TIANGEN®

EasyGeno Assembly Cloning Kit

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

www.tiangen.com/en

EasyGeno Assembly Cloning Kit

Cat. no. GVI201

Kit Contents

Contents	GVI201-01 (10 rxn)	GVI201-02 (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.

Introduction

The EasyGeno Assembly Cloning Kit enables rapid, directional cloning of one or multiple DNA fragments into any linearized vector based on sequence homology. The 2× EasyGeno Assembly Mix efficiently recombines homologous regions at the 3' ends to generate seamless circular plasmids, which can be directly transformed into competent cells. This method eliminates the need for restriction sites. By adding 15-25 bp homologous sequences to the 5' ends of PCR primers, both single- and multi-fragment assembly can be achieved with high efficiency.

The recombination reaction is performed under isothermal conditions at 50°C for 15 minutes. Combined with flexible PCR product preparation and rapid transformation procedures, the complete workflow—from DNA preparation to transformation and plating—can be accomplished within a few hours.

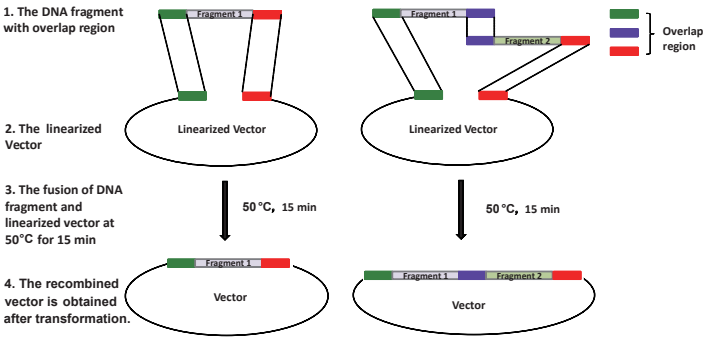


Figure1. Overview of the EasyGeno assembly method

Features

1. No restriction enzyme site limitation; rapid recombination of vector and insert can be achieved within 15 minutes based on sequence homology.
2. PCR products with correct and single bands can be used directly without purification for recombination.
3. Enables one-step directional assembly of 1-5 DNA fragments into any vector.
4. Homology-based assembly is well suited for high-throughput vector construction.
5. Optional 5-minute rapid transformation into *E. coli* competent cells.

Important Notes <u>Please read these notes before use.</u>

1. For single-fragment assembly, if the PCR product shows a single, specific band, it can be used directly without purification. However, the volume added should not exceed 20% of the total reaction volume, and the recombination efficiency may be lower than that obtained with purified PCR products.
2. For multi-fragment assembly, the recombination efficiency is generally lower than that of single-fragment assembly. Gel purification of PCR products is recommended prior to recombination.
3. If restriction enzyme sites need to be retained for downstream verification, it is recommended to introduce the missing restriction site sequences during primer design.

Protocol

1. Preparation of a Linearized Vector

Incomplete plasmid linearization may result in the formation of false (negative) clones. Therefore, it is recommended to linearize plasmids using double restriction enzyme digestion or PCR amplification, followed by gel extraction to purify the linearized vector. For PCR products amplified from a plasmid template, it is recommended to treat the reaction with Dpn I endonuclease to remove any residual template plasmid DNA.

2. Preparation of PCR products

High-fidelity DNA polymerases (e.g., *Pfu* DNA polymerase) are recommended for PCR amplification.

(1) Primer Design for Single-Fragment Cloning

The ends of the linearized vector can be blunt ends generated by restriction digestion (Fig. 2a), 3′ overhangs or 5′ overhangs (Fig. 2b), or blunt ends generated by PCR (Fig. 2a). During primer design, the homologous sequence should be aligned with the 3′-end sequence of the vector. For example, if restriction digestion produces a 5′ overhang, the homologous sequence (typically 15 nt) should be designed based on the corresponding 3′ end of the complementary strand. The forward primer consists of a 15-25 nt sequence homologous to the left arm of the vector plus the gene-specific forward sequence (approximately 22 nt). The reverse primer consists of a 15-25 nt sequence homologous to the right arm of the vector plus the gene-specific reverse sequence (approximately 22 nt).

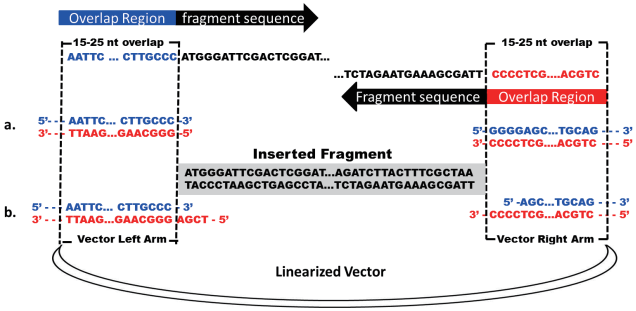


Figure2. Design PCR primers with overlap sequence

(2) Primer Design for Multi-Fragment Cloning

The primer design principles for the vector ends are the same as those for single-fragment cloning. For overlapping regions between fragments (see Fig. 3), the reverse primer of Fragment 1 and the forward primer of Fragment 2 should share a 15-25 nt overlapping sequence. Specifically, the reverse primer of Fragment 1 contains the overlap region (Region A) plus the fragment-specific reverse primer sequence, while the forward primer of Fragment 2 contains the same overlap region (Region A) plus the fragment-specific forward primer sequence. This principle is applied sequentially for additional fragments.

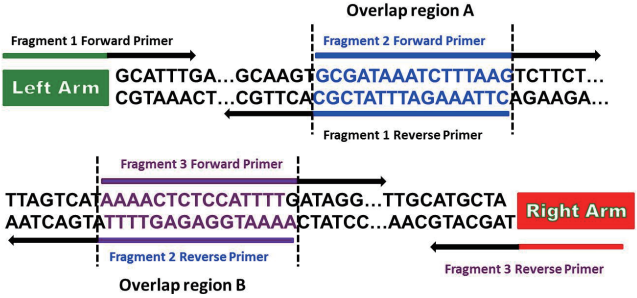
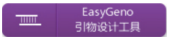


Figure 3. Design PCR primers for multiple fragments assembly into a linearized vector

(3) Primer Design Using the EasyGeno Primer Online Tool

EasyGeno EasyGeno Primer is a specialized primer design tool developed for use in conjunction with the EasyGeno Rapid Cloning Kit (GVI201). It is specifically designed to support the design of PCR primers for target DNA fragments in both single-fragment and multi-fragment directional cloning into vectors using EasyGeno technology.

To access the tool, please log in to the TIANGEN official website and click on 

from the homepage.

3. Preparation of the Recombinant Reaction System

(1) Carefully add the following components to the bottom of a PCR tube.

If any liquid adheres to the tube wall, briefly centrifuge to ensure that all contents are collected at the bottom of the tube.

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 = mass (ng) / [fragment length (bp) × 0.65 kDa]

* The recommended amount of vector is 50-100 ng.

** The recommended molar ratio of vector to each insert fragment is 1:2 to 1:3. If unpurified PCR products are used, it is recommended to add no more than 2 μl in a 10 μl reaction system.

*** For higher recombination efficiency, it is recommended to add the 2× EasyGeno Assembly Mix after the linearized vector and insert fragments have been added when setting up the recombination reaction.

(2) Incubate the reaction mixture at 50°C for 15 minutes.

For assemblies involving more than three fragments, it is recommended that the overlapping sequences in the primers be ≥20 nt, and the reaction time be extended to 60 minutes.

After the incubation, briefly centrifuge the tube and place it on ice, then proceed with the subsequent transformation step.

4. Transformation (Perform the following steps under sterile conditions).

(1) Standard Transformation Procedure

i. Place competent cells (DH5α, TOP10, or T-Fast) on ice to thaw. If aliquoting is required, transfer the freshly thawed cell suspension into sterile, pre-chilled microcentrifuge tubes and keep on ice.

Note: The recommended volume of competent cells per transformation is 50-100 µl, which can be adjusted according to experimental needs.

ii. Add 5-10 µl of the recombination product to the competent cell suspension (100 µl of competent cells can be saturated with 1 ng of supercoiled plasmid DNA). Gently flick the tube to mix and incubate on ice for 30 minutes.

Note: The volume of DNA added should not exceed one-tenth of the volume of the competent cells. The following procedure uses 50 µl of competent cells as an example.

iii. Place the tube in a 42°C water bath for 60-90 seconds, then immediately transfer it to ice and incubate for 2–3 minutes to cool the cells. Do not shake the tube during this process.

iv. Add 350 µl of sterile SOC or LB medium **(without antibiotics)** to each tube. Mix gently and incubate at 37°C with shaking at 180 rpm for 45 minutes to allow expression of the antibiotic resistance gene carried by the plasmid and recovery of the cells.

v. Mix the transformation reaction gently, then spread 100 µl of the transformed competent cells onto SOB or LB agar plates containing the appropriate antibiotic. Evenly distribute the cells using sterile spreading beads (GGB101) or a sterile spreader. Allow the plates to stand at room temperature until the liquid is absorbed, invert the plates, and incubate at 37°C for 12-16 hours.

Note: If a low colony number is expected based on prior experience, centrifuge the culture at 4,000 rpm for 10 minutes to collect the cells. Discard the supernatant, resuspend the pellet in 100-200 µl of SOC or LB medium, and then spread evenly onto SOB or LB agar plates containing the appropriate antibiotic.

The growth rate of colonies depends on the bacterial strain, the type of plasmid introduced, and the antibiotic resistance gene carried.

(2) Rapid Transformation Procedure

i. Place T-Fast competent cells on ice to thaw. If aliquoting is required, transfer the freshly thawed cell suspension into sterile, pre-chilled microcentrifuge tubes and keep on ice.

Note: The recommended volume of competent cells per transformation

is 50-100 µl, which can be adjusted according to experimental needs.

ii. After the competent cells are completely thawed, add 5-10 µl of the recombination product to the cell suspension (100 µl of competent cells can be saturated with 1 ng of supercoiled plasmid DNA). Gently mix without vortexing, and incubate on ice for 2 minutes.
Note: The volume of DNA added should not exceed one-tenth of the competent cell volume. The following procedure uses 100 µl of competent cells as an example.

iii. Incubate the transformation mixture in a 42°C water bath for 90 seconds, then immediately transfer the tube to ice and cool for 2 minutes. Do not shake the tube during this process.

iv. Add 200 µl of sterile SOC or LB medium **(without antibiotics)** to the transformation mixture and mix gently. After mixing, directly spread 200 µl of the transformed competent cells onto SOB or LB agar plates containing the appropriate antibiotic. Evenly distribute the cells using sterile spreading beads (GGB101) or a sterile spreader. Allow the plates to stand at room temperature until the liquid is absorbed, then invert the plates and incubate at 37°C for 6-9 hours.
Note: For vectors conferring ampicillin resistance, plate the cells directly after mixing. For vectors conferring kanamycin resistance (kanamycin sulfate) or other antibiotics, incubate the cells at 37°C with shaking at 180 rpm for 45-60 minutes before plating, then spread 100-200 µl onto the corresponding selective plates.

Analysis

1. Standard Analysis:

Inoculate a single colony into 1-5 ml of LB medium containing the appropriate concentration of antibiotic, and incubate overnight at 37°C with shaking. After preserving the bacterial culture, extract the plasmid and verify the inserted fragment by PCR or restriction enzyme digestion.

For PCR verification of the Control Insert DNA, please refer to the reaction conditions below:

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

Note: The annealing temperature for detection primers should be determined based on the primer sequences. It is recommended to use vector-specific primers for verification. The PCR extension time is recommended to be 1 kb/min.

2. Rapid Analysis:

Pick a colony and perform colony PCR directly (for detailed procedures, refer to *Molecular Cloning, 3rd Edition*).

3. Sequencing Verification:

After preliminary verification using standard or rapid methods, perform DNA sequencing to confirm the inserted fragment.