TOOLS UltraFast PCR Cloning Kit

For inserting any PCR product into any linearized vector at any site

Contents

Introduction

TOOLS UltraFast PCR Cloning Kit is designed for the fast and directional cloning of single or multiple DNA into any vector at any site, regardless of DNA fragment-end compatibility. This kit is applicable for efficient cloning of fragments of 50 bp to 10 kb. The vector is linearized at the cloning site. A small sequence overlapped with each end of the cloning site is added onto the insert through PCR. The insert and linearized vector, with overlapped sequences of 15–20 bp on both 5' and 3' ends, are mixed in an appropriate ratio and incubated with recombinase 2X UltraFast Mix at 50°C for 5–15 min. This kit is a novel cloning kit, applicable for homologous recombination with 1–5 fragments. The Kit is independent of DNA ligase, significantly reducing the self-ligated colonies. The enhanced recombinase and highly optimized buffer included in the 2X UltraFast Mix significantly improve the recombination efficiency and tolerance to impurities. The pFAST vector is compatible with most PCR products, enabling the specific PCR products to be used directly for recombination without treatment, which significantly simplifies the procedure.

Features

Fast cloning (fragments 50 bp to 10 kb)

High-throughput cloning

Seamless assembly

Site-specific mutagenesis

Kit Contents

Contents	TTC-CA15 (25rxn)
2X UltraFast Mix	125 µL
pFAST Vector, Linearized (50 ng/ul)* (5 u/µl)	25 µL
500 bp Control Insert (20 ng/ ul)	5 µL

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

Storage

All components of the Kit should be stored at –20°C. Repeated freeze–thaw cycles should be avoided.

Materials not supplied

1. PCR templates, primers, linearized vectors.
2. High-fidelity polymerase or other equivalent products.
3. Competent cells: chemically competent cells of the following strains:
    a. DH5α competent cells for conventional cloning, applicable to plasmids < 15 kb; and
    b. XL10 competent cells for long-fragment cloning, applicable to plasmids > 10 kb.
4. Other materials: ddH_2O, PCR tubes, and PCR instruments.

Work Flow

Quick guide

Amount of Linearized Vectors and Inserts

1. Simple homologous recombination and single-fragment homologous recombination:

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

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

2. Multifragment (2–5) homologous recombination:

   The optimal mass of a 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)

Recombination

1. The amount of DNA can be roughly calculated according to the above formula.

   Dilute linearized vectors and inserts before recombination to ensure loading accuracy.

   The volume of each component loaded should be no less than 1 μl.

2. Prepare the following reaction on ice:

Components	Recombination	Negative control-1	Negative control-2	Positive control
Linearized Vector	X μL	X μL	0 μL	1 μL
Insert (n ≤ 5)	Y1+Y2+...+Yn μL	0 μL	Y1+Y2+...+Yn μL	1 μL
2X Ultrafast Mix	5 μL	0 μL	0 μL	5 μL
ddH2O	To 10 μL	To 10 μL	To 10 μL	To 10 μL

3. Gently pipet up and down several times to mix thoroughly (DO NOT VORTEX). Spin briefly to bring the sample to the bottom of the tube before reaction.

4. Single-fragment homologous recombination: Incubate at 50°C for 5 min and chill the tube immediately at 4°C or on ice. Multifragment homologous recombination: Incubate at 50°C for 15 min and chill the tube immediately at 4°C or on ice.

- Note: Increase the volume of the reaction system to 20 μl if the total volume of the 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 recombination efficiency when the amount of DNA is between 300 and 400 ng. For multifragment homologous recombination, prolonging the recombination time to 15 min, but no more than 1h, can improve recombination efficiency.

Transformation

1. Place the competent cells on ice. Pipet 5–10 µl of the recombination product to 100 µl of competent cells, flip the tube several times to mix thoroughly (DO NOT VORTEX), and then place the tube on ice for 30 min.

The volume of transformation products should not be more than 1/10 the volume of competent cells.

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2. Heat-shock the tube at 42°C for 45 s and then immediately chill on ice for 2–3 min.
3. Add 900 µl of the SOC or LB medium (no antibiotics) to the tube. Then, shake at 37°C for 1 h at 200–250 rpm. Preheat the LB plate, containing an appropriate selection of antibiotics, at 37°C.
4. Centrifuge the culture at 5,000 rpm for 5 min and then remove 900 µl of the supernatant. Then, resuspend the pellet with 100 µl of the remaining medium and place it on an agar plate containing an appropriate selection of antibiotics. Incubate at 37°C for 12–16 h.

Simple Homologous Recombination

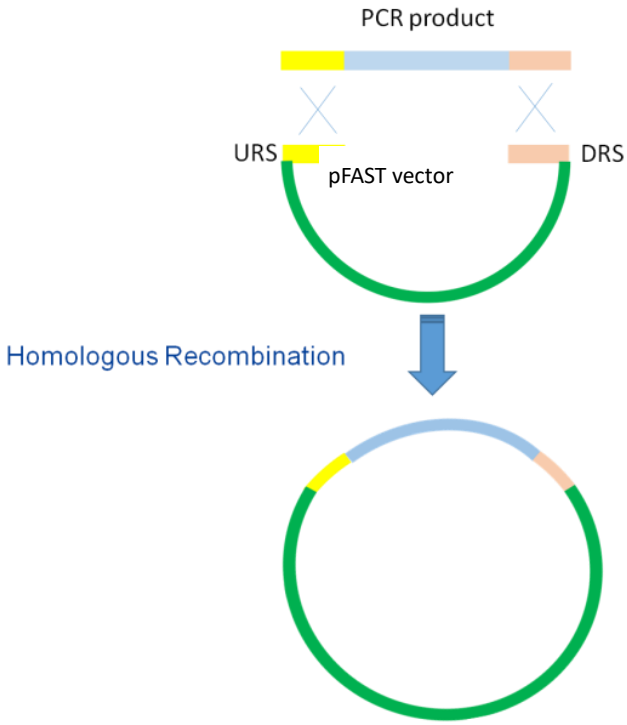


Figure 1

1. Acquisition of Inserts: Introduce upstream recombination sequences (URSs) and downstream recombination sequences (DRSs) into the 5'-end of Forward (F) & Reverse (R) primers, respectively, aiming to make the ends of amplified inserts and the pFAST vectors identical to each other.
2. Recombination: Mix pFAST vectors and inserts at an appropriate ratio and incubate with the 2X UltraFast mix recombinase at 50°C for 5 min to make two linearized DNA cyclized.

3. Transformation: The recombination products can be used for transformation directly.

Protocol

A. Preparation of the Inserts

1. Primer design principles for simple homologous recombination: introduce the URSs and DRSs into the 5'-end of F & R primers, respectively, aiming to make the ends of the amplified inserts and the pFAST vectors identical to each other (15 bp).

Please refer to the principle below:

Forward insert primer:

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

Reverse insert primer:

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

The specific sequences are as follows:

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

a. The pFAST vectors contain two EcoRI cutting sites, which can be used to verify the inserts by enzyme EcoRI digestion analysis. Other suitable enzyme cutting sites can be added between UDSs/DRSs and gene-specific forward/reverse amplification sequences.

b. Gene-specific forward/reverse amplification sequences refer to the sequences that amplify insertion. A temperature of 60°C–65°C is recommended.

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

When calculating the temperature of primers, the URS/DRS sequence and restriction enzyme cutting sites should be excluded; only gene-specific amplification sequences should be used.

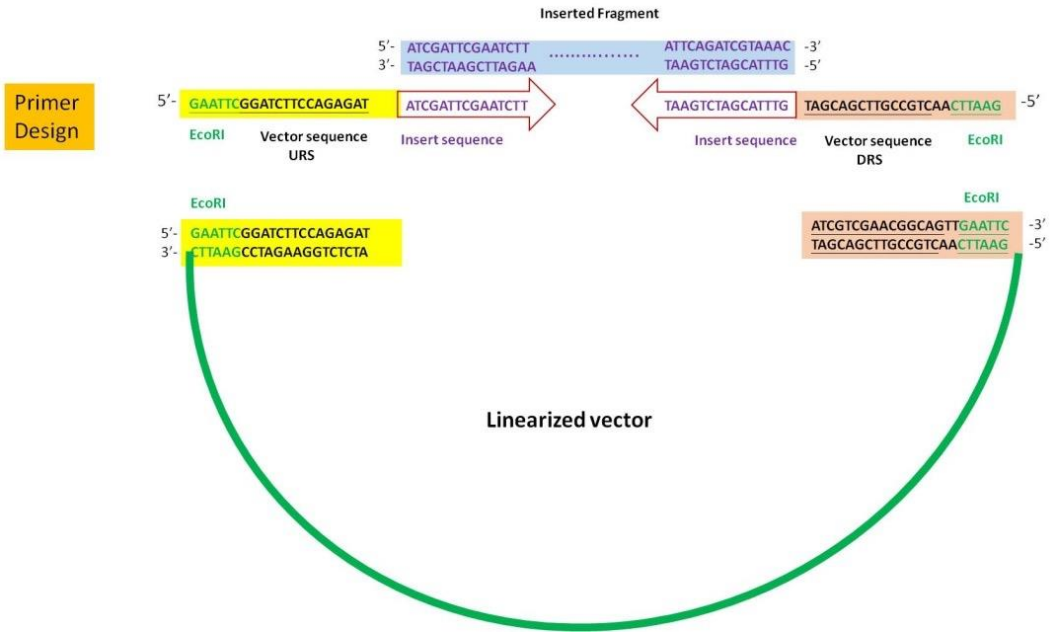


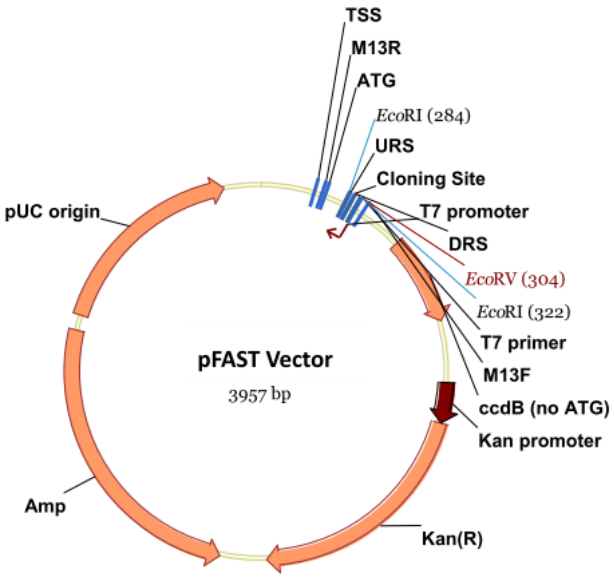
Figure 2

2. Take the control insert provided in this kit as an example. Primers designed for simple homologous recombination are shown in Figure 2.

3. PCR Amplification 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 recombination efficiency if A-tails are in the PCR products. To prevent possibly introducing mutations during PCR, high-fidelity polymerases are highly recommended.

The Sequence Information of the Vector



General primer (M13) used for pFAST vector sequencing.

To inquire about the complete pFAST vector sequences, please log into http://www.toolsbiotech.com.

Single-Fragment Homologous Recombination

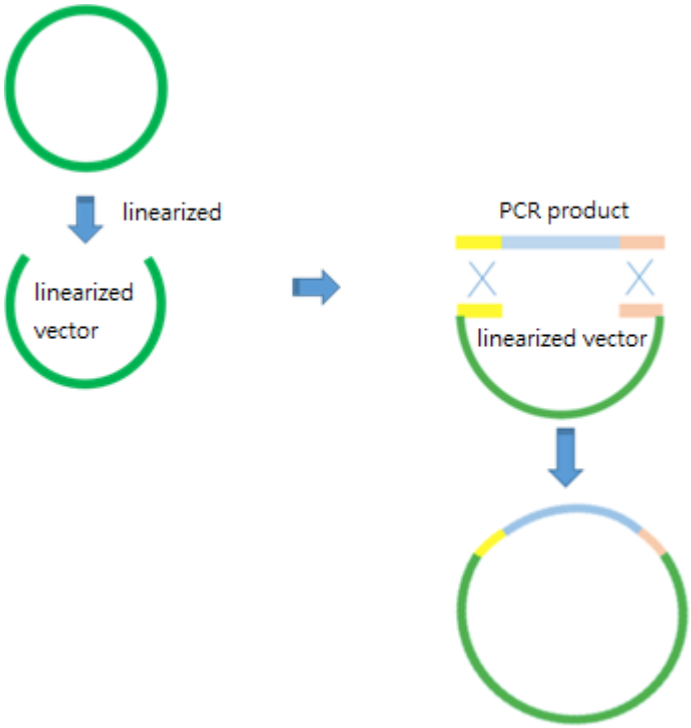


Figure 3

1. Preparation of linearized vectors: Linearized vectors can be obtained by digesting circular vectors with restriction enzymes or by reverse PCR.
2. Acquisition of Inserts: Introduce the homologous sequences of a linearized vector ends at approximately 15–20 bp (highlighted as blue and red) into the 5'-end of F & R primers, respectively, aiming to make the ends of amplified inserts and linearized vectors identical to each other.
3. Recombination: Mix the linearized vectors and inserts at an appropriate ratio and incubate with recombinase at 50°C for 5 min for the recombination reaction.
4. Transformation: The recombination products can be used directly for transformation.

B. Preparation of Linearized Vectors

1. Select an appropriate cloning site at which the vector will be linearized. It is recommended to select the cloning site from a region with no repetitive sequence and where the GC content of the region (within 20 bp upstream and downstream of the site) stays between 40% and 60%.
2. Vector linearization: The linearized vector can be obtained by digesting the circular vector with restriction enzymes or by reverse PCR.

Note:

- Double digestion is recommended because it yields complete linearization and a low false positive rate. If single digestion is adopted, a longer digestion time is necessary to reduce intact plasmid

residues and decrease the false positive rate.

- There is no DNA ligase activity in the reaction system of UltraFast and no occurrence of self-ligation of linearized vectors. 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 that linearization be performed again.
- When using reverse PCR amplification to obtain linearized vectors, it is highly recommended that a high-fidelity DNA polymerase for vector amplification be used to reduce the PCR error rate. It is also recommended that 0.1–1 ng of circular plasmids or prelinearized plasmids be used as PCR templates to reduce the false positive rate caused by residual circular plasmids in a 50-μl PCR reaction system.
- When the PCR templates are circular plasmids, digesting the amplification products with Dpn I to reduce the false positive rate caused by residual circular plasmids is recommended.

C. Acquisition of Inserts

The primer design principles for single-fragment homologous recombination:

Introduce homologous sequences of linearized vectors (15–20 bp, excludes restriction enzyme-cutting sites) into the 5'-end of both F & R primerto make the ends of amplified inserts and linearized vectors identical to each other.

Forward primer of the insert:

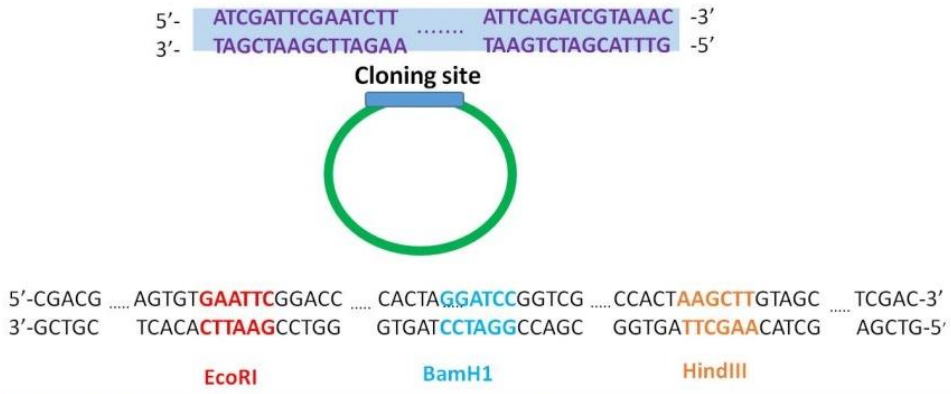
5' - homologous sequence of vector-upstream end + restriction enzyme-cutting site (optional) + gene-specific forward amplification sequence of insert - 3'

Reverse primer of the insert:

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

Note:

- Gene-specific F & R amplification sequences refer to the sequences that amplify the insert. A temperature of 60°C–65°C is recommended.



- Homologous sequences of vector-upstream or -downstream ends are the sequences at the ends of a linearized vector (for homologous recombination). GC content of 40%–60% is recommended.

An example of a primer design is shown in Figure 4.

RE site	Forward primer sequence	Reverse primer sequence
	15-20 bp homologous sequence - RE site - Insert sequence	Insert sequence - RE site - 15-20 bp homologous sequence
EcoRI & HindIII	5'CGACG……AGTGTGAATTCATCGATTCGAATCTT-3'	3'TAAGTCTAGCATTTGTTCGAACATCG……AGCTG-5'
BamH1	5'CGACG……CACTAGGATCCATCGATTCGAATCTT-3'	3'TAAGTCTAGCATTTGCCTAGGCCAGC……GGTGA-5'

RE site	Forward primer sequence	Reverse primer sequence
	15-20 bp homologous sequence - Insert sequence	Insert sequence - 15-20 bp homologous sequence
No RE site	5'CGACG……AGTGTATCGATTCGAATCTT-3'	3'TAAGTCTAGCATTTGCATCG……AGCTG-5'

Restriction sites will be fully retained during cloning. If the length of a primer exceeds 40 bp, PAGE purification of synthesized primers is recommended, which will improve recombination efficiency.

PCR of the inserts: high-fidelity polymerases are highly recommended.

Multifragment Homologous Recombination

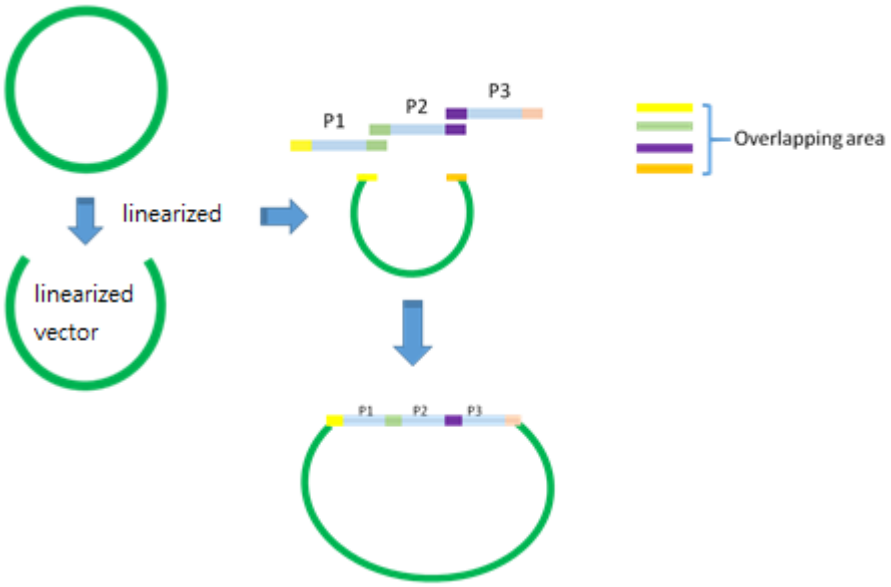


Figure 5

1. Preparation of linearized vectors: A linearized vector can be obtained by digesting the circular vector with restriction enzymes or by reverse PCR.

2. Acquisition of inserts: Obtained by PCR amplification. Introduce homologous sequences of –15–20 bp (highlighted in dark blue, green, light blue, and red) into the 5'-end of the primers, aiming to make the ends of the amplified insert and linearized vector identical.
3. Recombination: Mix the linearized vectors and all inserts at an appropriate ratio and incubate with recombinase at 50°C for 15 min to complete the recombination reaction
4. Transformation: The recombination products can be used directly for transformation.

A. Preparation of Linearized Vectors

Select an appropriate cloning site on the vector to be linearized. It is recommended to select a cloning site from regions with no repetitive sequences and where the GC content of the region (within 20 bp up- and down-stream of the site) stays between 40% and 60%.

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

Note:

- Double digestion is recommended because it yields complete linearization and a low false positive rate. If single digestion is adopted, a longer digestion time is necessary to reduce intact plasmid residues and decrease the false positive rate.
- There is no DNA ligase activity in the UltraFast system, and no occurrence of self-ligation of linearized vectors. 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 reverse PCR amplification to obtain a linearized vector, it is highly recommended that a high-fidelity DNA polymerase be used for vector amplification to reduce the PCR error rate. It is also recommended that 0.1–1 ng of circular plasmids or prelinearized plasmids be used as PCR templates to reduce the false positive rate caused by residual circular plasmids in a 50-µl PCR reaction system.
- When the PCR templates are circular plasmids, digesting the amplification products with Dpn I to reduce the false positive rate caused by residual circular plasmids is recommended.

B. Acquisition of Inserts

1. The primer design principles for multifragment homologous recombination: Introduce certain sequences (15–20 bp, excluding restriction enzyme-cutting sites) into the 5'-end of primers, aiming to make certain amplified inserts and linearized vectors share homologous sequences with each other.

An example of three fragments, A, B, and C (0.5, 1, and 2 kb, respectively) inserted into a pFAST vector is shown as follows:

Primer design principles of inserts A and C:

Forward primer of insert A:

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

Reverse primer of insert C:

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

Note:

- Gene-specific F & R amplification sequences refer to the sequences that amplify insertion. A temperature of 60°C–65°C is recommended.
- The homologous sequences (for homologous recombination) of the vector-upstream or -downstream end are the sequences at the ends of the linearized vector. GC content of 40%–60% is recommended.
- Three types of the primer design of insert B are shown in Figure 6.

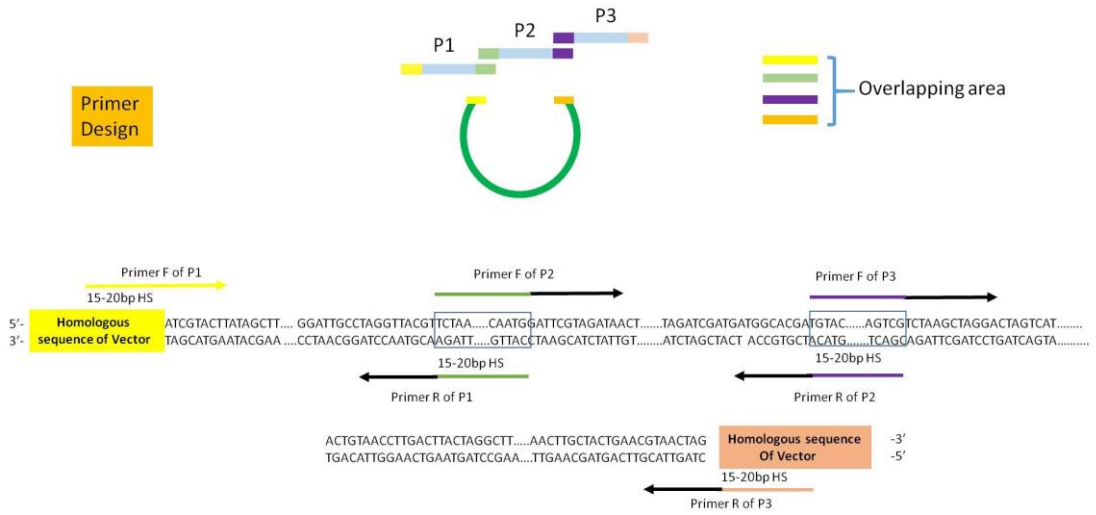


Figure 6

a. Introduce homologous sequences (15–20 bp) from the 3’-end of fragment A into the 5’-end of the forward primer;

b. Introduce homologous sequences (15–20 bp) from the 5’-end of fragment C into the 5’-end of the reverse primer;

c. Introduce homologous sequences from A and C (total: 15–20 bp) into the 5’-end of the F & R primer, respectively;

Note:

- For the primer design of fragments of A and C, refer to Figure 6 (primer design for a single-fragment homologous recombination).
- If the length of the primer exceeds 40 bp, PAGE purification of the synthesized primers is recommended, which will improve recombination efficiency. When calculating the temperature

of the primers, the homologous sequence of vector ends and restriction enzyme-cutting sites should be excluded and only gene-specific amplification sequences should be used.

2. PCR of the inserts:

Inserts can be amplified by any PCR polymerase (i.e., conventional Taq DNA polymerase or high-fidelity DNA polymerase). It will not interfere with the recombination efficiency if there are A-tails in the PCR products. To prevent introducing possible mutations during PCR, amplification with high-fidelity polymerases is highly recommended.

Troubleshooting

A. Primer design notes

1. It is recommended that the included software for primer design be used.
2. Linearized vectors can be obtained through double digestion, single digestion, and reverse PCR; of these, double digestion is recommended.
3. The three parts of primers are the homologous sequences (15–20 bp, excluding restriction sites and base residues; the content of GC is 40%–60%), restriction sites (optional according to experimental need), and specific primers (when calculating the temperature of primers, the homologous sequence should be excluded).

B. Few clones or no clones formed on the plate

1. Improper primer design: primer includes 15–20 bp homologous sequences (excluding restriction sites); the content of GC is 40%–60%.
2. The amount of linearized vectors and amplified inserts is too low/high in the recombination reaction or the ratio of fragments is not appropriate. Please use the specific amount and ratio recommended.
3. Contamination in vectors and inserts inhibits recombination: The total volume of unpurified vectors and inserts digested should be ≤ 2 μl (≤ 1/5 of the total volume of the recombination reaction system). Gel extraction purification is recommended to purify the vector and insert. It is recommended that the purified DNA be dissolved in ddH_2O.
4. The low efficiency of the competent cells: Ensure the transformation efficiency of competent cells is $>10^8$ cfu/μg. Transform a 1-ng vector and pick 1/10 of that to transform, which will yield 1,000 colonies on the plate. Then, the transformation efficiency of competent cells can be estimated as 10^8 cfu/μg. The volume of transformation products should not be more than 1/10 of the volume of competent cells. Choose competent cells used for cloning (such as DH5α/XL10), not those for expressing.

C. Incorrect/no inserts found in the colony plasmids

1. Nonspecific amplification is mixed with target inserts: Optimize the PCR reaction system to improve amplification specificity, purify the PCR products with a gel recovery kit, or select more colonies for verification.
2. Incomplete linearization of the vector: Use negative controls to confirm the complete linearization of vectors, improve the amount of restriction endonucleases, prolong the digesting time, or purify the digesting products before the recombination reaction.
3. Plasmids with the same resistance with vectors mixed in reaction system: When the PCR templates for amplification of vectors or inserts are circular plasmids, digesting the amplification products

with Dpn I or purifying them by gel recovery can both effectively reduce or even eliminate the residues of cyclic plasmid templates.

D. No electrophoretic bands in colony PCR

1. Improper primer: it is recommended that at least one common sequencing primer of the vector be used.
2. Inappropriate PCR system or program: No bands of targets or empty plasmids. It is recommended that the PCR reaction system or program be optimized; extract plasmids as PCR templates or use enzyme digestion for confirmation.
3. Unsuccessful recombination: There is only the band of empty plasmid after colony PCR, which indicates an unsuccessful recombination and incomplete linearization of the vector. One of the approaches to overcome such a situation is to optimize the enzyme digestion system.

This product is for research only, not for diagnostic or clinical use.