-bp PCR fragment corresponds to approximately 0.062 pmols

3.2 Performing Sample Reaction: The table below describes how to set up the reactions, including the controls if needed.

Reagent	**Cloning Sample (µL)**	**Positive Control (µL)**	**Negative Control (µL)**
2X Raybio® Assembly Premix	5	5	5
Linearized vector	X	-	X
PCR fragment 1 (P1)	Y1	-	-
PCR fragment 1 (P2)…	Y2…	-	-
Positive control vector (2.6 kb, 20 ng/µL)	-	1	-
Positive control template (0.5 kb, 20 ng/µL)	-	1	-
Sterile water	5-X-Y1-Y2…	3	5-X

Note: *If the final reaction volume exceeds 10 µl, double the amount of 2× Raybio Assembly Premix and add ddH2O to bring the final volume to 20 µl.*

Mix the reaction gently and spin it down. Incubate the reaction at a 37 ℃ water bath for 10-25 minutes. After the incubation time is complete, place the tubes on ice. The reaction products can be directly used to transform bacterial competent cells or stored at -20 ℃ for future use.

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4. **Bacterial transformation**

4.1 Take 100 µL of high-efficiency competent cells and thaw on ice. Add the reaction from *Step 3.2*, and gently mix. Incubate on ice for 25 minutes.

4.2 Heat shock in a 42 ℃ water bath for 45 seconds without shaking (shaking will reduce transformation efficiency dramatically). Immediately transfer the tubes to ice and let them stand for 2 minutes.

4.3 Add SOC or LB medium prewarmed to 37 °C to bring the final volume to 500 µl. Incubate at 37°C for 1 hour with shaking (160-225 rpm).

4.4 Centrifuge the transformation reaction at 3,000 rpm for 1 minute. Discard the supernatant and resuspend each pellet in 100 µl fresh LB medium. Spread each sample on LB agar plates containing the appropriate antibiotics. Plate the positive control (pUC19) sample on LB agar plate with 50 µg/ml ampicillin. Incubate LB agar plates overnight at 37°C.

4.5 Analyze the transformant colonies by colony PCR, plasmid restriction enzyme digestion, and DNA sequencing.

Typical Data

Below are the typical results of one and three fragment cloning into a 4.5 kb vector using this kit. The target gene was amplified: 1) in full length (~800 bp), 2) split into 3 fragments (150, 300, 350 bp). The recombinant reaction products generated using this kit were transformed into bacteria DH5α high-efficiency competent cells. For one fragment cloning, about 200 colonies appeared on the LB selection plate (Figure 4A). For three fragment cloning, about 50 colonies appeared on the plate (Figure 4B). No colonies grew on the negative control plate (Figure 4C). Seven colonies were randomly selected from Plates A and B, PCR colony screening using F1 forward primer from the vector and R1 reverse primer from the target gene showed that all colonies were positive (Figure 4 D & E). Further DNA sequencing confirmed all constructs were correct (data not shown).

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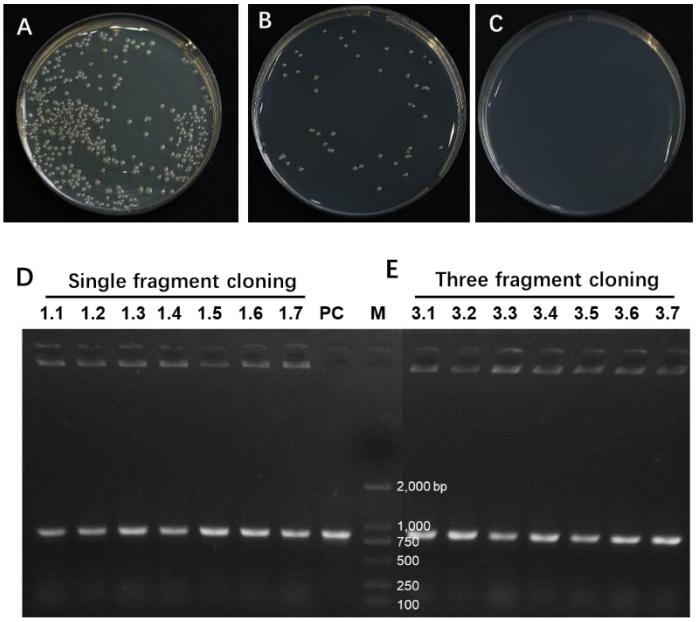

Figure 4. Bacterial competent cell transformation and PCR colony screening of transformants.

The kit assembled reaction was transformed into bacteria and selected on an LB agar plate with ampicillin antibiotic. A: one fragment cloning; B: 3 fragment cloning; C: negative control without PCR fragment. Seven colonies from plate A and plate B were randomly selected for colony PCR screening using the F1 forward primer from the vector and R1 reverse primer from the target gene for the full-length amplification (~800 bp). Both single fragment cloning (D) and 3 fragment cloning (E) showed all colonies were positive. PC: positive control, using the separate full-length cDNA plasmid as the PCR template.

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Troubleshooting Guide

Problem	Solution
No or few bacterial colonies in transformation plates	• Check the competent cell efficiency, it should be >1×10^8 cfu/μg plasmid. Otherwise, use freshly prepared competent cells. • Make sure the regions of homology were long enough, ranging from 15-20 nt. • Check vector concentration, we recommend using 50-200 ng vector, depending on its size. • Use the appropriate molar ratio of the vector and fragment (see section “Procedures”).
Clones contained incorrect insert.	• If your PCR product was not a single distinct band, it needs gel purification to isolate the correct size of the PCR product.
Most clones had no inserts.	• Make sure the vector was completely linearized. It is important to remove any uncut vector prior to use. If necessary, recut your vector and rerun gel purification. • Always include a negative control. If more colonies were present on the negative control plate than on your sample plates; discard all plates, and there is no need to verify it again. However, if the colonies on your sample plates were 2-3 times more than negative control plate, pick more colonies from the sample plates and identify them by PCR screening. • If your insert was amplified from a plasmid, the closed circular DNA may have carried over during the purification process and contaminated the cloning reaction. To ensure the complete removal of any plasmid contamination, we recommend pre-linearizing the template DNA before performing PCR.

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