MCLAB

Choo-Choo PCR Cloning® kit

User Manual

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Contents

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Description

Choo-Choo PCR Cloning® Kit is a highly efficient directional PCR cloning product for rapid, ligase- and restriction enzyme-independent cloning of PCR products. It allows you to clone any PCR fragment into any linearized vector at any location. By a simple incubation on ice, the end of a PCR-generated DNA fragment can precisely fuse to another DNA (vector) end with 6 bp (or more) overlap. The system is so robust: up to 8 PCR-generated DNA fragments can be assembled and cloned into one piece up to 10 kb by one step;
The system is highly efficient: 98-100% positive clones.
The function of Choo-Choo PCR Cloning® depends on our proprietary enzyme systems.There is no need of restriction enzyme digestion, ligation, and blunt-end polishing. You may limit any extra bases in the final construct. The linearized vector can be generated by PCR or restriction enzyme digestion. The PCR fragments can be generated by Taq DNA polymerase or other high fidelity DNA polymerase. The addition of A by Taq DNA polymerase is not required or has no effect on cloning efficiency. If the PCR product is amplified from plasmid template, it needs gel purified to reduce the background. In addition for PCR cloning, the Choo-Choo PCR Cloning® Kit has other functions, such as for adaptor, linker and tag addition before and after the inserts, and for gene synthesis.

List of Components

• Store Box 1 at -20°C.
• Store Box 2 at -80°C.
The Choo-Choo Cloning Kits are available in 20 reaction and 100 reaction sizes.

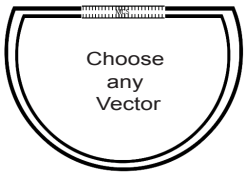
Cat. No. CCK-20 Choo-Choo Cloning Kit (20 reactions)
Box 1:
• 10x Choo-Choo Cloning Reaction Buffer 40 µl
• Choo-Choo Cloning Enzyme Mix 40 µl
Box 2:
• Choo-Choo Blue chemical Competent cell 20 tubes x 50 µl

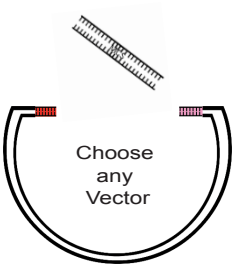
Cat. No. CCK-100 Choo-Choo Cloning Kit (100 reactions)
Box 1:
• 10x Choo-Choo Cloning Reaction Buffer 200 µl
• Choo-Choo Cloning Enzyme Mix 200 µl
Box 2:
• Choo-Choo Blue chemical Competent cell 100 tubes x 50 µl

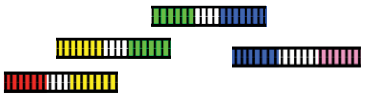
Figure 1

The Choo-Choo Cloning Method. During the 45 min incubation, the Choo-Choo Cloning enzyme mix containing our proprietary enzymes and recombination proteins creates single-stranded regions at the end of the vector and PCR product, which are then linked due to the 6-20 bp homologies. The resulted recombinant clone can be used to transform *E. coli*.

Continued
Choo-Choo® Cloning Kits Illustration show







up to 8 PCR fregment

Choo-Choo® Cloning Kits Enyzme Mix

45 mins

Transformation...

400-500 clear and big clones.

Additional Materials Required

The following materials are required but not supplied:

LB (Luria-Bertani) medium (pH 7.0)

1.0% Bacto-tryptone 10 g
0.5% Yeast extract 5 g
1.0% NaCl 10 g

For 1 liter, dissolve ingredients in 950 ml of deionized H2O. Adjust the pH to 7.0 with 5M NaOH and bring the volume up to 1 L. Autoclave on liquid cycle for 20 min at 15 lb/in2. Store at room temperature or at 4°C.

LB/antibiotic plates

Prepare LB medium as above, but add 15 g/L of agar before autoclaving. Autoclave on liquid cycle for 20 min at 15 lb/in2. Let cool to ~55°C, add antibiotic (e.g., 100 ug/ml of ampicillin), and pour into 10 cm plates. After the plates harden, then invert and store at 4°C.

SOC medium

2% Tryptone
0.5% Yeast Extract
10 mM NaCl
2.5 mM KCl
10 mM MgCl2•6H2O
20 mM glucosa

1. For 1 liter, dissolve 20 g of tryptone, 5 g of yeast extract, and 0.5 g of NaCl in 950 ml of deionized H2O.
2. Prepare a 250 mM KCl solution by dissolving 1.86 g of KCl in deionized H2O for a total volume of 100 ml. Add 10 ml of this stock KCl solution to the solution prepared in Step 1.
3. Adjust pH to 7.0 with 5 M NaOH, and then bring the volume to 980 ml with deionized H2O.
4. Prepare a 1 M solution of MgCl2 by dissolving 20.33 g of MgCl2•6H2O in deionized H2O for a total volume of 100 ml.
5. Autoclave both solutions on liquid cycle at 15 lb/in2 for 20 min.
6. Meanwhile, make a 2 M solution of glucose by dissolving 36 g of glucose in deionized H2O for a total volume of 100 ml. Filter-sterilize this solution.
7. Let the autoclaved solutions cool to about 55°C, then add 10 ml of the filter-sterilized 2 M glucose solution and 10 ml of 1 M MgCl2. Store at room temperature or 4°C.

Gel extraction kit

PCR and Experimental Preparation

PLEASE READ ENTIRE PROTOCOL BEFORE STARTING.

A. PCR Primer Design

Primer design and quality are critical for the success of the Choo-Choo Cloning reaction. You can join two or more fragments, e.g. vector and insert (or multiple inserts), as long as they share 6-20 bases of homology at each end. The best result is for 12-15 bp. Figure 2a outlines the guidelines for universal primer design for vector using single restriction site. Figure 2b outlines the guidelines for universal primer design for vector using double restriction sites. Therefore, design PCR primers that will generate the homologous region in the PCR product during the amplification.

Every Choo-Choo cloning primers must serve two purposes: it should contain a homologous sequence and be gene specific. The 6-20 base pairs towards the 5' end of the primer must match the 6-20 base pairs at the linear end of the DNA fragment to which it will be joined. The 3' end of the primer is the gene specific portion of the primer. The 3' end of the primer must have a melting temperature (Tm) suitable for PCR. Please note that the Tm should be calculated based upon the 3' (gene-specific) end of the primer, and not the entire primer. If the calculated Tm is too low, increase the length of the gene-specific portion of the primer until you reach a Tm of between 58º–65º C. The Tm difference between the forward and reverse primers should be ≤ 4º C, or you will not get good amplification. We generally use desalted oligos in PCR reactions.

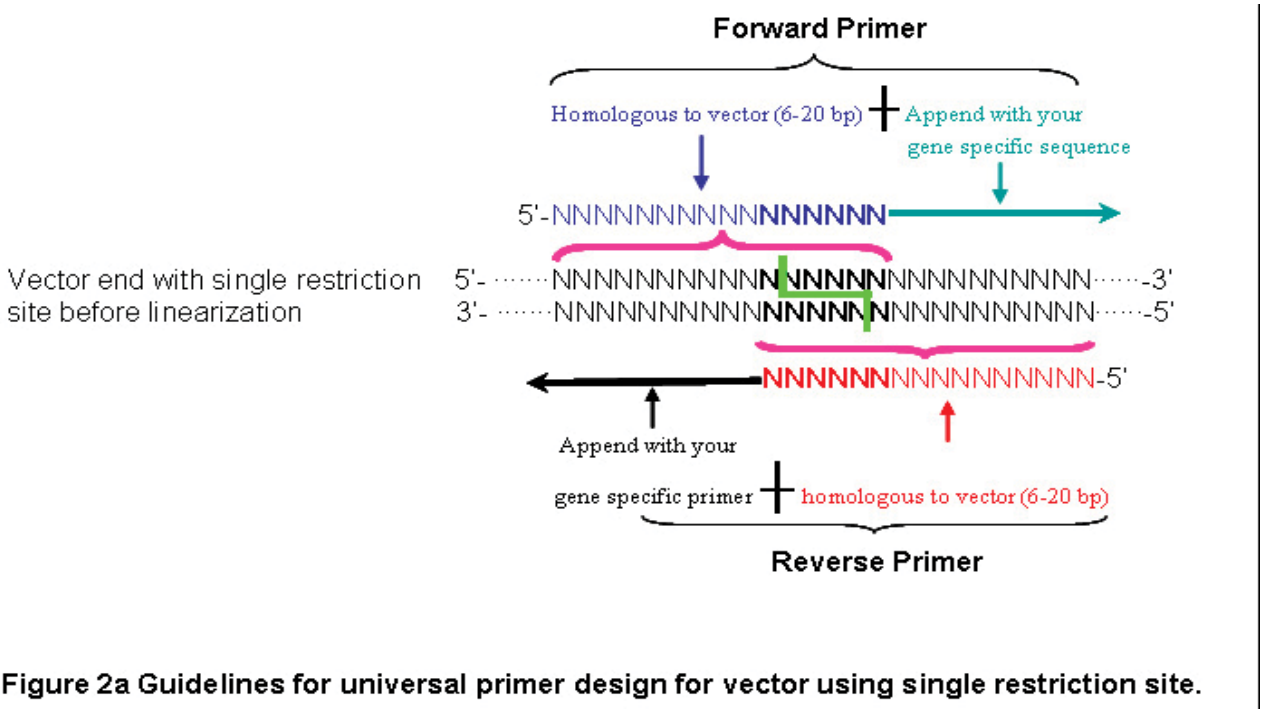


Figure 2a Guidelines for universal primer design for vector using single restriction site.

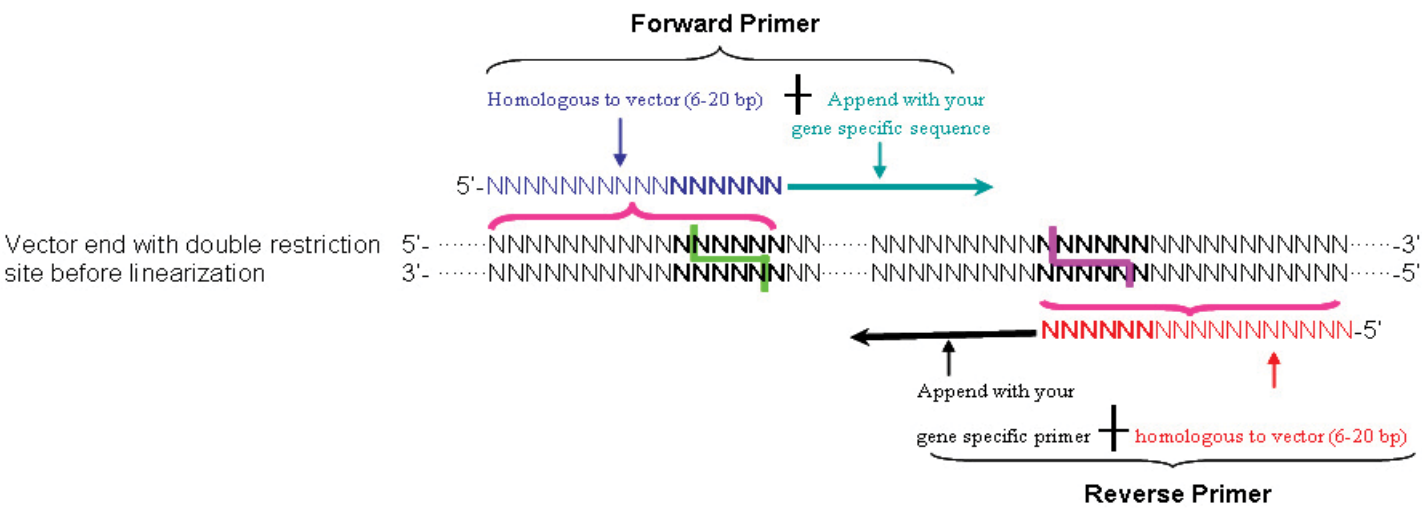


Figure 2b Guidelines for universal primer design for vector using double restriction site.

B. Preparation of linearized Vector by Restriction Digestion

To achieve a successful Choo-Choo Cloning reaction, you must first generate a very pure linearized vector (with very low background of uncut vector present). Restriction enzymes will generate different amounts of background, due to differences in cutting efficiency. Generally speaking, two enzymes cut better than any single enzyme. We recommend that you select two enzymes for linearizing your vector. Efficiency of digestion will always be better if the restriction enzyme sites are as far apart as possible. In addition, increasing the enzyme digestion time and digestion reaction volume will reduce the background.

Prepare a linearized vector as follows.

1. We recommend cutting the vector with two different enzymes to reduce background, unless there is only one site available for cloning.

Vector 2-5 µg
10x Enzyme buffer 5 µl
Restriction enzyme 2.5–5.0 Units
Deionized water to 50 µl

2. Incubate your restriction digest as directed by the restriction enzyme supplier. For many enzymes, incubation from 3 hours to overnight can increase linearization and reduce background.
3. After digestion, purify the linearized vector using any available gel extract kit.
4. [Control] Check the background of your vector by transforming 5–10 ng of the linearized and purified vector into Choo-Choo Blue Competent Cells (See the following transformation procedure).

If the background is high, continue digesting the vector for a longer time after the addition of more restriction enzyme(s). Incubate 2 hours to overnight. Gel purify the remainder of the vector and transform

C. PCR Amplification of Insert

It is important to use only 10–50 ng of plasmid DNA as a PCR template. However, if you are amplifying a pool of cDNA, the amount of template DNA depends on the relative abundance of the target message in your mRNA population. For best results, we recommend using Pfu, AFU DNA Polymerase and other Highfidelity polymerase.

When PCR cycling is complete, analyze your PCR product by electrophoresis on an agarose/EtBr gel to confirm that you have obtained a single DNA fragment and to estimate the concentration of your PCR product. Quantify the amount of DNA by measuring against a known standard or molecular weight marker ladder run on the same gel.

Choo-Choo® Cloning Reaction and Transformation Procedure

1. Set up the following 13 ul Choo-Choo® Cloning reaction:

Reagent	Volume / Amount
10x buffer	1.5 µl
Linearized vector	? µl[1] (40-100 ng)
Insert	? µl[2]
Water	Add to a final volume of 13 µl

[1] The linearized can be obtained from PCR or by restriction enzyme digestion.
[2] The ratio of insert to vector should be 2-5:1.

2. Add 2 µl of Choo-Choo Cloning Kit Enzyme Mix into to the reaction tube, mix by short spin, and incubate on ice for 45 min.
3. Add whole reaction into 50 µl Choo-Choo Golden Chemical Competent E. coli cells, and incubate on ice for 15 min.
4. Heat-shock the cells for 45 seconds at 42°C without shaking, then incubate on ice for 2 min.
5. Add 300 µl of room temperature S.O.C. Medium to the cells.
6. Cap the tubes and shake at 37°C for 1 hour.
7. Spread whole content from each transformation on prewarmed on LB plates with respective antibiotics.
8. Incubate plates overnight (about 16 h) at 37°C.
9. Pick ~10 defined colonies for analysis.