MacBlunt™ PCR Cloning Kit Manual

Shipping and Storage
MacBlunt™ PCR Cloning Kits are shipped on dry ice. Each kit contains a box with cloning reagents and an attached bag with Eco-Blue Competent Cells (optional). Store the box at –20˚C and the bag at –70˚C.

Types of Cloning Kits
MacBlunt™ PCR Cloning kits are available in three kit sizes, with or without competent cells.

Product	Reaction Size	Competent Cells	Catalog No.
	10	Eco-Blue	MCB-10C
	20	Eco-Blue	MCB-20C
MacBlunt™ PCR Cloning Kit	20	None	MCB-20N
	50	Eco-Blue	MCB-50C
	50	None	MCB-50N

pMAC1 plasmid vector	10 μg		PMAC-100
Eco-Blue	20 x 50 μl		EcoBL-20

Kit Contents

Component	Storage Conditions	10 rxn	20 rxn	50 rxn
pMAC1-Blunt vector (40 ng/μl)	-20˚C	10 μl	20 μl	50 μl
5X Buffer Mix A	-20˚C	20 μl	40 μl	100 μl
5X Enzyme Mix B	-20˚C	20 μl	40 μl	100 μl
T4 DNA Ligase	-20˚C	10 μl	20 μl	50 μl
Nuclease-free water	-20˚C	1 ml	1 ml	1 ml
Test Insert (use 2 μl)	-20˚C	10 μl	10μl	10 μl
Eco-Blue Competent Cells	-70˚C	10x 50μl	20x 50μl	50x50μl

Materials Supplied by the User
In addition to general microbiological equipment, you will need the following reagents and equipment.

- Sterile microfuge tubes or PCR tubes
- 42˚C water bath
- 75˚C water bath or programmable thermocycler
- LB plates containing 50-100μg/ml ampicillin
- 40 mg/ml X-gal in dimethylformamide
- 100 mM IPTG in water
- 37˚C non-shaking incubator

Introduction

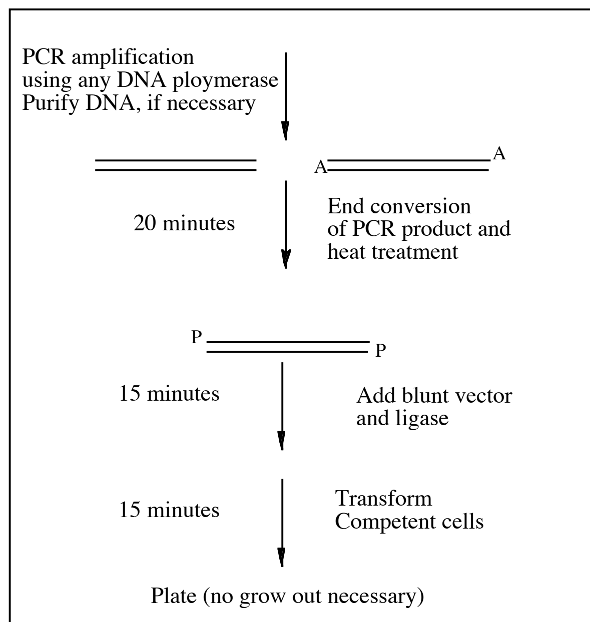
The MacBlunt™ Cloning Kits are designed for simplified cloning of PCR-generated DNA, from any type of DNA polymerase. Many protocols for PCR cloning rely on the single 3'-A addition by Taq polymerase and a vector with a single T overhang. If a high-fidelity, proof-reading enzyme is used, the PCR DNA must be tailed with Taq polymerase prior to cloning. In the MacBlunt™ protocol, PCR fragments are converted to blunt ends and the 5' ends are phosphorylated in a brief (15 min) reaction. The PCR DNA is then ligated into a blunt-ended, de-phosphorylated vector, followed by transformation into competent cells. The entire protocol, from PCR product to plating transformations, can be performed in less than one hour, resulting in >95% of white colonies having the correct insert.

How It Works

In the end conversion reaction, the PCR product is treated with Polynucleotide Kinase and ATP in addition to T4 DNA Polymerase and dNTPs. The kinase phosphorylates the 5' ends of the PCR product while the proofreading and polymerase activity of T4 Pol produces blunt ends. Following end conversion, the reaction is heated to 75˚C for five minutes to destroy the kinase activity.

A blunt-end linear vector with the 5' ends de-phosphorylated is then added along with T4 DNA ligase. The vector ends are unable to ligate to themselves due to the de-phosphorylation. The ends of the treated PCR insert are able to ligate to the vector, creating a circular, transformable plasmid. Insertion of the PCR fragment disrupts expression of the lacZ alpha peptide on the plasmid. Transformation of the ligated DNA into competent cells followed by plating on LB agar with ampicillin is easily visualized by blue/white screening.

In addition to PCR products, this kit is also suitable for cloning cDNA, sheared DNA, or restriction fragments using the same protocol.



Procedure steps

A. Insert Preparation

The MacBlunt™ cloning procedure works best when the DNA fragment to be cloned is gel isolated. Crude PCR preparations contain residual Taq polymerase, free primers, and other potential DNA fragments such as primer-dimers. All of these can interfere with the cloning efficiency. We recommend the MacConnell BroadBander Kit™ (Cat. BRB-100) for purification from an agarose gel or straight from the PCR reaction itself.

Precipitation of PCR fragments can also be used to prior to cloning. Add 1/10 volume of 3M Na Acetate and 1 volume isopropyl alcohol to the PCR mix and vortex briefly. Centrifuge 5 minutes in a microfuge at maximum speed. Carefully remove the supernatant with a pipet tip and wash the pellet with 80% ethanol. Dry the pellet and resuspend in a small volume (10 µl) of TE (10mM Tris pH 8, 1mM EDTA).

Note: If the PCR DNA to be cloned is from an ampicillin-resistant plasmid, gel isolation of the PCR product is necessary to remove the template prior to transformation.

The cloning procedure will generate positive clones over a range of insert concentrations. A standard reaction contains 20 ng of a 500 bp insert (0.06 pmol). Larger fragments will require more mass to equal the same molarity of ligatable ends.

B. End Conversion and Ligation

A PCR tube works well for the following reaction because the heat-denaturaton step can be performed in a thermocycler.

1. Add the following components in order:

0.5-2.0 µl	PCR product (or 2 µl Control Insert)
2 µl	5X Buffer Mix A (A on tube top)
2 µl	5X Enzyme Mix B (B on tube top)
4 - 5.5 µl	Nuclease-free water
10 µl	Total Volume

Mix gently by pipeting up and down.

2. Incubate the reaction 15 minutes at room temperature (22-24 ˚C).
3. Heat the reaction to 75 ˚C for 5 minutes to inactivate the kinase. This can be performed in a thermocycler if the appropriate tube was used.
4. Cool the reaction tube on ice for 1 minute so as not to heat inactivate the ligase in the following steps. If liquid has condensed on the lid of the tube, centrifuge briefly to bring the entire reaction to the bottom of the tube.

5. Add 1 μl of pMAC1 blunt vector (C on tube top) (40 ng) , and then 1 μl T4 DNA ligase (D on tube top) directly to the conversion reaction. Mix by gently pipeting up and down.
6. Incubate the reaction at room temperature (22-24 ˚C) for 15 minutes. Incubate for longer periods of time (up to 1 hr) for increased numbers of transformant colonies. Longer ligation times may be required for larger inserts (≥ 3 kb).

D. Transformation

1. Remove one tube of Eco-Blue Single Competent Cells from the –70 ˚C freezer for every reaction and immediately place on ice. Allow the cells to thaw on ice for ~5 minutes. Flick the tube to see that they have thawed, and to resuspend the cells, then return to ice.

Set aside one LB agar plate containing 50-100 μg/ml ampicillin (or carbenicillin) for every transformation. For blue/white selection of transformant colonies, spread X-gal/IPTG solution onto the plates and allow it to soak in prior to plating cells.

X-gal/IPTG solution: Mix 900 μl of 40 mg/ml X-gal in dimethylformamide with 100 μl of 100 mM IPTG in sterile water. Use 50 μl per 82 mm plate.

2. Add 5 μl - 12 μl of the ligated reaction directly to the competent cells. Mix gently and return to ice. Repeat for additional samples.
3. Incubate on ice for 10 minutes.
4. Heat the tubes for exactly 45 seconds in a 42 ˚C water bath and immediately return to ice. Do not shake the tubes.
5. Incubate on ice for two minutes.
6. Spread the entire transformation onto the prepared agar plates using a sterile spreader and allow the liquid to soak in. There is no need to grow out the transformation prior to plating.
7. Invert the plate and grow at 37˚C overnight.

E. Screening

White colonies are selected for screening for plasmids with inserts. These colonies contain plasmids with a disrupted lacZ alpha peptide. A small number of blue colonies will also be present as a background due to self-ligation of the non-dephosphorylated vector.

The PCR fragment becomes inserted into the EcoRV site of the vector, and thus destroys the site in the process. This site is flanked by two Eco RI sites and several other unique sites. The vector contains priming sites for M13 forward and reverse, as well as T3 and T7. See pMAC-1 vector map below.

Screening can be performed by:

- Colony PCR with PCR primers, or forward/reverse, T3/T7 primers.
- Plasmid mini-prep followed by restriction digest

pMAC1™-Blunt
2370 bp