



Cold Fusion Cloning Kit

Cat. #s 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 revolutionary cloning approach for rapid, efficient and precise cloning of any PCR fragments to any linearized vector in one single step. The PCR fragments can be generated by Taq DNA polymerase or other high fidelity DNA polymerases, with primers that are designed to have at least 15 bases of homology at their linear ends to where you want the product to “fuse”. The linearized vector can be generated by PCR or restriction enzymes (single or double cut). One tube simple reaction format, 5 minute incubation at room temperature then 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 95% positive cloning rate.

II. 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

III. Applications

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

IV. List of components**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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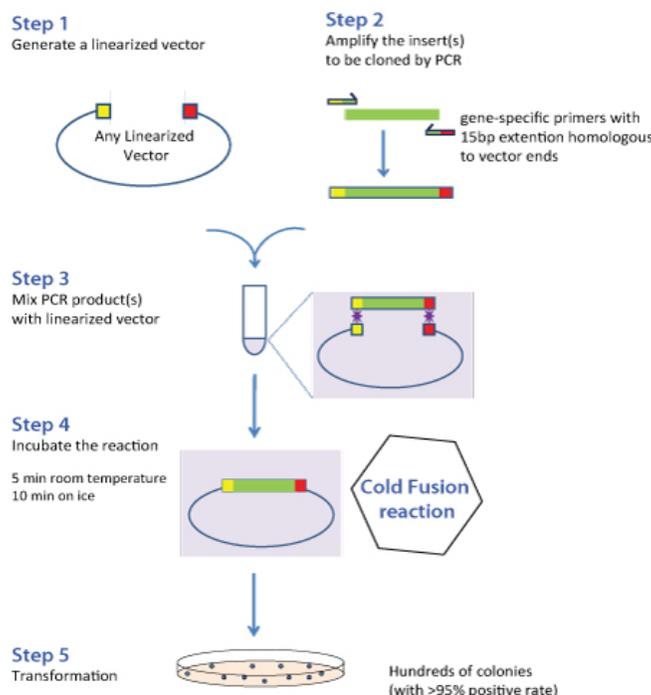
Cat. No. MC101A-1

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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V. Storage

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

VI. Protocol



A. 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 enzymes (single or double digest) and should be purified using a gel or PCR purification kit.

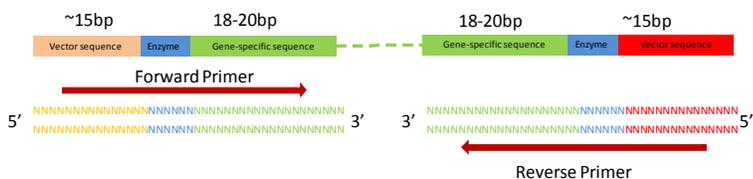
Due to the digestion efficiency, different restriction enzymes will generate different background. In general, two enzyme digestion is better than any single enzyme. The further the restriction sites are apart, the better digestion efficiency yields. Increasing the enzyme digestion time and the digestion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

Check the background of your vector by transforming 1 μ g (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 2 μ g vector in 50 μ l reaction overnight. Use QIAGEN's QIAquick Spin Gel Extraction kit for gel purification and elute the DNA with 30 μ l dH₂O.

B. PCR Amplification of Target DNA

To successfully clone any DNA fragment into a linearized vector, PCR primers must be designed to have at least 15 bases of homology with the end of the linearized vector. Thus, a primer will consist of a 15-base vector homology sequence at the 5'-end, and optional restriction site in the middle, and the gene-specific sequence at the 3'-end. The guidelines for primer design is shown in the graph below.



(Note: The restriction site in the middle of the primer can be the same or different one used to linearize the vector. You can also add any other sequence in the middle for frame adjustment or tag addition. For multiple DNA fragment joining, it is recommended that each PCR product shares at least 18 base pairs of homology.)

The PCR fragments can be generated by Taq DNA polymerase or other high fidelity DNA polymerase. The melting temperature (T_m) should be calculated based on the 3' (gene-specific) end of the primer, NOT the entire primer.

Primers and primer dimers produced in PCR reactions are inhibitory to the Cold Fusion cloning reaction. If the PCR produces a single specific band (from an agarose gel), the PCR product can be purified by simply using a PCR purification kit. If the PCR produces multiple bands, the specific DNA band desired should be purified by a gel purification kit to remove non-specific DNA bands and avoid false-positive clones.

C. Reaction Set up

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/ μ l)	1 μ l*
PCR insert(s) (20-200ng/ μ l)	1 μ l* for each PCR product
dH ₂ O	μ l
5x master mix	2 μ l
total	10 μ l

- **Positive control reaction**

Linearized vector (positive control)	1 μ l
500bp PCR insert (positive control)	1 μ l
dH ₂ O	6 μ l
5x master mix	2 μ l
total	10 μ l

- **Negative Control**

Linearized destination vector (10-100ng/ μ l)	1 μ l*
dH ₂ O	7 μ l
5x master mix	2 μ l
total	10 μ l

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

For reaction with larger volumes of vector and insert (>8 μ l of vector + insert), double the amount of reaction buffer and enzyme, and add dH₂O for a total volume of 20 μ l.

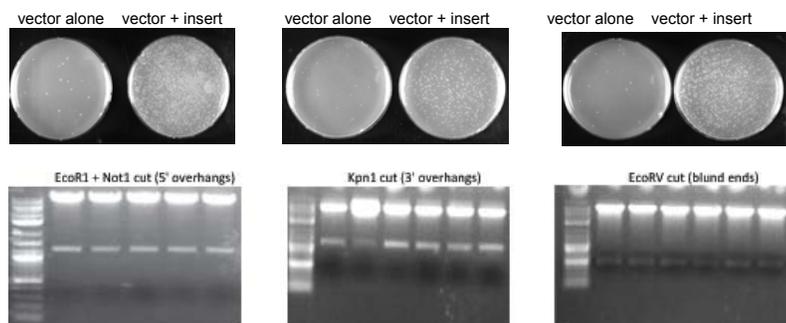
When using Cold Fusion cloning kit for the first time, we strongly recommend that you perform the positive and negative control reaction in parallel with your Cold Fusion cloning reaction. The positive control 500bp PCR insert and linearized vector provided in the kit has already been purified. There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

D. Reaction Incubation

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

E. Transformation

1. Add 50 μ l 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 250 μ l S.O.C medium or LB broth
6. Incubate at 37°C for an hour
7. Take 100 μ l culture spread on pre-warmed (37°C) culture plate containing selected antibiotic
8. Incubate the plate at 37°C to grow.

VII. Examples**A. Cloning Single DNA Fragment****B. 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 from single or two fragments cloning.

VIII. Troubleshooting		
Problems	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 and using different method to purify your PCR product.
	Low DNA concentration in reaction	It is imperative to obtain a high DNA concentration possible in your Cold Fusion reaction.
	There are inhibitory contaminants from PCR product or from linearized vector	Both of the PCR product and the linearized vector should be purified.
	Transform with too much reaction mixture	Do not add more than 10 μ l of reaction mixture to 50 μ l 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 thawed. 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/ μ g are recommended.
	Wrong antibiotic or too much antibiotic in the media	Choose the plate with appropriate concentration of 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.
Problems	Probable cause	Solution
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.