Cold Fusion Cloning Kit

Cat. #s MC010A-1, MC100A-1, MC101A-1

User Manual

Store the master mixture and positive controls at -20˚C
Store the competent cells at -80˚C.

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I. Introduction

The Cold Fusion technology is a simple, rapid and highly efficient PCR cloning kit. It allows you to directly clone any PCR product(s) to any linearized expression vector, at any site. The PCR fragments can be generated by Taq DNA polymerase or other high fidelity DNA polymerases, with primers that are designed to have ~15 bases of homology at the linear ends where the DNA-of-interest will "fuse". The linearized vector can be generated by PCR or restriction enzyme digest (single or double cut). With a one tube simple reaction format, a 5 minute incubation at room temperature, followed by 10 minutes on ice, your PCR product(s) rapidly and accurately fuse into the linearized vector in the desired orientation. The kit is so robust that multiple DNA fragments can be assembled simultaneously and cloned into one construct in a single step. The system is highly efficient, with more than a 95% positive cloning rate.

A. Key Features

- Cloning is simple, rapid, accurate and directional
- Clone any insert, at any site within any vector
- Restriction enzyme, phosphatase and ligase free system
- Broad PCR size
- Joining multiple fragments at once
- High efficiency with > 95% positive clones

B. Applications

- PCR cloning into any vector
- Gene transfer from one vector to another
- Add adaptor, linker and tag before or after the insert
- Gene synthesis
- High throughput cloning

C. List of Components

Cat. No. MC010A-1

5x Master Mixture (20rxns)	20µl
Linearized vector, positive control	5µl
500bp PCR insert, positive control	5µl
Competent cells ($1x10^9$ cfu/µg)	10 tubes
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Cat. No. MC100A-1

5x Master Mixture (20rxns)	40µl
Linearized vector, positive control	5µl
500bp PCR insert, positive control	5µl
Competent cells ($1x10^9$ cfu/µg)	20 tubes
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Cat. No. MC101A-1

5x Master Mixture (50rxns)	100µl
Linearized vector, positive control	10µl
500bp PCR insert, positive control	10µl
Competent cells ($1x10^9$ cfu/µg)	50 tubes
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D. Storage

Store the master mixture and positive controls at -20˚C. Store the competent cells at -80˚C.

E. Other Reagents Needed

- Gene-specific primers
- dNTPs, Taq or other high fidelity polymerase, and corresponding buffers for PCR
- QIAquick PCR Purification Kit (Cat # 28106, Qiagen)
- QIAquick Gel Extraction kit (Cat # 28704, Qiagen)
- SOC or LB Broth for transformation of bacteria
- LB + 100 µg/ ml Ampicillin plates

F. Technical Information

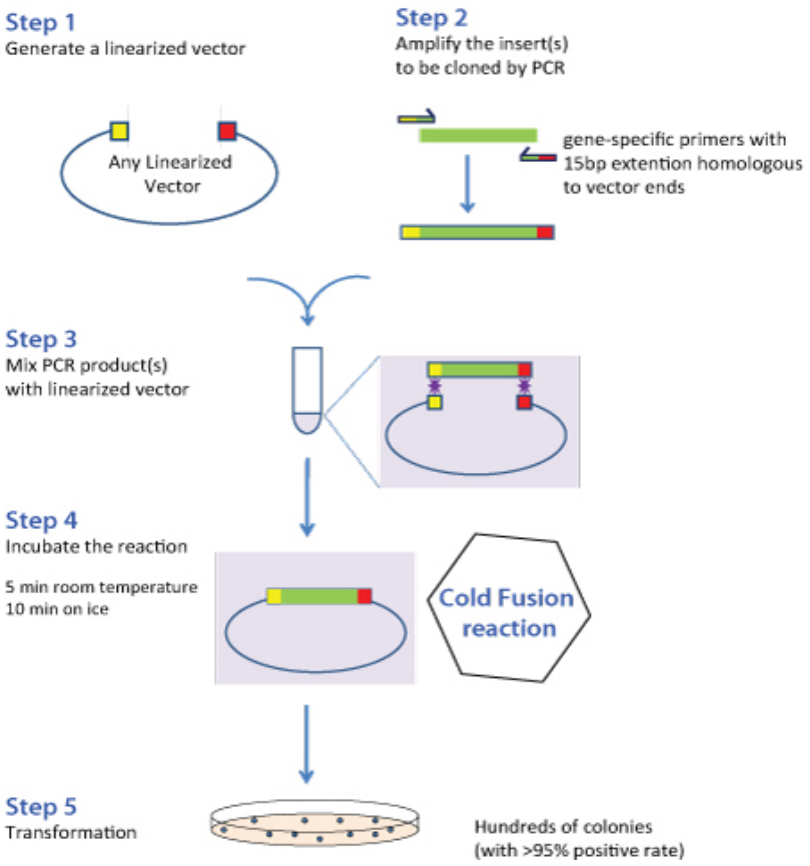
The competent cells provided with the Cold Fusion Cloning Kit has the following genotype:

F' {proAB+ lacIq lacZ Δ M15 Tn10(TetR) Δ (ccdAB)} mcrA Δ (mrr-hsdRMS-mcrBC) ϕ 80(lacZ) Δ M15 Δ (lacZYA-argF) U169 endA1 recA1 supE44 thi-1 gyrA96 relA1 tonA panD

Due to the presence of the Tn10 transposon element, which encodes the tetracycline-resistance gene (TetR), the competent cell will be able to grow in LB-Agar plates containing 10 µg/ml of tetracycline. Cloning of inserts into plasmids containing a TetR marker and transforming the competent cells in the kit will likely lead to the failure of the cloning reaction. In this case, we would recommend another high-quality competent cell that does not have built-in tetracycline resistance (TetR) for best results.

II. Protocol

A. Overview



B. Preparation of Linearized Vector

Complete linearization of the vector is critical to achieve a successful Cold Fusion cloning reaction. Incomplete linearization of the vector will result in high background. The linearized vector can be generated by PCR or restriction enzyme digest (single or double digest) and should be purified using a gel or PCR purification kit.

Due to the digestion efficiency, different restriction enzymes will generate different levels of background. In general, two enzyme digestion is better than a single enzyme digestion. The further the restriction sites are apart, the better the digestion efficiency. Increasing the enzyme digestion time and the digestion reaction volume will also help reduce the background. For many enzymes, we recommend incubate the digestion reaction between 3 hours and overnight in order to increase linearization and reduce background.

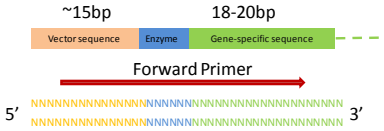
Check the background of your vector by transforming 1μg (10-100ng) linearized and purified vector into competent cells. If the background is high, continue digesting the remaining vector for a longer time after the addition of more restriction enzyme(s).

We recommend digesting 2μg vector in 50μl reaction overnight. Use QIAGEN's QIAquick Spin Gel Extraction kit for gel purification and elute the DNA with 30μl dH_2O.

C. Primer Design

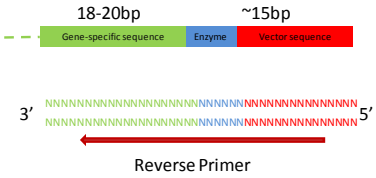
Forward Primer Design

The forward primer should contain 18-20 bases complimentary to the 5' end of your gene-of-interest plus 15 bases corresponding to the vector. Depending on the purpose of your cloning, your primers might also need to contain a Kozak sequence and/ or ATG start site.



Reverse Primer Design

Reverse primers should be made to the negative strand of DNA. Depending on the vector you are using, the 3' end of the insert may need to contain a stop codon (TAG, TAA, or TGA). The 3' primer should contain 18-20 bases complimentary to the 3' end of your gene-of-insert, plus 15 bases corresponding to the vector.



Introducing Restriction Sites

A restriction site can be introduced in the middle of the primer and can be the same as or different than the one used to linearize the vector. For multiple DNA fragment joining, it is recommended that each PCR product shares ~ 18 base pairs of homology.

D. PCR Requirements

- The PCR fragments for the cDNA-of-interest should be generated using *Taq* DNA polymerase or other high fidelity DNA polymerase.
- The melting temperature (Tm) should be calculated based on the gene-specific ends of the primer, NOT the entire primer.
- Specific PCR reaction conditions should be optimized for the cDNA-of-interest.
- After completion of the PCR reaction, gel purify the appropriate band to remove any extra primers or primer dimmers that will inhibit the Cold Fusion reaction. We recommend the QIAquick PCR Purification Kit (Cat # 28106, Qiagen).

E. Cold Fusion Reaction

Set up the following reaction in a 1.5 ml sterile reaction tube by mixing the following reagents gently and then spin down briefly to collect the reagents at the bottom of the tube.

Cloning reaction

Linearized destination vector (10-100ng/µl)	1µl*
PCR insert(s) (20-200ng/µl) (for each PCR Product)	1µl*
dH_2O	_µl

5x master mix	2μl
total	10μl

Positive control reaction

Linearized vector (positive control)	1μl
500bp PCR insert (positive control)	1μl
dH2O	6μl
5x master mix	2μl
total	10μl

Negative Control

Linearized destination vector (10-100ng/μl)	1μl*
dH_2O	7μl
5x master mix	2μl
total	10μl

* 2:1 or 1:1 molar ratio of insert: vector works well in the Cold Fusion reaction.

For reactions with larger volumes of vector and insert (>8μl of vector + insert), double the amount of reaction buffer and enzyme, and add dH_20 for a total volume of 20μl.

When using Cold Fusion cloning kit for the first time, we strongly recommend that you perform the positive and negative control reactions in parallel with your Cold Fusion cloning reaction. The positive control 500bp PCR insert and linearized vector provided in the kit have already been purified. There is no treatment needed prior to the cloning reaction.

Cold Fusion Reaction Incubation

1. 5 minutes at room temperature
2. 10 minutes one ice

F. Transformation

1. Add 50μl Cold Fusion competent cells to the cloning mixture
2. Incubate on ice for 20 minutes
3. Heat shock at 42˚C for 50 seconds
4. Transfer on ice for 2 minutes
5. Add 250μl S.O.C medium or LB broth
6. Incubate at 37˚C for an hour
7. Take 100μl culture spread on pre-warmed (37˚C) culture plate containing Ampicillin
8. Incubate the plate at 37˚C overnight

III. Examples

A. Primer Design for Positive Control Reaction

1 Original Vector Sequence. Linearize with EcoRI and BamHI

5'- ACGACGTTGAAAACGACGGCGAGTGAATTCGAGCTCGGTACCCGGGGATCCTCTAGAGTCGACCTGCAGGCATGCAAGCT -3'

2 Construct-of-interest shown below. Black = Vector sequence. Blue = Insert sequence. Red = restriction site

5'- Forward Primer -3'

5'- ACGACGTTGAAAACGACGGCGAGTGAATTC atggagagcg acgagagcgg cctgcccgcc atggagatcg agtgccgcat

caccggcacc ctgaacggcg tggagttcga gctggtgggc ggcggagagg gcacccccaa gcagggccgc atgaccaaca agatgaagag

caccaaaggc gccctgacct tcagccccta cctgctgagc cacgtgatgg gctacggctt ctaccacttc ggcacctacc ccagcggcta

cgagaacccc ttcctgcacg ccatcaacaa cggcggctac accaacaccc gcatcgagaa gtacgaggac ggcggcgtgc tgcacgtgag

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caagatcatc cgcagcaacg ccaccgtgga gcacctgcac cccatgggcg ataacgtgct ggtgggcagc ttcgcccgca ccttcagcct

gcgcgacggc ggctactaca gcttcgtggt ggacagccac atgcacttcaag GGATCCTCTAGAGTCGACCTGCAGGCATGCAAGCT -3

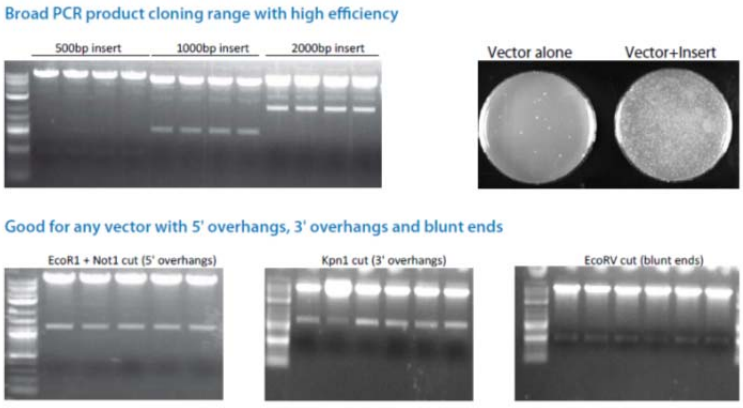
3'-cctgtcggtg tacgtgaagttc CCTAGGAGATCTCAGCTGGAC- 5'

3'- -5' Reverse Primer

3 Forward primer Sequence: 5'-AAAACGACGGCCAGTGAATTCatggagagcgacgagagcggc-3'

Reverse primer Sequence: 5'- CAGGTCGACTCTAGAGGATCCcttgaagtgcatgtggctgtc -3'

B. Cloning a single DNA Fragment



C. Joining Multiple DNA Fragments

Note: For multiple DNA fragments cloning, depending on the number and the size of each insert, you may obtain fewer colonies than those with one or two fragments.

IV. Troubleshooting

Problem	Probable cause	Solution
1. No or few colonies obtained from the transformation	Primer sequences are incorrect	Check primer sequences to ensure that they provide 15 bases of homology with the region flanking the insertion site.
	Suboptimal PCR product	Optimize your PCR amplification reactions so that you generate pure PCR products. Use a different method to purify your PCR product.
	Low DNA concentration in reaction	It is imperative to obtain as high a DNA concentration as possible in your Cold Fusion reaction.
	Inhibitory contaminants from PCR product or linearized vector	Both the PCR product and the linearized vector should be purified.
	Transform with too much reaction mixture	Do not add more than 10μl of reaction mixture to 50μl of competent cells. Too much reaction mixture inhibits the transformation.
	Low quality or poor handling of competent cells	Handle the competent cells gently. Do not re-freeze cells after thawing. Quality of competent cells may be tested by transforming a circular plasmid to determine cells' competency. Competent cells with a transformation efficiency of $1x10^9$ cfu/ µg are recommended.
	Wrong antibiotic or too much	Choose plates with the appropriate concentration of

Problem	Probable cause	Solution
	antibiotic in the media	the right antibiotic.
2. Large numbers of colonies contain no insert	Incomplete linearization of your vector	It is critical to remove any uncut vector prior to use in the Cold Fusion reaction. If necessary, re-digest your vector and gel purify.
	Contamination of cloning reaction with plasmid with the same antibiotic resistance	If you insert was amplified from a plasmid, circular DNA may have carried through purification and contaminated the cloning reaction. We recommended gel purifying your PCR product or linearizing the template DNA before performing PCR.
2. Large numbers of colonies contain no insert	Plates are too old or contained incorrect antibiotic	Make sure that your antibiotic plates are fresh. Check the antibiotic resistance of your fragment.
3. Clones contain incorrect insert	PCR products contain non-specifically amplified artifacts	Optimize your PCR reaction to improve the specificity. Screen more colonies for the correct clones.

V. Technical Support

For more information about SBI products and to download manuals in PDF format, please visit our web site:

http://www.systembio.com

For additional information or technical assistance, please call or email us at:

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VI. Licensing and Warranty

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