Version 3.2

User Manual

Product name: Choo-Choo Cloning™ Kits

Cat #: CCK-10, CCK-20, CCK-096, CCK-384

Description:

Choo-Choo Cloning™ is a highly efficient directional PCR cloning kit designed for rapid cloning of one or multiple PCR fragments without the need for ligase or restriction enzyme. It enables to clone any PCR fragment into any linearized vector at any location. By simply incubating on ice, the ends of a PCR-generated DNA fragment can be precisely fused into a DNA vector with 6 bp (or more) overlapping homologous sequence. The system is very robust: up to 8 PCR-generated DNA fragments can be assembled into one piece (up to 10 kb) and cloned into a vector of choice in one step. The system is highly efficient with 98-100% positive colonies.

The function of Choo-Choo Cloning™ depends on MCLAB's proprietary enzyme systems. There is no need for restriction enzyme digestion, ligation, and blunt-end polishing. Any extra unwanted bases can be eliminated from 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 an "A" by *Taq* DNA polymerase is not required or has no effect on cloning efficiency. If the PCR product is amplified from a plasmid template, a gel purification step is needed to reduce the background. In addition to PCR cloning, the Choo-Choo Cloning™ Kit is versatile and can be used for other applications, such as adaptor, linker and tag addition before and after the inserts, and gene synthesis.

List of Components:

Cat #	Box1		Box 2
	Choo-Choo Cloning™ Enzyme Mix	10x Choo-Choo Cloning™ Reaction Buffer	Choo-Choo Blue chemical Competent cell
CCK-10 (10 reactions)	20 µl	20 µl	10 tubes x 50 µl
CCK-20 (20 reactions)	40 µl	40 µl	20 tubes x 50 µl
CCK-100 (100 reactions)	200 µl	200 µl	100 tubes x 50 µl

- Store Box 1 at -20°C.
- Store Box 2 at -80°C.

Figure: The Choo-Choo Cloning™ Method.

During the 45 min incubation, the Choo-Choo Cloning™ enzyme mix containing MCLAB's proprietary enzymes and recombinant proteins creates single-stranded regions at each end of the vector and PCR fragments, which are then linked together due to the 6-20 bp overlapping homologous sequences. The resulting construct can be directly transformed into *E. coli* competent cells.

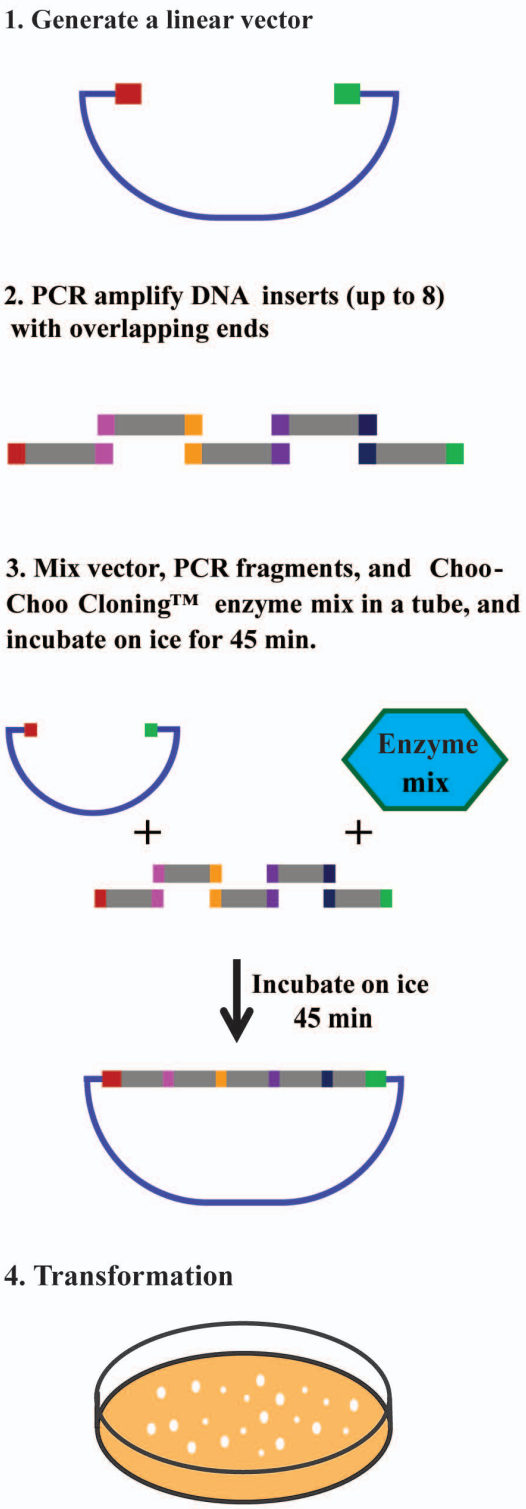


Figure 1. Experimental workflow of mutiple PCR fragments insertion into a vector using the Choo-Choo Cloning™ Kit.

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

1. For 1 liter, dissolve ingredients in 950 ml of deionized H_2O. 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
2. Prepare LB media as described above, then add 15 g/L of agar in to LB media. 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 hardened, invert and store at 4°C.

SOC medium

- 2% Tryptone
- 0.5% Yeast Extract
- 10 mM NaCl
- 2.5 mM KCl
- 10 mM $MgCl_2 \bullet 6H_2O$
- 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 H_2O.
2. Prepare a 250 mM KCl solution by dissolving 1.86 g of KCl in deionized H_2O 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 H_2O.
4. Prepare a 1 M solution of $MgCl_2$ by dissolving 20.33 g of $MgCl_2 \bullet 6H_2O$ in deionized H_2O 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 H_2O 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 filter-sterilized 2 M glucose solution and 10 ml of 1 M $MgCl_2$. Store at room temperature or 4°C.

PCR and Experimental Preparation

PLEASE READ THE ENTIRE PROTOCOL BEFORE STARTING.

A. PCR Primer Design

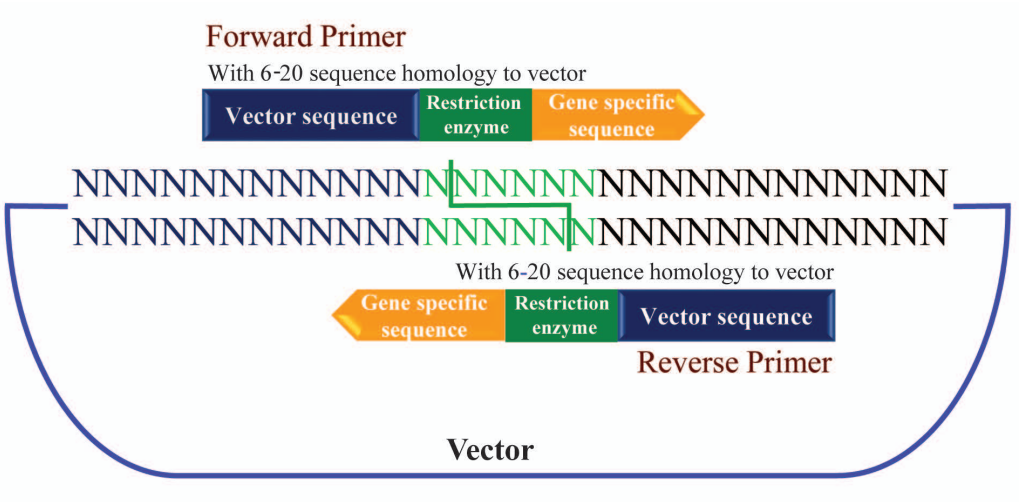
Primer design and quality are critical for the success of Choo-Choo Cloning™ reaction. Two or more fragments, e.g. vector and insert (or multiple inserts) can be joined together as long as they share 6-20 bases of homology at each end. The best result can be obtained from 12-15 bp overlapping range.

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.

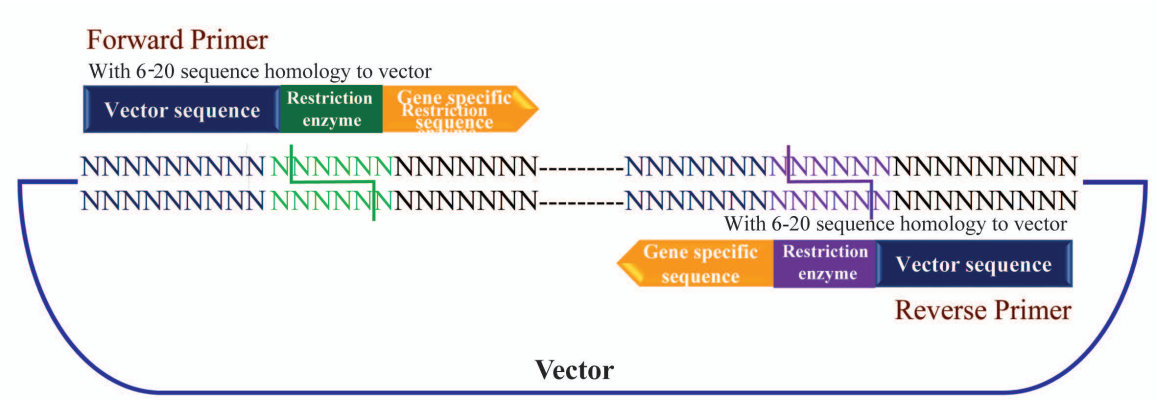
Every Choo-Choo Cloning™ primers must serve two purposes: it should contain a homologous sequence and be gene specific. The 6-20 base pairs towards the 5′ end of the primer must match the 6-20 base pairs at the linear end of the DNA fragment to which it will be joined. The 3′ end of the primer is the 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 a Tm of between 58°–65° C is reached. The Tm difference between the forward and reverse primers should be ≤ 4° C, otherwise, a good amplification will not be achieved. Desalted oligos in PCR reactions are recommended to use.

Figure 2. Guidelines for universal primer design.



2a. For vector using single restriction site.



2b. For vector using double restriction sites.

B. Preparation of linearized Vector by Restriction Digestion
To carry out a successful Choo-Choo Cloning™ experiment, a pure linearized vector must be generated first (with very low background of uncut vector present). Restriction enzymes may introduce certain amount of background due to difference in cutting efficiencies. In general, two enzymes cut better than any single enzyme. We recommend to select two enzymes to linearize the vector. Efficiency of the digestion is always 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 the restriction digest as directed by restriction enzyme suppliers. For many enzymes, incubation from 3 hours to overnight can increase linearization and reduce background.
3. After digestion, purify the linearized vector using an available gel extract kit.
4. [Control] Check the background of the 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 period of time after additional amount of restriction enzyme(s) added. Incubate the digestion from 2 hours to overnight, then gel purify the remainder of the vector and transform into *E. coli* competent cells.

C. PCR Amplification of Insert
It is important to use only 10–50 ng of plasmid DNA as a PCR template. However, if a pool of cDNA to be amplified, the amount of template DNA depends on the relative abundance of the target message in the mRNA population. For the best results, we recommend using Pfu, AFU DNA Polymerase and other high fidelity polymerase.
When PCR cycling is complete, analyze the PCR product by electrophoresis on an agarose/EtBr gel to confirm that a correct DNA fragment is obtained, and to estimate the concentration of the 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 15 µl Choo-Choo Cloning™ reaction:

Reagent	Volume/Amount
10X Choo-Choo Reaction Buffer	1.5ul
Choo-Choo Cloning Kit Enzyme Mix	2ul
Linearized Vector	40-100 ng
Insert	5ul
Water	Up to 15ul

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

2. Incubate the reaction on ice for 45 min.
3. Apply the whole reaction content to 50 µl Choo-Choo Golden Chemical Competent *E. coli* cells, and incubate on ice for 15 min.
4. Heat-shock the cells for 1 min at 42°C without shaking, then incubate on ice for 2 min.
5. Add 200 µl of room temperature S.O.C. media to the cells.
6. Cap the tubes and shake at 37°C for 1 hour.
7. Spread the whole content from each transformation on pre-warmed LB plates with respective antibiotics.
8. Incubate plates overnight (about 16 h) at 37°C.
9. Pick ~10 defined colonies for analysis.