1. Introduction

GenieClone DNA Assembly Cloning Kit allows for the highly efficient cloning and assembly of 1-5 DNA fragments in as little as 15 minutes into any vector at any site. This system utilizes ligation-independent technology and a novel GenieClone recombinase to significantly reduce self-ligating colonies. The enhanced GenieClone Recombinase and highly optimized buffer included in the 2x GenieClone Mix significantly improve the recombination efficiency and the tolerance to impurities. The pCE-ONE vector is compatible with most PCR products, enabling the specific PCR fragments to be used directly for recombination without any pre-treatment.

Utitizing a simple procedure, the vector is first linearized at the cloning site. A small overlapping sequence at each end of the cloning site is added to the insert through PCR. The insert and the linearized vector both with overlapping 5'- and 3' (15 bp - 20 bp) sequences are mixed in an appropriate ratio and incubated with GenieClone Recombinase at 50℃ for 5 - 15 min before transformation of competent cells for successful DNA cloning and assembly.

2. Applications

- Fast Cloning
- High-throughput Cloning
- Seamless Assembly
- Site-specific Mutagenesis

3. Quick Workflow

3.1 Quantity of Linearized Vectors and Inserts

- Simple homologous recombination and single-fragment homologous recombination:
  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)

- Multi-fragment (2 - 5) homologous recombination:
  The optimal mass of 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)

3.2 Recombination

1. The amount of DNA can be roughly calculated according to the above formula. Dilute linearized vectors and inserts before recombination to assure the 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)	$Y_1 + Y_2 \ldots + Y_n$ µl	0 µl	$Y_1 + Y_2 \ldots + Y_n$ µl	1 µl
2X GenieClone Mix	5 µl	0 µl	0 µl	5 µl
ddH_2O	To 10 µl	To µl	To 10 µl	To 10 µl

3. Gently pipette up and down for several times to mix thoroughly (DO NOT VOTEX!). 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.
Multi-fragment homologous recombination: Incubate at 50°C for 15 min and chill the tube immediately at 4°C or on ice.
▲Increase the volume of reaction system to 20 µl if the total volume of vector and insert is more than 5µl. For single-fragment homologous recombination, increasing the recombination time to 15min may be helpful to improve the recombination efficiency when the amount of DNA is between 300 ng and 400 ng. For multi-fragments homologous recombination, prolonging the recombination time to 15min, but no more than 1h, can improve the recombination efficiency.

3.3 Transformation

1. Place the competent cells on ice (i.e., DH5α Competent at least >10^8 cfu/µg).

2. Pipet 5 - 10µl of the recombination products to 100 µl competent cells, invert the tube several times to mix thoroughly (DO NOT VOTEX), and then place the tube on ice for 30 min. ▲The volume of transformation products should not be more than 1/10 of the volume of competent cells.

3. Heat-shock the tube at 42°C for 45sec and then immediately chill on ice for 2-3 min.

4. Add 900µl of SOC or LB medium (without antibiotics) to the tube. Then, shake at 37°C for 1h at 200 - 250 rpm.

5. Preheat the LB plate which contains appropriate selection antibiotic at 37°C.

6. Centrifuge the culture at 5,000 rpm for 5 min and discard 900 µl supernatant. Then, re-suspend the pellet with 100 µl of remaining medium and plate it an agar plate containing appropriate selection antibiotic.

7. Incubate at 37°C for 12 - 16 hours.

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4. Package Information

Components	MORV0004 – 25 rxn	MORV0004– 50 rxn
2X GenieClone Mix	125 µl	2 x 125 µl
pCE-ONE Vector, Linearized (50 ng/ul)*	25 µl	50 µl
500 bp Control Insert (20 ng/µl）	5 µl	µl

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

5. Storage

All components should be stored at -20°C. Avoid repeat freezing and thawing.

6. Materials Required But Not Supplied

PCR templates, primers, linearized vectors.

High-fidelity polymerase: Genie Fusion Ultra High-Fidelity DNA Polymerase (MRV0001) or other equivalent products.

Competent cells: chemically competent cells by cloning strains:

DH5α competent cells (at least >10^8 cfu/µg) for conventional cloning –> applicable to plasmids ＜15 kb

XL10 competent cells for long-fragment cloning –> applicable to plasmids ＞10 kb

Other materials: ddH2O, PCR tubes, PCR instrument, etc.

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7. Simple Homologous Recombination

7.1 Workflow.

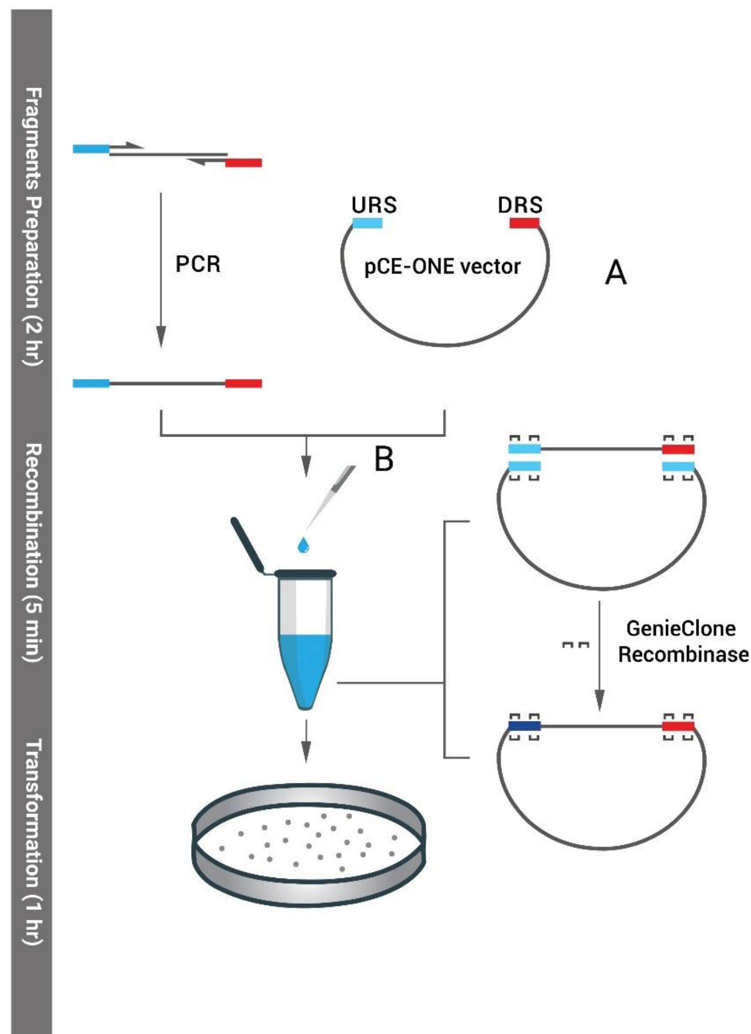

Figure 1: Mechanism of GenieClone Simple Homologous Recombination

A. Acquisition of Inserts: Introduce URS/DRS sequences (highlighted in blue and red) into 5'-end of Forward (F) & Reverse (R) primers, aiming to make the ends of amplified inserts and pCE-ONE vector identical to each other.

B. Recombination: Mix pCE-ONE vectors and inserts at an appropriate ratio and incubate with GenieClone Recombinase at 50℃ for 5min to make two linearized DNA cyclized.

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

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7.2 Preparation of the Inserts

1. Primer-design principles for simple homologous recombination: introduce URS and DRS sequence into 5'-end of Forward (F) & Reverse (R) primers, respectively, aiming to make the ends of amplified inserts and pCE-ONE vectors identical to each other (15bp). It is recommended to use Assay Genies software, Genie Clone Design (available on request techsupport@reagentgenie.com), to design primers. The Genie Clone Design automatically generates amplification primers of insert. You can also design the primers manually, please refer to the principle below:

Forward primer of insert:

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

Reverse primer of insert:

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

a. URS is the abbreviation of Upstream Recombination Sequence; DRS is the abbreviation of Downstream Recombination Sequence. The specific sequences are as follows: URS: 5' - GGATCTTCCAGAGAT - 3', DRS: 5' - CTGCCGTTCGACGAT - 3'.

b. pCE-ONE vector contains two ECO RI cutting sites, which can be used for verifying the inserts by enzyme ECO RI digestion analysis. Other suitable enzyme cutting site can also be added between UDS/DRS sequence and gene specific forward/reverse amplification sequence.

c. Gene specific forward/reverse amplification sequence refers to the sequence to amplify the insertion. The Tm of 60℃ - 65℃ is recommended.

d. If the length of primer exceeds 40 bp, PAGE purification of synthetized primers is recommended, which will benefit the recombination efficiency. When calculating the Tm of primers, the URS/DRS sequence and restriction enzyme cutting site should be excluded and only gene specific amplification sequence should be used.

2. Take the control insert provided in this kit as an example. Primers design for simple homologous recombination are as follows:

Forward primer of inserts:

5' – GGATCTTCCAGAGAT + ATCTCGCACATTGCC- 3'

URS   Gene specific forward amplification sequence of insert

Reverse primer of inserts:

5' –CTGCCGTTCGACGAT + GTTGCCGCATTATCG – 3'

DRS   Gene specific reverse amplification sequence of insert

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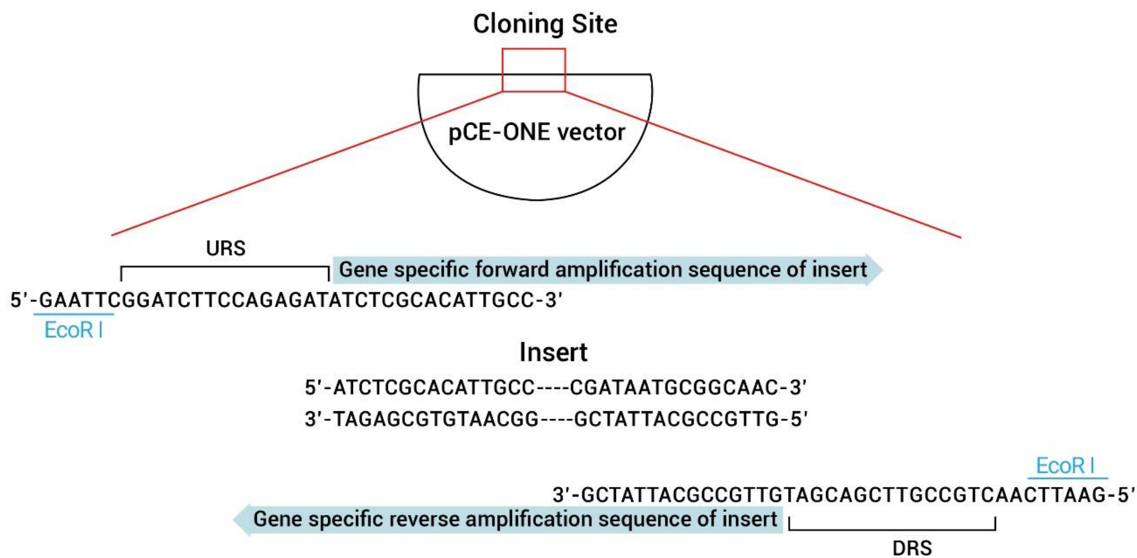
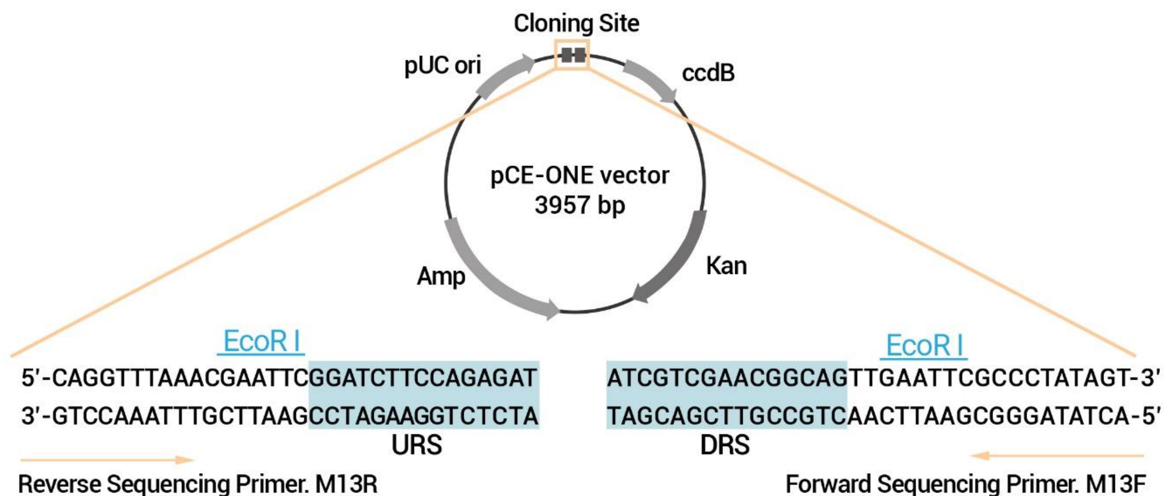


Figure 2: Primer Design for GenieClone Simple Homologous Recombination

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 whether there are A-tails in the PCR products or not. To prevent possible mutations introduced during PCR, high-fidelity polymerases (i.e Genie Fusion Ultra High-Fidelity DNA Polymerase (MRV0001) are highly recommended.

7.3 The Sequence Information of the Vector



2. General primer can be used for pCE-ONE vector sequencing.

Contact techsupport@reagentgenie.com for pCE-ONE vector sequence information.

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8. Single-fragment Homologous Recombination

8.1 Workflow

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

B: Acquisition of Inserts: Introducing homologous sequences of linearized vector ends about 15 bp - 20 bp (highlighted as blue and red) into 5'-end 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 and incubate with GenieClone Recombinase at 50°C for 5 min for recombination reaction and to make two linearized DNA cyclized.

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

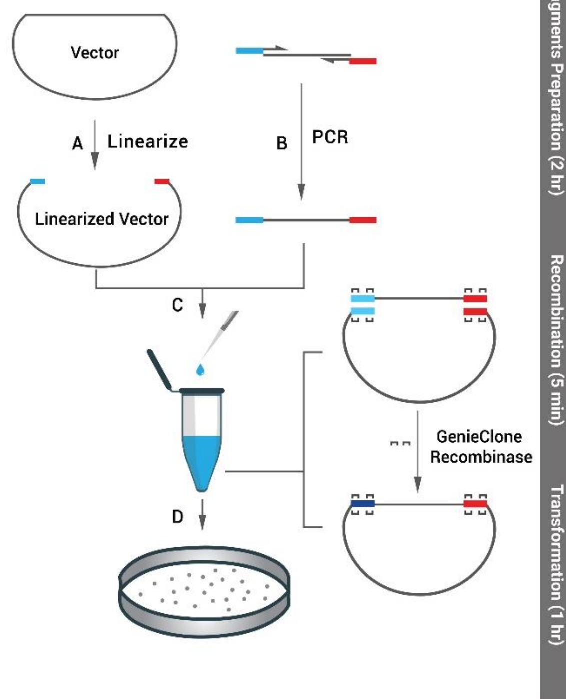

Figure 3: Mechanism of GenieClone Homologous Recombination of Single Fragment:

8.2 Preparation of Linearized Vectors

1.Select an appropriate cloning site on the vector that will be linearized. It is recommended to select the cloning site from regions with no repetitive sequence and the GC content of the certain region (within 20 bp up 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.

- Double digestion is recommended because it brings complete linearization and 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 GenieClone and no occurrence of self-ligation of linearized vector. 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 to generate linearization again.
- When using reverse PCR amplification to obtain linearized vector, it is highly recommended to use a high-fidelity DNA polymerase (i.e. Genie Fusion Ultra High-Fidelity DNA Polymerase (MRV0001) for vector amplification to reduce the PCR error rate. It is also recommended to use o.1 ng - 1 ng circular plasmids or pre-linearized plasmids as PCR templates to reduce the false positive rate caused by residual circular plasmids in a 50 µl PCR reaction system.

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*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.

8.3 Acquisition of Inserts

1. The primer-design principles for single-fragment homologous recombination Introduce homologous sequences of linearized vector (15 bp - 20 bp, excludes restriction enzyme cutting sites) into 5'-end of both Forward (F) & Reverse (R) primer, respectively, aiming to make the ends of amplified inserts and linearized vectors identical to each other.

Forward primer of insert:

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

Reverse primer of insert:

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

*Gene specific forward/reverse amplification sequence refers to the sequence to amplify the insert. Tm of 60℃- 65℃ is recommended. 8.2 Preparation of Linearized Vectors 8.3 Acquisition of Inserts 5.

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

It is recommended to use Assay Genies software, Genie Clone Design (available on request techsupport@reagentgenie.com), to design primers. The Genie Clone Design automatically generates amplification primers of insert. You can also design the primers manually. An example is shown in Fig 4.

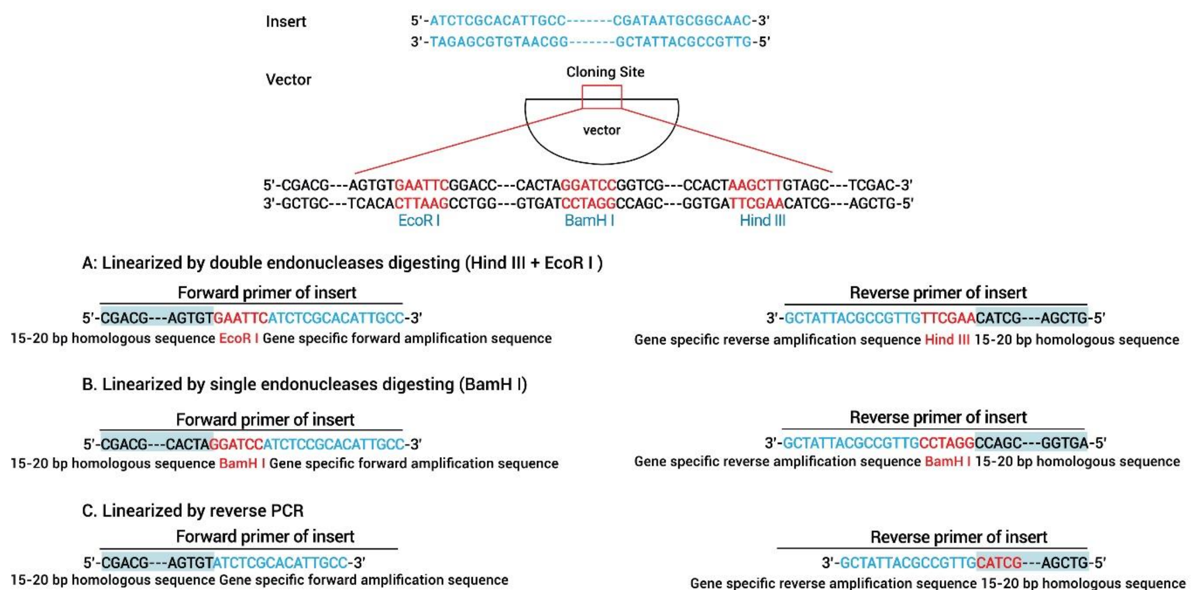

Figure 4:Primer Design for Single-fragment Homologous Recombination

*Restriction sites will be fully retained during cloning.

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

2. 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 recombination efficiency whether there is A-tail in the PCR products or not. To prevent possible mutations introduced during PCR, high-fidelity polymerases (Genie Fusion Ultra High-Fidelity DNA Polymerase (MRV0001) are highly recommended.

9 Multi- fragment DNA Assembly (Homologous Recombination)

9.1 Workflow

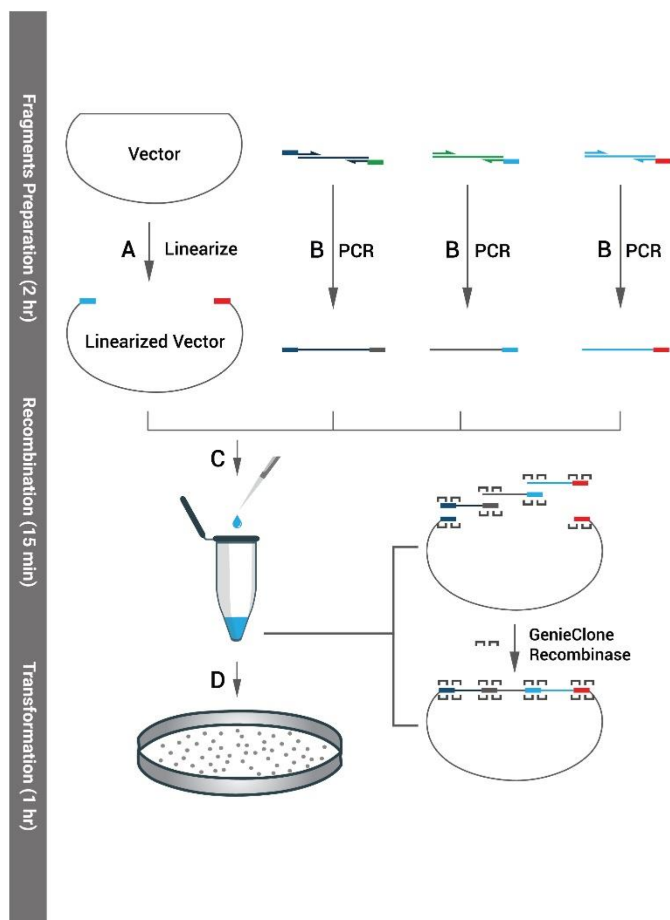

Figure 5: Mechanism of GenieClone Multi-fragment Homologous Recombination

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

B: Acquisition of Inserts: obtained by PCR amplification. Introduce homologous sequences of 15 bp - 20 bp (highlighted in dark blue, green, light blue and red) into 5'-end of the primers, respectively, aiming to make the ends of amplified insert and linearized vector, one kind insert and another, identical to each other.

C: Recombination: Mix the linearized vectors and all inserts at an appropriate ratio and incubate with GenieClone Recombinase at 50℃ for 15min to complete recombination reaction and make several linearized DNA cyclized.

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

9.2 Preparation of Linearized Vectors

1.Select an appropriate cloning site on the vector that will be linearized. It is recommended to select the cloning site from regions with no repetitive sequence and the GC content of the certain region (within 20 bp up 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.

- Double digestion is recommended because it brings complete linearization and 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 GenieClone, and no occurrence of self-ligation of linearized vector. 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 linearized vector, it is highly recommended to use a high-fidelity DNA polymerase (i.e., Genie Fusion Ultra High-Fidelity DNA Polymerase (MRV0001) for vector amplification to reduce the PCR error rate. It is also recommended to use o.1 ng –1 ng circular plasmids or pre-linearized plasmids 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.

9.3 Acquisition of Inserts

1. The primer-design principles for multi-fragment homologous recombination: introduce certain sequences (15 bp - 20 bp, excludes restriction enzyme cutting sites) into 5’-end of primers, aiming to make the certain amplified insert and linearized vector, one kind insert and another share homologous sequences with each other.

It is recommended to use Assay Genies software, Genie Clone Design (available on request techsupport@reagentgenie.com), to design primers. The Genie Clone Design automatically generates amplification primers of insert. You can also design the primers manually. An example of three fragments, A, B, C (0.5 kb, 1 kb and 2 kb) inserted into pCE-ONE 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’

*Gene specific forward/reverse amplification sequence refers to the sequence to amplify the insertion. Tm of 60℃ ~ 65℃ is recommended.

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*The homologous sequences (for homologous recombination) of 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 primer design of insert B:
  a. Introduce homologous sequences (15 bp - 20 bp) from 3'-end of fragment A into 5'-end of forward primer;
  b. Introduce homologous sequences (15 bp - 20 bp) from 5'-end of the fragment C into 5'-end of reverse primer;
  c. Introduce homologous sequences from A and C (total 15 bp - 20 bp) into 5'-end of forward / reverse primer, respectively.

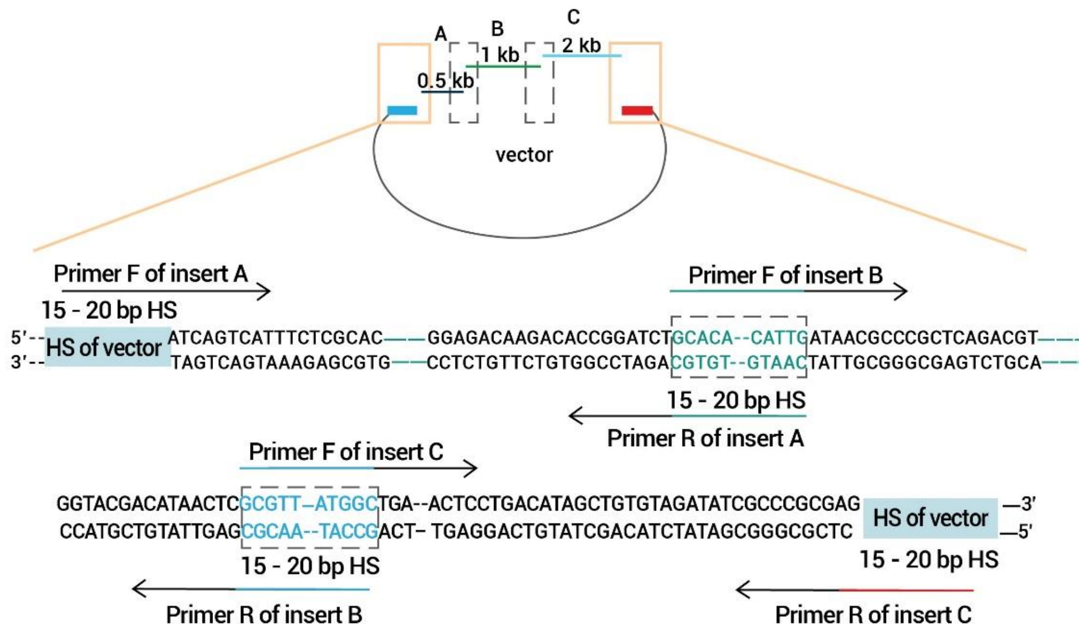

Figure 6: Primer Design for Multi-fragment Homologous Recombination

HS：Homologous Sequences Primer F: Forward primer Primer R: Reverse primer

*Primer design of fragments of A and C (Refer to Fig4. Primer design for single-fragment homologous recombination).

*If the length of primer 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 and restriction enzyme cutting site should be excluded and only gene specific amplification sequence 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 whether there are A-tails in the PCR products or not. To prevent possible mutations introduced during PCR, amplification with a high-fidelity polymerases (i.e. Genie Fusion Ultra High-Fidelity DNA Polymerase (MRV0001) are highly recommended.

10 Protocol

10.1 Quantity of Linearized Vectors and Inserts

1. Determination of DNA concentration

If the linearized vectors and inserts have been purified by high quality gel DNA recovery kit, and there is no obvious nonspecific band or smear after gel electrophoresis, instruments based on absorbance, i.e., Onedrop, can be used to determine the DNA concentration, but the results of concentration are only reliable when A260/A280 value is between 1.8 and 2.0. For samples with DNA concentration lower than 10 ng/μl, the detection results may differ significantly between instruments (Nanodrop, Onedrop, Qubit® and PicoGreen® is recommended) based on the A260.

2. The calculation of amount of vectors and inserts

GenieClone DNA Assembly Cloning Kit containing pCE-ONE vector is applicable for homologous recombination with 1 - 5 fragments. For single-fragment homologous recombination, the optimal amount of vector required is 0.03 pmol, the optimal amount of insert required is 0.06 pmol (optimal molar ratio of vector to insertion is 1:2). For multi-fragment homologous recombination, the optimal amount of inserts and linearized vectors are both 0.03 pmol (the radio of vector to insert is 1:1). Their mass can be roughly calculated according to the following formula:

- Simple homologous recombination
  The optimal mass of insert required = [0.04 × number of base pairs] ng (0.06 pmol)

- Single-fragment homologous recombination
  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 to a vector of 5 kb, the optimal mass of vector is 0.02 × 5000 =100 ng, and that of insert is 0.04 × 2000 = 80 ng.

- Multi-fragment homologous recombination
  The optimal mass of 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)
  For example, when cloning inserts of 0.5 kb, 1 kb and 2 kb to a vector of 5 kb, the optimal mass of vector and three inserts are as follows：
  The optimal mass of linearized vector required: 0.02 × 5000 = 100 ng;
  The optimal mass of insert of 0.5 kb required: 0.02 × 500 = 10 ng;
  The optimal mass of insert of 1 kb required: 0.02 × 1000 = 20 ng;
  The optimal mass of insert of 2 kb required: 0.02 × 2000 = 40 ng.

  a. For single-fragment homologous recombination: The mass of amplified insert should be more than 20 ng. When the length of the insert is larger than that of the vector, the calculation method of the optimal mass of vector and insert should be inverted.
  b. For multi-fragment homologous recombination: The mass of each insert should be more than 10 ng. When the optimal mass calculated by the above formula is under 10 ng, just use 10ng.
  c. The amount of linearized vectors should be between 50 ng - 200 ng. When the optimal amount calculated using the above formula is beyond these ranges, just choose the maximum or minimum amount for recombination.

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d. For simple or single-fragment homologous recombination: if there are no obvious nonspecific bands or smear shows in gel electrophoresis, the DNA can be directly used without purification and the total volume of vectors and inserts should be ≤ 2 µl (1/5 of the total volume of recombination reaction system), which will reduce the recombination efficacy (Purification is recommended before recombination).

10.2 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 recombination, 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] (n≤5)	$Y_1 + Y_2 ... + Y_n$ µl	0 µl	$Y_1 + Y_2 ... + Y_n$ µl	1 µl
2X GenieClone Mix	5 µl	0 µl	0 µl	5 µl
ddH_2O	To 10 µl	To µl	To 10 µl	To 10 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 templates are circular plasmids which share the same antibiotic resistance with the cloning vector.
d. Positive controls are used to exclude materials and operation factors.

3. Gently pipette up and down for several times to mix thoroughly (DO NOT VOTEX!). 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 immediately chill the tube at 4°C or on ice.

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

- It is recommended to use an instrument with high accurate temperature controlling system (i.e., a PCR instrument) for the reaction.
- If the total volume of vector and insert is more than 5 µl, the volume of reaction system can be increased to 20 µl. For single-fragment homologous recombination, if the amount of DNA is between 300 ng and 400 ng, the time of recombination can be prolonged to 15min to improve the recombination efficiency. For multi-fragment of 4 - 5 homologous recombination, the time of recombination can be prolonged to 30 min to improve the recombination efficiency, but not more than 1h.
- The recombination product can be stored at -20°C for one week. Thaw the product before transformation.

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10.3 Transformation

1. Place the competent cells on ice (i.e., DH5α Competent cells).

2. Pipet 5 - 10 µl of the recombination products to 100 µl of competent cells, flip the tube for several times to mix thoroughly (DO NOT VOTEX!), and then place the tube still on ice for 30 min.

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

3. Heat-shock the tube at 42℃ for 45 sec and then immediately chill on ice for 2 - 3 min.

4. Add 900µl of SOC or LB medium (without antibiotics) to the tube. Then, shake at 37℃ for 1h at 200 rpm - 250 rpm.

5. Place the LB plate which contains appropriate selection antibiotic at 37℃.

6. Centrifuge the culture at 5,000 rpm for 5 min, discard 900 µl of supernatant. Then, re-suspend the pellet with 100 µl of remaining medium and plate it on an agar plate which contains appropriate selection antibiotic.

7. Incubate at 37℃ for 12 -16 hours.

10.4 Selection of Positive Colonies

After overnight culture, hundreds of mono-colonies will form on the transformation plate of recombination reaction, whereas fewer of those on the transformation plate of negative control.

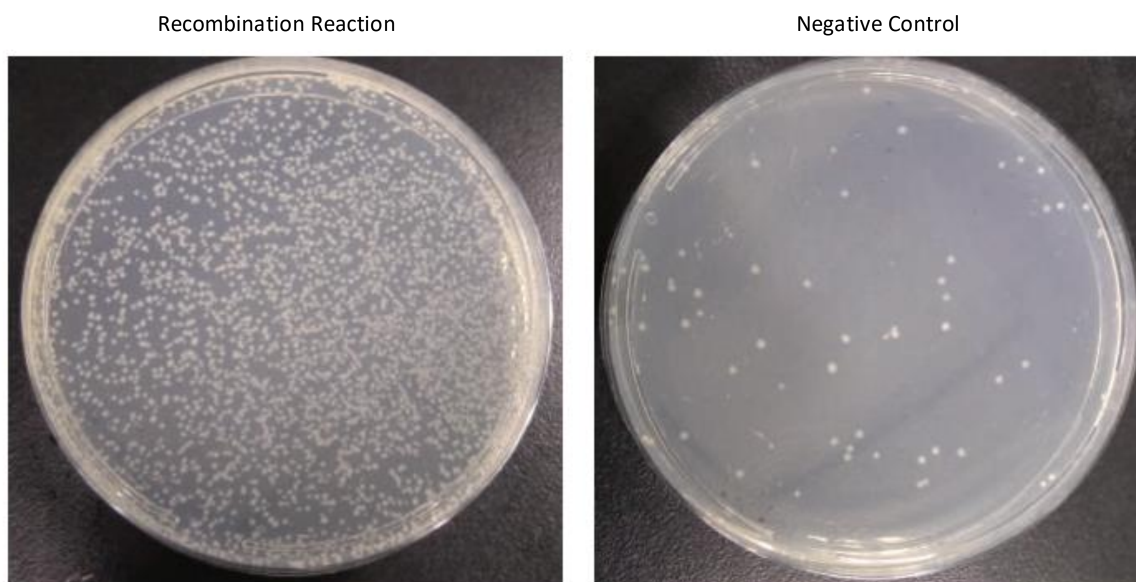

Figure 7: Plate Incubated Overnight

Pick several mono-colonies from the plate of recombination reaction for colony PCR with at least one common sequencing primer of the vector. If the colony is positive, there should be a band which length is slightly bigger than that of insert (Fig 8).

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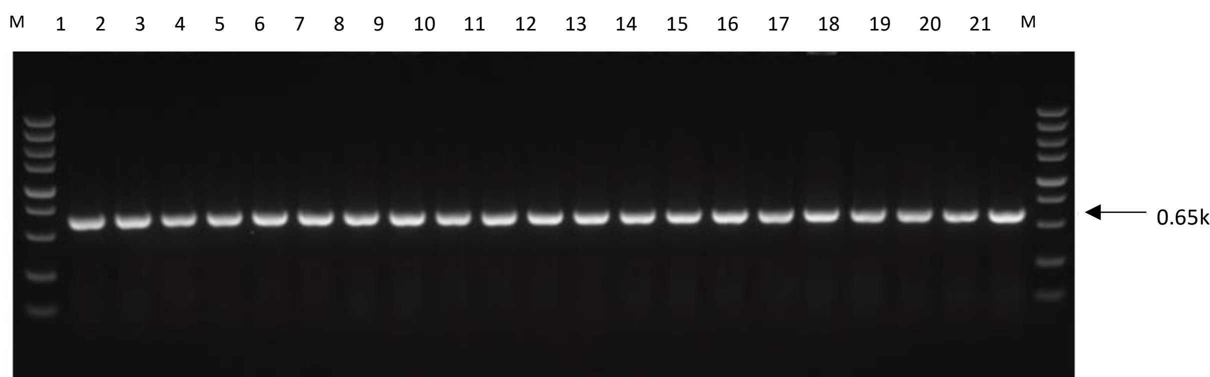

Figure 8: Agarose Gel Electrophoresis of Colony PCR Products

M: 5kb Marker; 1 - 21: 21 Positive Colonies

Inoculate the remaining medium of positive clones into fresh LB medium and culture overnight. Then, extract the plasmids for further enzyme digestion analysis (Fig 9) or DNA sequencing.

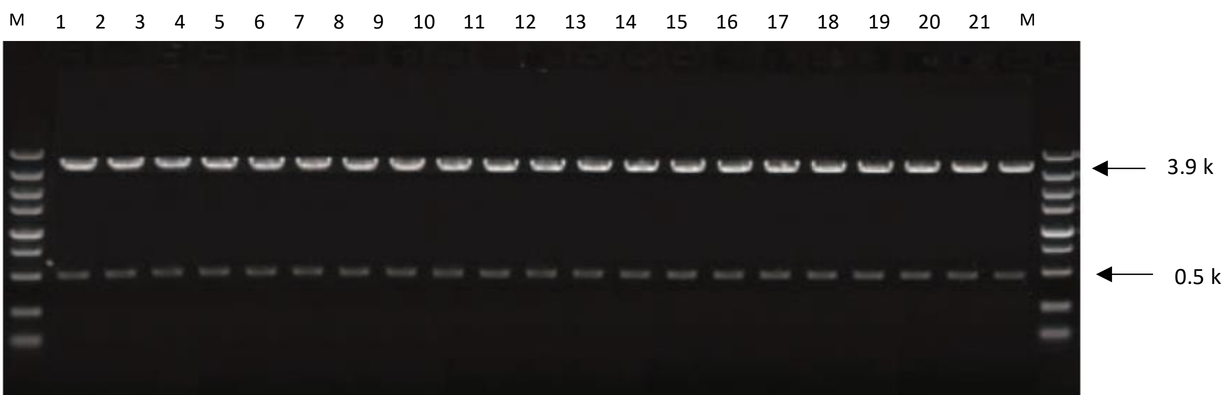

Figure 9:Agarose Gel Electrophoresis of Enzyme Digestion Products

M: 5kb Marker; 1 - 21: 21 Positive Colonies

11 Tips

1. Place the recombination products on ice and transform it to competent cells directly. Commercial super competent cells (transformation efficiency over 10^8 cfu/μg) are highly recommended. The volume of transformation products should not be more than 1/10 of the volume of competent cells.

2. GenieClone DNA Assembly Cloning Kit is applicable for efficient clone of fragments of 50 bp - 10 kb.

3. Usages of inserts and vectors
   Cloning fewer or short fragments (< 5 kb)

- Linearized vectors, prepared by restriction digestion, can be directly used for recombination after inactivating the restriction enzymes. Inactivation is available for most restriction enzymes, please refer to instruction of specific inactivation methods.

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- For linearized vectors prepared by reverse PCR, if the amplification templates are pre-linearized and PCR products show singer 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 electrophoresis and the templates are not circular plasmids which share the same antibiotic resistance with the cloning vector, high specific PCR products can be directly used. For recombination without further purification, please refer to Table 1 / Table 2 for the usages of linearized vectors and inserts prepared in different ways.

Table 1: Usages of Linearized Vectors

Method of Linearization		Temple 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		

Table 2: Usage 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
	Pre-linearized plasmid, genomic DNA, cDNA	Use directly	Gel recovery
Non-specific Amplification	Gel recovery		

- When using enzyme- digestion products or amplified products directly for recombination, the volume should be ≤ 2μl (≤ 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 deactivate Dpn I, so as to prevent cloning vectors from degradation when recombination.

- Cloning multiple fragments (4 - 5) or large fragment (> 5 kb)

It is recommended to purify the linearized vectors and amplified inserts with high quality gel DNA recovery kit before recombination, so as to improve the DNA purity and eliminate residual circular vectors.

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12 Troubleshooting

1. Primers design notes

1.1 It is recommended to use the software Genie Design for primer design.

1.2. The linearized vector can be obtained by double digestion, single digestion and reverse PCR, among which, double digestion is recommended.

1.3. Three parts of primers: homologous sequences (15 bp – 20 bp, exclude restriction sites and base residues, the content of GC is 40% - 60%)+ restriction sites (optional according to experiment need) + specific primers (when calculating the Tm of primers, the homologous sequence should be excluded).

2. Few or no clones formed on the plate

2.1. Improper primer design: primer includes 15 bp - 20 bp homologous sequences (exclude restriction sites); the content of GC is 40% - 60%.

2.2. The amount of linearized vectors and amplified inserts are too low/high in the recombination reaction or the ratio of fragments is not appropriate. Please use the amount and radio as specification recommended.

2.3. Contamination in vector and insert inhibits the recombination: The total volume of unpurified vector and insert digested should be ≤ 2µl (≤ 1/5 of the total volume of recombination reaction system). Gel extraction purification is recommended to purify the vector and insert. It is recommended to dissolve the purified DNA in ddH2O.

2.4. The low efficiency of the competent cell: Make sure the transformation efficiency of competent cells is >10^8 cfu/µg. Transform 1 ng vector and pick 1/10 of that to transform, and there are 1000 colonies growing 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 engineered for protein expression. 12 Troubleshooting 11

3. Incorrect/no inserts found in the colony plasmids

3.1. Non-specific amplification is mixed with target inserts: Optimize the PCR reaction system to improve the amplification specificity; purify the PCR products with a gel recovery kit; select more colonies for verification.

3.2. Incomplete linearization of the vector: Approaches to overcome such situation include using negative controls to confirm the complete linearization of vectors, improving the amount of restriction endonuclease, prolonging the digesting time, and purifying the digesting products before the recombination reaction.

3.3. 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.

4. No electrophoretic bands in colony PCR

4.1. Improper primer: it is recommended to use at least one common sequencing primer of the vector.

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4.2. Inappropriate PCR system or program: No bands of targets or empty plasmids. It is recommended to optimize the PCR reaction system or program; extract plasmids as PCR templates or use enzyme digestion for confirmation.

4.3. Unsuccessful recombination: There is only the band of empty plasmid after colony PCR, which indicates the unsuccessful recombination and incomplete linearization of the vector. One of the approaches to overcome such situation is to optimize the enzyme digestion system.

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Notes:

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