Pro Ligation-Free Cloning MasterMix

Cat. No. G4004

Store all components at -20°C.

Product Description

abm's Pro Ligation-Free Cloning MasterMix is a ready-to-use solution designed for rapid, efficient and seamless DNA assembly regardless of fragment length or end compatibility. It enables one-step assembly of multiple DNA fragments with overlapping ends in a single isothermal reaction making it ideal for synthetic biology and molecular biology applications. To support accurate fragment amplification prior to assembly, the kit includes MegaFi™ Pro Fidelity 2X PCR MasterMix to enable robust, high-fidelity PCR.

Product Component	Quantity	Part. No.
Pro Ligation-Free Cloning 2X MasterMix	50 rxn (500 µl)	P4004-1
Positive Control Insert	5 µl	P4004-2
Positive Control Vector	5 µl	P4004-3
MegaFi™ Pro Fidelity 2X PCR MasterMix	1.25 ml	P887-1

Additional Materials Required (not supplied)

Material	Recommended Product	Cat. No.	Required For
PCR Purification Kit	Column-Pure Gel and PCR Clean-Up Kit	D516	Part B
Competent *E. coli* cells	ProClone™ Competent Cells	E003	Part D

Protocol

Part A – Design Primers and Prepare Overlapping Fragments

1. Linearize vector backbone by restriction digestion or inverse PCR. Load 2-5 µl of vector product on an agarose gel to confirm correct linearization.
2. Design PCR primers to amplify insert fragments with 15-25 bp of overlapping homology to each adjacent component (see Appendix for details).
3. Using the primers from Step 2, amplify each fragment using MegaFi™ Pro Fidelity 2X PCR MasterMix according to the table below.

MegaFi™ Pro Fidelity 2X PCR MasterMix reaction set up:

Component	Volume
MegaFi™ Pro Fidelity 2X PCR MasterMix	12.5 µl
Forward Primer (10 µM)	1 µl
Reverse Primer (10 µM)	1 µl
Template DNA	Variable (1-10 ng Plasmid DNA)
Nuclease-free H_2O	Up to 25 µl

Gently mix the reaction components and briefly centrifuge, then transfer tube to a thermal cycler using the following conditions:

Step	Temperature	Time
Initiation Denaturation	95°C	5 min
Annealing/Extension (25-35 Cycles)	95°C 50-70°C 72°C	15 sec 15 sec 20-30 sec/kb
Final Extension	72°C	5 min

4. Load 2-5 µl of product on an agarose gel to confirm correct amplification.

Part B – Purify Overlapping Fragments

1. Purify each fragment and vector using a standard spin column PCR Purification Kit.
   •If non-specific or undigested products were observed in Part A, use gel extraction to excise and purify the desired fragment(s).
2. Quantify concentration and purity of each fragment using a Nano Spectrophotometer.

Part C – DNA Assembly of Overlapping Fragments

1. Set up the below reactions on ice.

Component	Volume	Positive Control
Pro Ligation-Free Cloning 2X MasterMix	10 µl	10 µl
Linearized Vector or Positive Control Vector	50-100 ng	1 µl
Each Fragment or Positive Control Insert	3:1 insert-vector molar ratio	1 µl
Nuclease-free H_2O	Up to 20 µl	8 µl

2. Use the formula below to calculate the insert-vector molar ratio.

$$\text{insert (ng)} = 3 \times \frac{\text{insert (bp)}}{\text{vector (bp)}} \times \text{vector (ng)}$$

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3. Mix the reactions by pipette and transfer to a thermal cycler. Incubate at 50°C: 15 min for 1-2 fragments or 60 min for 3+ fragments.
   •Incubate the Positive Control Reaction for 15 min.
4. Store samples on ice or at -20°C until transformation.

Part D – Transformation of Assembly Mixture and Screening

1. Transform mixture from Part C into competent *E. coli* cells, plate cells on appropriate antibiotic LB agar and incubate overnight at 37°C.
   •Plate the Positive Control Reaction on LB agar ampicillin.
2. The following day assess transformation plates and screen by colony PCR.
3. Confirm correct DNA assembly by plasmid extraction followed by restriction digestion and/or sequencing.
   •Assess the Positive Control Reaction for correct assembly by the appearance of pink-colored colonies following transformation as the insert contains reporter mCherry. Alternatively, restriction digestion of extracted plasmid DNA can be performed with enzymes HindIII and EcoRI resulting in 2.6 + 0.8 kb fragments.

Troubleshooting

•**No colonies following transformation**: re-assess primer design, concentration and purity of DNA fragments, insert-vector molar ratio calculation, antibiotic used and transformation efficiency of competent *E. coli* cells.
•**Colonies do not pass screening**: vector was not completely linearized, presence of non-specific amplicons, antibiotic plates are old or expired.

Appendix

Primer Design for Overlapping Fragments

1. Begin by designing the assembled DNA construct using sequence viewer software.
2. Identify the junctions between each DNA fragment (e.g. junctions between grey vector and blue fragment) and choose 15-25 bp of overlapping sequence (e.g. indicated purple boxes). It is critical that each overlapping sequence is unique to allow for the correct order of assembly.
3. Design the 3' region of each primer to be specific to the insert (e.g. blue arrows), ensuring appropriate melting temperature and minimal secondary structures for optimal PCR amplification.
4. Add the 5' overlapping region to each primer (e.g. grey portion of the blue arrows).

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