enzynomics

EZ-Fusion™ Cloning Kit

User Manual

EZ-Fusion™ Cloning Kit (Cat.EZ015)
EZ-Fusion™ Cloning core Kit (Cat.EZ016)
EZ-Fusion™ Cloning Kit (*Dry Type*) (Cat.EZ019)
EZ-Fusion™ Cloning core Kit (*Dry Type*) (Cat.EZ020)

Contents

I. Introduction

EZ-Fusion™ Cloning Kit is designed for rapid and efficient cloning of PCR-amplified DNA fragments into any cloning vector including commercial and customized ones. It is also possible to insert one or more DNA fragments into a cloning vector in a defined orientation. EZ-Fusion™ Cloning Kit allows terminal 18 base pair overlapping homologous DNA at the ends of linearized vectors (usually by restriction enzymes) and insert DNA fragments (usually PCR-amplified) to precisely recombine to generate cloning products. In addition, the EZ-Fusion™ Cloning Kit can be used to clone very long DNA fragments with high efficiency.

II. Advantages and features

- Efficient cloning of a broad range of insert DNA fragments
- Cloning insert DNA fragment into any location of a cloning vector
- Cloning multiple insert DNA fragments simultaneously in a single reaction
- Ligase-independent, thus eliminating background
- No alteration in DNA sequence of final cloning products

III. EZ-Fusion™ Cloning Kit components

(1) EZ-Fusion™ Cloning Kit components

EZ-Fusion™ Cloning Kit	Cat.EZ015S	Cat.EZ015M	Cat.EZ015L
	10 rxns	20 rxns	40 rxns
5X EZ-Fusion™ Cloning PreMIX	20 µl	40 µl	80 µl
pUC19 control vector, linearized (50 ng/µl) (positive control)	10 µl	20 µl	40 µl
2 kb control insert (40 ng/µl) (positive control)	10 µl	20 µl	40 µl
Sterile water	0.5 ml	1 ml	2 ml
DH5α chemically competent *E. coli*	100 µl x 11 ea	100 µl x 21 ea	100 µl x 42 ea

EZ-Fusion™ Cloning Kit *(Dry Type)*	Cat.EZ019S	Cat.EZ019M	Cat.EZ019L
	8 rxns	16 rxns	96 rxns
EZ-Fusion™ Cloning DryMIX	8 tubes	16 tubes	96 tube
pUC19 control vector, linearized (50 ng/µl) (positive control)	10 µl	20 µl	20 µl
2 kb control insert (40 ng/µL) (positive control)	10 µl	20 µl	20 µl
Sterile water	0.5 ml	1 ml	1 ml
DH5α chemically competent *E. coli*	100 µl x 11 ea	100 µl x 21 ea	100 µl x 105 ea

※ EZ-Fusion ™ Cloning Core Kit (Cat.EZ016) and EZ-Fusion ™ Cloning Core Kit (Cat.EZ020) are also available, which exclude only DH5α chemically competent *E. coli.*

(2) EZ-Fusion™ Cloning Kit storage condition

DH5α Chemically competent *E. coli* should be stored at -80℃ and other components should be stored at -20℃.

IV. Quick Protocol for EZ-Fusion™ Cloning Kit

1. Determine a cloning site in a target vector and generate linearized vector DNA by restriction enzyme digestion or inverse PCR (Refer to Fig. 1. step 1 “Preparation of Linearized Vector”).

2. Prepare a pair of primers (forward/reverse) to PCR-amplify target DNA fragments to be cloned. Each primer should contain gene specific 20-nucleotides and additional 18-nucleotides (restriction enzyme site 6 nt + end sequence of vector 12 nt) that have homology to each end of the linearized vector prepared above. (Refer to the illustration in Fig. 1. step 2 “Primer Design”)

3. Carry out PCR reaction to amplify target DNA. Confirm amplification of PCR products in agarose gel electrophoresis (Refer to Fig. 1. step 3).

4. Purify PCR products. Omit this step if the PCR amplification is satisfactory.

5. Set up the following cloning reaction with vector and insert DNA prepared

Component	Volume
5X EZ-Fusion™ Cloning PreMIX (EZ-Fusion™ Cloning DryMIX)	2 µl (1 tube)
[a)] Linearized vector	x µl
[a)] Purified PCR fragment	y µl
Sterile water	up to 10 µl

[a)] It is better to prepare the DNA with higher concentration or dissolved in distilled water when the combined volume of vector and insert DNAs are larger than 6 µl (EDTA and glycerol reduce cloning efficiency).

6. Incubate the reaction mixture for 15 min at 37°C, then place the reaction tube on ice.
   * If the reaction continues for more than 15 minutes, the cloning efficiency is reduced. If you react at 60 minutes, the cloning efficiency can be reduced to 1/10 of that at 15 minutes. Please keep the reaction time exactly.

7. Use the reaction mixture to transform the competent *E. coli* cells. Cloning reaction mixtures can store at -20°C for few days.

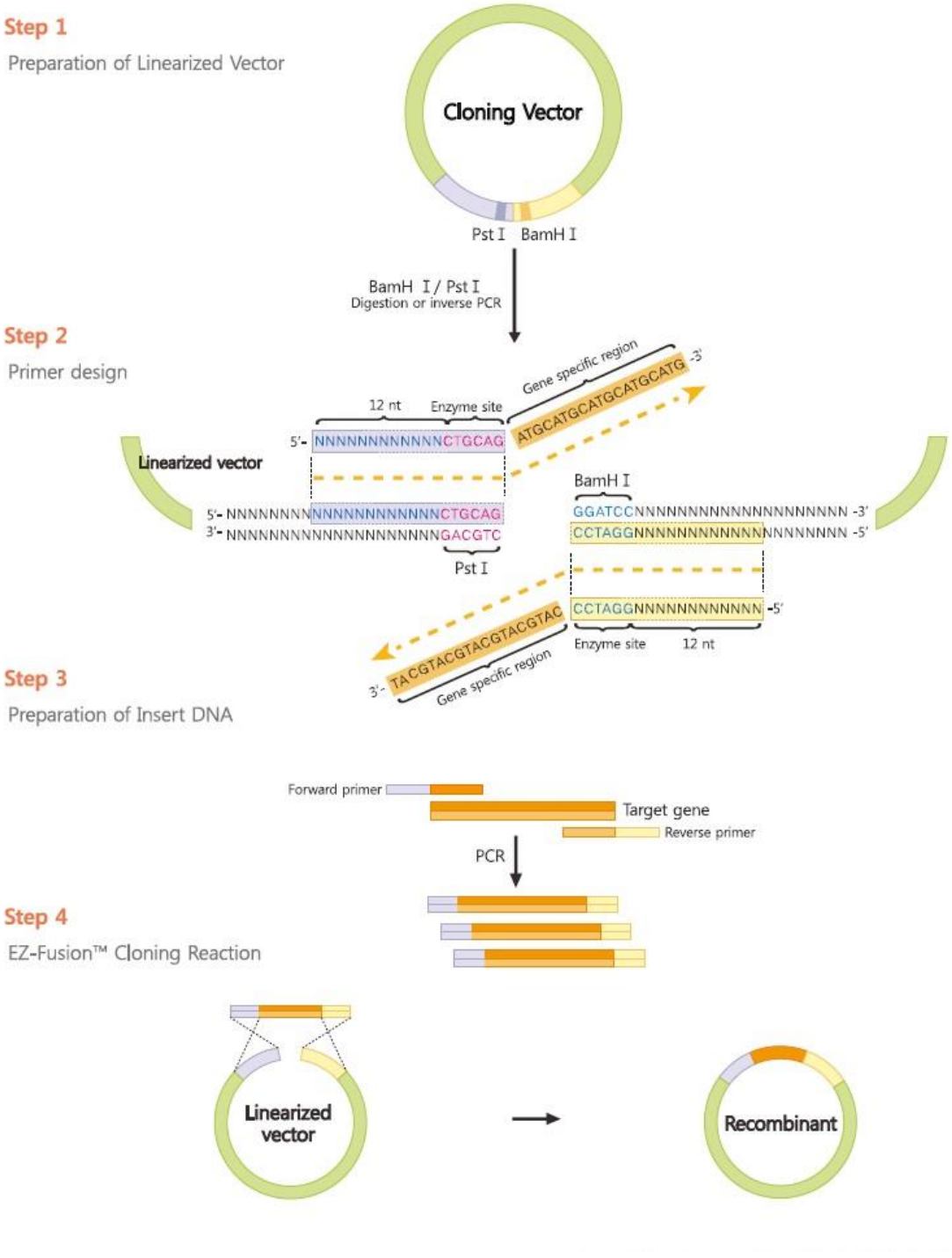

Fig. 1. Summary of EZ-Fusion™ Cloning Protocol.

V. Detailed Protocol for EZ-Fusion™ Cloning Kit

(1) Preparation of a linearized vector

A linearized vector can be generated by conventional methods using one or two restriction enzymes. PCR-amplified DNA can be cloned into the cleaved region in the linearized vector. Inverse PCR can also be used to generate a linearized vector.

Efficient cleavage of vector is critical to significantly reduce the background level of false colonies. High-transformation efficiency of undigested residual supercoiled or circular plasmids could give rise to high background levels. Therefore, caution should be taken in vector digestion. Use recommended conditions to prepared linearized vectors as exemplified below.

1. Avoid single-enzyme digestion to prepare a linearized vector. If possible, use two restriction enzymes to generate linearized vector. Double digestion can markedly reduce the supercoiled or circular plasmid DNA. In addition, insert cannot be cloned into vector linearized by a single enzyme in double digestion and do not give rise to background due to low transformation efficiency. Therefore, double digestion markedly reduces background colony formation.

2. Conduct the restriction digestion according to the conditions given by the restriction enzyme supplier. Incubate 3~12 hours with high-purity restriction enzymes to reduce the background colony formation. Enzynomics provides high-purity restriction enzymes.

3. After digestion, purify the linearized vector using PCR-product purification Kit. This step may not be required if the restriction enzyme used can be completely inactivated by heating.

(2) PCR primer design

The quality of primers is critical for successful DNA amplification. Specific and efficient amplification of target DNA fragment ensures successful cloning. The 5' side of the primers must contain 18 base-pair stretches homologous to one of the two ends of linearized cloning vector. Cloning takes place via recombination between the homologous DNA sequences at each end.

※ Two important features of primers for EZ-Fusion™ Cloning Kit

① 5' region of the primer must contain 18-nt which is identical to the very end of linearized vector (restriction recognition sequence: 6-nt + Vector homology sequence: 12-nt).

- The length of 'Restriction recognition sequence' is dependent on the selected restriction enzyme.
- The length of 'vector homology sequence' can be varied from 9 to 12-nt.

② 3' region of the primer must contain the specific sequence for amplifying the gene of interest (Refer to the illustration in Fig. 1. step 2 "Primer Design").

③ The Primer design program is available on our homepage (www.enzynomics.com). (Technical > Tool > EZ-Fusion™ Cloning Kit primer design program)

(3) PCR amplification and purification of insert DNA fragment

The 3' part of the primer intended for PCR amplification can be designed like ordinary primers for PCR use. Their length is within 20 nucleotides with a GC-content between 40-60% and T_m between 58~65℃. The difference in T_m between two primers should not exceed 4℃ as of ordinary PCR primers. The last five nucleotides at the 3' end of each primer should contain no more than two guanines (G) or cytosines (C) to reduce nonspecific DNA amplification.

EZ-Fusion™ Cloning Kit can be used to clone any insert amplified by thermostable polymerases. We recommend *n*Pfu-Forte (Cat. P410) to reduce errors in amplified DNA.

Analyze DNA by gel electrophoresis to verify specific DNA amplification and estimate the amount and concentration of amplified DNA. Estimate DNA concentration by using size standard DNA as reference on an agarose gel. Use spin-column purification if the insert DNA amplified shows a specific single band in the gel (caution: If the selection marker of the cloning vector is the same as that of plasmid used for PCR template, it is recommended to treat the PCR reaction mixture with Dpn I to destroy the template DNA. Dpn I treatment reduces the background colony formation.) If non-specific bands are also present in the agarose gel, purify target PCR products using a gel extraction method.

(4) EZ-Fusion™ Cloning reaction and transformation procedure

Use both positive and negative controls to monitor proper performance of the EZ-Fusion™ Cloning Kit. Negative control is the reaction carried out with linearized vector only. Reactants for positive control are included in EZ-Fusion™ Cloning Kit.

① Refer to the below to set up the cloning reaction

EZ-Fusion™ cloning reaction			
Component	cloning reaction	Positive control	Negative control
[a] Cloning vector (restriction-enzyme digested)	50~200 ng	1 µl (Control Vector)	1 µl
[b] Insert DNA (PCR-amplified DNA)	10~200 ng	2 µl (Control Insert)	
5X EZ-Fusion™ Cloning PreMIX	2 µl	2 µl	2 µl
[c] Distilled water	up to 10 µl	up to 10 µl	up to 10 µl

[a] Cloning vector

: We recommend using 50 ~ 100 ng for under 10 kb and 50 ~ 200 ng for more than 10 kb.

[b] Insert DNA
: Please use vector and insert ratio in 1: 2 ratio.
: insert amount = (insert size) / (vector size) x (vector amount) x 2

[c] If the volume of vector and insert DNA is more than 6 µl, cloning reaction may be inhibited depending on the composition of the buffer containing the DNA (EDTA, Glycerol, etc.). In this case, prepare DNA again at high concentration or use DNA dissolved in sterilized water.

② Incubate the reaction mixture at 37℃ for 15 min, then place the tube on ice.

③ 2.5 µl of the EZ-Fusion™ reaction mixture is used transformation to chemically competent *E.coli* cells with. Grow transformed cells on plates containing appropriate antibiotics according to the selection marker in the vector used. We recommend that both positive and negative controls should be included for every experiment. Use competent *E.coli* cells with transformation efficiency over 1 x 10^8 cfu/µg

(5) Expected results

Several hundred of colonies will grow on positive control plates if you use competent cells with efficiency of 1 x 10^8 cfu/µg. The negative control normally produces only a few colonies in a single plate.

- If the number of colonies on both negative and positive plates are two low (usually ~10), this is either because an excess amount of the EZ-Fusion™ reaction mixture is used for transformation or because qualities of vector DNA and/or the primers are low.

- If hundreds of colonies appear on the negative control, this is due to incomplete vector digestion.

VI. Troubleshooting guide

If you do not obtain the expected results as described above, use the following guide to solve the problem.

A. No colonies appear after transformation

Description	Solution
Low transformation efficiency	Do not add more than 10 µl of the EZ-Fusion™ reaction mixture to 100 µl competent cells.
	Do not exceed incubation time of EZ-Fusion™ cloning reaction more than 15 minutes. Prolonged incubation inhibits cloning efficiency.
	For a few strains, it may be better to dilute EZ-Fusion™ reaction mixture in TE buffer up to 100 folds.
	Check transformation efficiency. Use competent cells with efficiency over $\geq$ 1 x 10^8 cfu/µg.
Low quality DNA fragments	EZ-Fusion™ reaction becomes more efficient with higher concentration of DNA. Thus, it is important to prepare highly concentrated DNA for EZ-Fusion™ reaction. The amount of cloning vectors used varies ranging from 100 to 400 ng according to their size If gel extraction is used to purify the insert DNA, it is important to prepare highly concentrated DNA solution to minimize contaminants originated from gel. The combined volume of purified vector and insert should not exceed 6 µl for EZ-Fusion™ reaction.
	Check primer sequences and confirm that they contain 15 base homology with the vector at the insertion site.

B. Large numbers of colony formation or contain no insert

Description	Solution
A large number of colonies without insert DNA are formed	It is important to remove any uncut vector prior to use in the EZ-Fusion™ reaction. If necessary, recut vector and gel purify.
	If the insert DNA was amplified using a plasmid as template, the template plasmid may have been carried over through purification to contaminate the cloning reaction. a) We recommend linearizing the template DNA prior to performing PCR. b) If you use spin-column to purify the insert DNA, it is recommended to treat the PCR reaction mixture with Dpn I to destroy the template DNA. Dpn I treatment reduces the background colony formation.
	Be sure that antibiotics-containing plates are fresh (<1 month old from preparation). Check the antibiotic resistance marker of your plasmid DNA.

C. Clones that contain incorrect insert DNA

증상	해결 방법
colonies contain incorrect insert DNA	If PCR products do not appear as a single distinct band, it is necessary to gel purify the target PCR product.