EasyGeno Assembly Cloning Kit

For fast, and directional cloning of the single or multiple DNA into any vector

www.tiangen.com/en

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Cat. no. 4992812/4992813

Kit Contents

Contents	4992812 (10 rxn)	4992813 (20 rxn)
2×EasyGeno Assembly Mix	50 µl	2× 50 µl
Linearized pUC19 Control (50 ng/µl)	10 µl	10 µl
2 kb Control Blunt Insert (50 ng/µl)	10 µl	10 µl
ddH_2O	1 ml	2 × 1 ml
HandBook	1	1

Storage

EasyGeno assembly cloning kit should be stored at -30~-15°C. Avoid repeated freezing and thawing. The shelf life is one year.

Introduction

EasyGeno Assembly Cloning Kit is designed for the fast, and directional cloning of the single or multiple DNA into any vector, regardless of DNA fragment end compatibility. The core component of this kit is the 2×EasyGeno Assembly Mix, which fuse DNA insert(s) and linearized vector based on the 15~25 bp overlap sequence at their ends. The 15~25 bp overlap sequence can be engineered by designing primers for amplification of desire DNA fragments. During the EasyGeno assembly reaction, the DNA insert(s) and linearized vector will be accomplished as a closed loop plasmid by sticky-ends creating, gaps filling, and ends linking at the overlap regions. And the product of the assembly reaction can be directly to transform competent cells and then select colonies.

EasyGeno Assembly Cloning Kit joins DNA fragments in a one-step isothermal reaction, only for 15 min at 50°C.

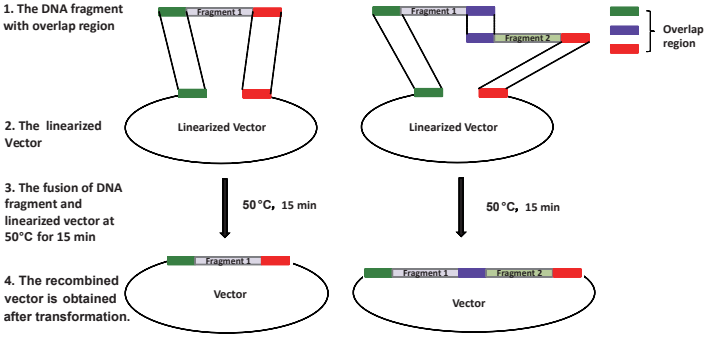

Figure1. Overview of the EasyGeno assembly method

Features

1. The rapid recombination of vector and fragment can be realized by sequence homology for 15 min without restriction of enzyme site.
2. The PCR product of the inserted fragment is correct and single, and can be directly recombined with the vector without purification.
3. 1-5 inserted fragments are recombined into any vector in only one step.
4. Fragment ligation based on homologous recombination is more suitable for high throughput vector construction.
5. *E.coli* sensitive cells can be transformed selectively for 5 minutes.

Important Notes Please read these notes before use.

1. For the single fragment recombination, if only one PCR band is shown, PCR product purification is not necessary. You can add unpurified PCR product directly into EasyGeno assembly reaction for up to 20% of the total volume. Note that the recombination efficiency is lower than the purified PCR product.
2. EasyGeno assembly kit could achieve oriented-assembly of 1-5 inserts into a vector. For multiple inserts assembly, it is recommended to purify the target fragments by gel-extraction.
3. If you want to retain the enzyme digesting site for subsequent identification, it is recommended to add the missing enzyme digestion site sequence.

Protocol

1. Preparation of a Linearized Vector

To achieve a successful assembly reaction, you must first generate a linearized vector. The linearized vector can be generated using restriction enzymes (single or double digests) or by PCR. Then, recover the vector by gel recovery. For PCR products amplified with plasmid template, it is recommended to digest the remaining plasmid template with Dpn I endonuclease.

2. Preparation of PCR products

It is recommended to use high fidelity DNA polymerase for PCR amplification, such as *Pfu* DNA polymerase (4992760), Ultra HiFidelity PCR Kit, etc.

a. Design PCR primers for a single DNA fragment assembling into a linearized vector

Successful insertion of a PCR fragment requires that the ends of PCR insert share 15~25 bases of homology with the ends of the linearized vector. This sequence homology is added to the insert through the PCR primers. During the assembly reaction, the conjunction fragments will be fused at the 3' end of overlap DNA double strands, so the 15~25 nt overlap sequence to be incorporated into each primer, starts at the 3' end of DNA double strands of a linearized vector (as shown in figure 2). The priming gene-specific sequence is added to the 3' end of the primer after the overlap sequence. The priming sequence should meet the criteria required for the template annealing during PCR amplification.

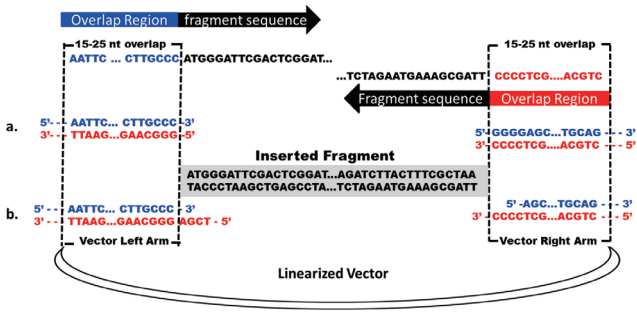

Figure2. Design PCR primers with overlap sequence

b. Design PCR primers for multiple DNA fragments assembling into a linearized vector

To achieve efficient assembly of multiple PCR fragments into a linearized vector, we suggest using a 15~25 nt overlap sequence. It is recommended to perform DNA fragment assembly firstly in silico and create a final sequence display both DNA strands. As shown in figure 3, first, mark the junctions between the fragment 1, 2 and 3. Next, select a 15~25 bp sequence at each junction to serve as the overlap region between the two adjacent fragments. Finally, add the overlap sequences to the 5' end of gene-specific primers. The reverse primer of Fragment 1 and the forward primer of Fragment 2 share the 15~25 nt overlap sequence. The reverse primer of Fragment 1 contains the overlap region A at the 5' end and Fragment 1-specific region at 3' end. And the forward primer of Fragment 2 contains the overlap region A at the 5' end and Fragment 2-specific region at 3' end. And so on, for the reverse primer of Fragment 2 and the forward primer of Fragment 3. It is noted that the forward primer of Fragment 1 and the reverse primer of Fragment 2 share overlap sequence with left and right arm of the linearized vector.

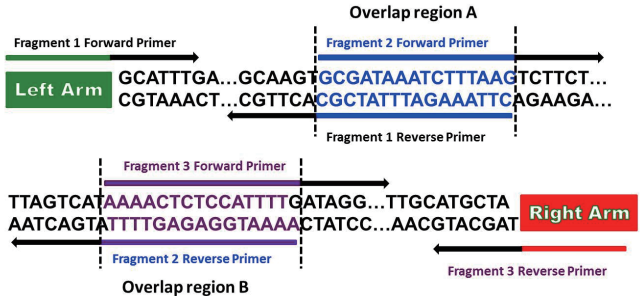

Figure 3. Design PCR primers for multiple fragments assembly into a linearized vector

c. Design PCR primers using online tool EasyGeno Primer

EasyGeno primer is an online tool, available at www.tiangen.com/en. It can be used to design PCR primers with overlap sequence between the adjacent DNA fragments and for their assembly into a linearized vector.

In some case, it might be appropriate to further manually alter primer sequence to adapt them for the use in more complex assembly. So it is necessary to understand the general principle of primer design used in the EasyGeno assembly.

Primer design and quality are critical for the success of the assembly reaction. PCR primers used for the EasyGeno assembly must have two sequence components:

- An overlap sequence, required for the assembly of adjacent fragments, which is added at the 5' end of the primer.
- A gene-specific sequence, required for template priming during PCR amplification, which is added at the 3' end of the primer.

3. EasyGeno assembly reaction

a. Set up the following reaction in ice (centrifuge briefly to collect the liquid to the tube bottom):

components	Amounts	Negative control	Positive control
Linearized vector	0.01~0.25 pmol *	0.01~0.25 pmol	Linearized pUC19 Control, 1 µl
Insert fragment	0.01~0.25 pmol**	0.05~0.5 pmol	2 kb Control Blunt Insert, 2 µl
2×EasyGeno Assembly Mix	5 µl***	---	5 µl***
ddH_2O	Up to 10 µl	Up to 10 µl	Up to 10 µl

pmol =(weight in ng)/ (base pairs × 0.65 kDa)

* Optimized weight of the linearized vector in EasyGeno assembly reaction is 50 ~100 ng.

** 2~3 fold of excess inserts is suggested. Use 5~10 times of inserts if size is less than 200 bp. For assembly of 2~5 inserts, the equimolar ratio is suggested. For unpurified PCR products, it is suggested to add less than 2 µl insert in 10 µl reaction system.

*** To guarantee a higher efficiency, it is recommended to add the linearized vector and inserts firstly, and then add 2×EasyGeno Assembly Mix.

4. Chemical Transformation Protocol

All procedure must be done in asepsis environment.

a. Transformation Step

i. Remove tube(s) of TOP10 or DH5α or T-Fast competent cells from storage and place in an ice bath until just thawed.

Note: It is recommended to use 50~100 µl competent cells for each transformation, so please aliquot the competent cells accordingly.

ii. Carefully add 5~10 µl assembly reaction mixture to 50~100 µl competent cells (100 µl of competent cells can be saturated by 1 ng of plasmid DNA). Gently flick the tubes to mix and place them on ice for 30 min.

Note: The volume of assembly reaction mixture should not exceed 1/10 of the competent cells. The following protocol is for 50 µl competent cells.

iii. Heat-shock the cells for 60~90 s in a water bath at exactly 42°C (do not shake). Immediately return the tubes to ice for 2~3 min (do not shake).

iv. Add 350 µl room temperature SOC or LB culture medium per tube (not containing antibiotic), and then incubate for 45 min at 37°C with shaking (~180 rpm).

v. Mix bacterium in the tube completely. Then plate 100 µl transformation culture onto each SOB or LB agar plate containing antibiotic to ensure good separation of colonies for subsequent single-colony isolation. Smear bacterium completely with asepsis elbow glass stick or glass beads. After the surface of plate is dry, then put the plate at 37°C for 12~16 hours.

Note: If based on previous experience the number of clones is small, the bacteria can be collected by centrifuging the bacterial solution at 4,000 rpm for 10 min. Discarding the medium, and add 100~200 µl SOC or LB medium to resuspend the bacteria, and transferring to the SOB or LB solid agar medium containing the corresponding antibiotics to smear evenly.

The growth rate of the competent colony depends on the type of strain, the type of plasmid transformed and the resistance gene.

b. Rapid Transformation Step

This method applies only to ampicillin resistant vector transformation.

i. Remove tube(s) of T-Fast competent cells from storage and place in an ice bath until just thawed.

Note: It is recommended to use 50~100 µl competent cells for each transformation, so please aliquot the competent cells accordingly.

ii. Carefully add 5~10 µl assembly reaction mixture to 50~100 µl T-Fast competent cells (100 µl of competent cells can be saturated by 1 ng of plasmid DNA). Gently flick the tubes to mix and place them on ice for 2 min.

Note: The volume of assembly reaction mixture should not exceed 1/10 of the competent cells. The following protocol is for 100 µl competent cells.

iii. Heat-shock the cells for 90 s in a water bath at exactly 42°C (do not shake). Immediately return the tubes to ice for 2 min (do not shake).

iv. Add 200 µl room temperature SOC or LB culture medium per tube (not containing antibiotic). Mix bacterium in the tube completely. Then plate 200 µl transformation culture onto each SOB or LB agar plate containing ampicillin antibiotic to ensure good separation of colonies for subsequent single-colony isolation. Smear bacterium completely with asepsis elbow glass stick or glass beads. After the surface of plate is dry, then put the plate at 37°C for 6~9 hours.

5. Detection

a. General detection: Inoculate the colony into 1~5 ml liquid LB medium (containing antibiotics), and culture at 37°C overnight with shaking. Save bacterium strain and extract plasmid. To detect whether the fragment has inserted rightly using PCR or enzyme restriction.

For PCR detection of the positive control (2 kb Control Blunt Insert), the follow program can be used:

95°C 2 min
94°C 30 sec
55~65°C 30 sec } 30 cycles
72°C 1 kb/min
72°C 5 min
4°C ∞

Note: The annealing temperature of the detection primer is based on its sequence, and it is suggested to use vector primer for detection, and the PCR extension time is suggested to be 1 kb/min.

b.Quick detection: To detect whether the fragment has inserted rightly, use bacterium PCR directly.

c.Sequencing: Sequence the fragment after general or quick detection.