

mRNex® Plus One Step Cloning Kit

Cat. no. LM301002

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Product Information

Product	mRNex® Plus One Step Cloning Kit
Catalog No.	LM301002
Storage conditions	Dry ice transportation. Store at -20°C, valid for 1 year.
Size	5T/20T/50T
Application	Rapid cloning; directional cloning; site-directed mutagenesis.

2. Product Description

The Plus One Step Cloning Kit is a simple, fast, and efficient DNA directional cloning product. This kit can efficiently clone PCR products into any site of any vector, with fragment sizes ranging from 50 bp to 10 kb. The vector is linearized, and 15-25 bp homologous sequences corresponding to the ends of the linearized vector are introduced at the 5' ends of the forward and reverse PCR primers of the insert fragment. As a result, the 5' and 3' ends of the PCR product of the insert fragment will have identical sequences corresponding to the ends of the linearized vector. The PCR product and linearized vector, under the action of recombinase, can undergo a reaction at 50°C for 20 minutes, after which transformation is performed to complete the directional cloning. The cloning efficiency can reach over 95%.

3. Product Component

Cat No.	Component	Size		
		5T	20T	50T
LM301002-A	2 × Fast Clone Enzyme Premix	50 µL	200 µL	500 µL
LM301002-B	500 bp Control Insert (25 ng/µL)	5 µL	5 µL	5 µL
LM301002-C	pUC 19 Control Vector, linearized (50 ng/µL)	5 µL	5 µL	5 µL

4. Notes

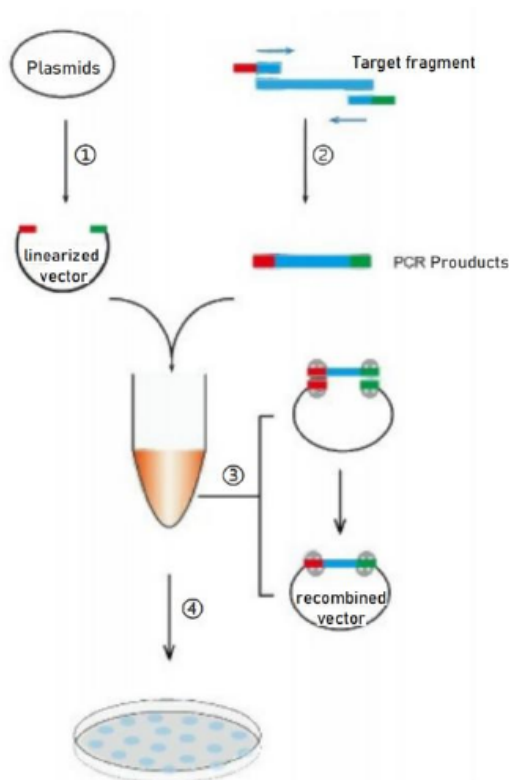
4.1. Materials to be prepared by the user:

- Prepared samples: Prepare the linearized vector and insert fragment.
- Prepared reagents (partial list): ① Super competent cells: Transformation efficiency > 10⁸ cfu/µg. ② High-fidelity enzyme. ③ Colony PCR mix. ④ Nucleic acid dye.
- Prepared instruments and consumables (partial list): PCR machine, horizontal electrophoresis tank, gel cutter, EP tubes, etc.;

4.2. For your safety and health, please wear a lab coat and disposable gloves during operation.

4.3. This product is for research use only!

5. Experimental Procedure



① Vector linearization: Use enzyme digestion or inverse PCR to prepare the linearized vector.

② Preparation of insert fragment: Introduce 15-25 bp homologous sequences of the vector ends at the 5' end of the forward and reverse primers of the target fragment; PCR amplify the target fragment so that the 5' and 3' ends of the amplified product are fully consistent with the sequences at both ends of the linearized vector

③ Recombinant reaction: Mix the linearized vector and insert fragment in proportion, and perform the recombination reaction at 50°C for 15 minutes under the catalysis of the recombination enzyme to complete the recombination.

④ Transformation and plating: Directly transform the recombinant product and plate it.

Figure 1. mRNex® one-Step Rapid Cloning Kit Experimental Workflow Diagram.

5.1. Preparation of Linearized Vectors

Choose an appropriate cloning site and linearize the vector. It is recommended to select cloning sites with no repetitive sequences and a uniform GC content. The GC content of the 25 bp region upstream and downstream of the cloning site should ideally be within the range of 40%-60%. The linearized vector can be obtained via restriction enzyme digestion or inverse PCR amplification.

5.1.1. Enzyme Digestion for Linearized Vector Preparation

5.1.1.1. Double-enzyme digestion for linearization: Complete linearization with low transformation background. This is recommended.

5.1.1.2. Single-enzyme digestion for linearization: Poor linearization. The digestion time may be appropriately extended to reduce the transformation background.

Note: False-positive clones without the insert may result from the transformation of unlinearized circular vectors. If the false-positive clone rate is high, it is recommended to re-prepare the linearized vector.

5.1.2. Inverse PCR Amplification for Linearized Vector Preparation

It is recommended to use high-fidelity polymerase (2×mRNex® Plus PCR Master Mix-With Dye, Cat. LM201002-2) for vector amplification to reduce the introduction of amplification mutations. The PCR amplification template should preferably use pre-linearized plasmids to avoid the impact of residual circular plasmid templates on cloning efficiency.

Our recombination reaction system is compatible with almost all enzyme digestion and conventional PCR systems. When the vector digestion products or inverse PCR amplification products have high purity, purification may not be necessary, and the recombination reaction can be directly performed. However, if the purity is low and there is a possibility of residual unlinearized circular plasmids, it is recommended to use a high-quality kit to perform gel recovery purification on the linearized vector. This will improve the product purity and remove some of the unlinearized circular vectors, which helps to improve recombination efficiency.

5.2. PCR Amplification for Insert Preparation

5.2.1. Primer Design for Amplification

mRNex® Primer Design Strategy:

This method involves introducing 15 – 25 bp (excluding restriction sites) of sequences homologous to the linearized vector termini at the 5' ends of both the forward and reverse PCR primers. As a result, the 5' and 3' ends of the PCR product will carry sequences identical to the corresponding termini of the linearized vector.

Forward Primer Design for Insert Amplification:

5' — Homologous sequence from the upstream vector terminus + restriction site (optional retention or removal) + gene-specific forward primer sequence — 3'

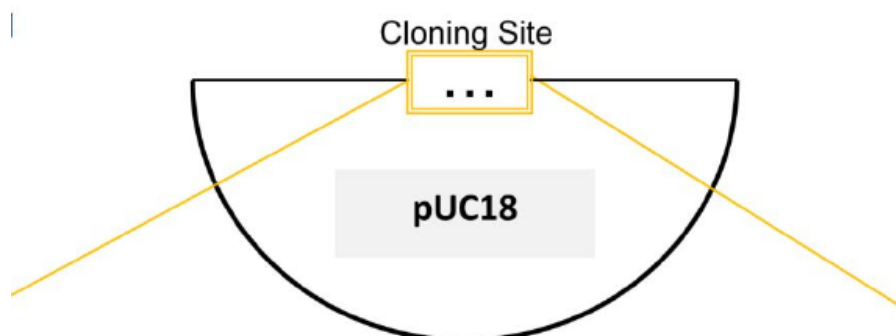
Reverse Primer Design for Insert Amplification:

3' — Gene-specific reverse primer sequence + restriction site (optional retention or removal) + homologous sequence from the downstream vector terminus — 5'

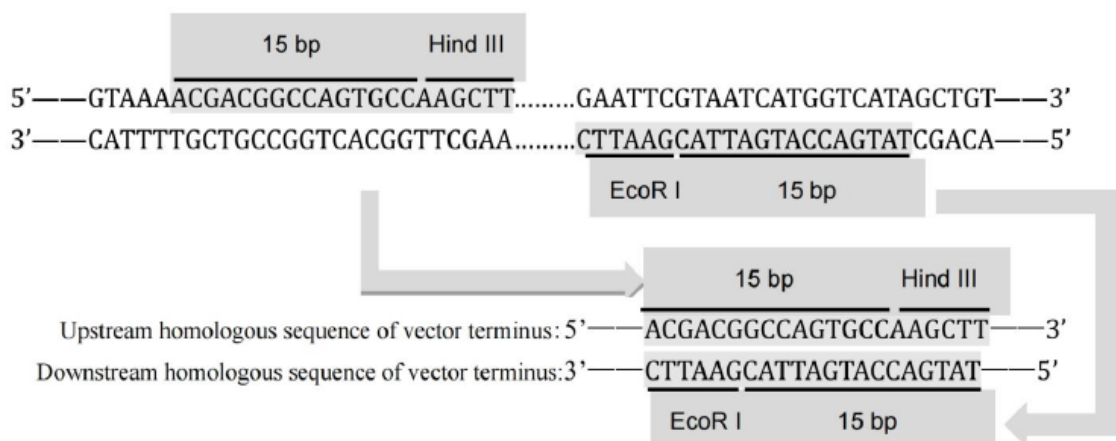
Note:

- 1)The gene-specific forward/reverse primer sequence refers to the conventional primer sequence used for amplifying the insert fragment.
- 2)The upstream/downstream homologous sequences correspond to the terminal sequences of the linearized vector (used for homologous recombination). A GC content between 40% and 60% is recommended.

It is recommended to use the Professional cloning primer design tool to automatically generate primers for insert amplification. For manual primer design, please refer to the following example:

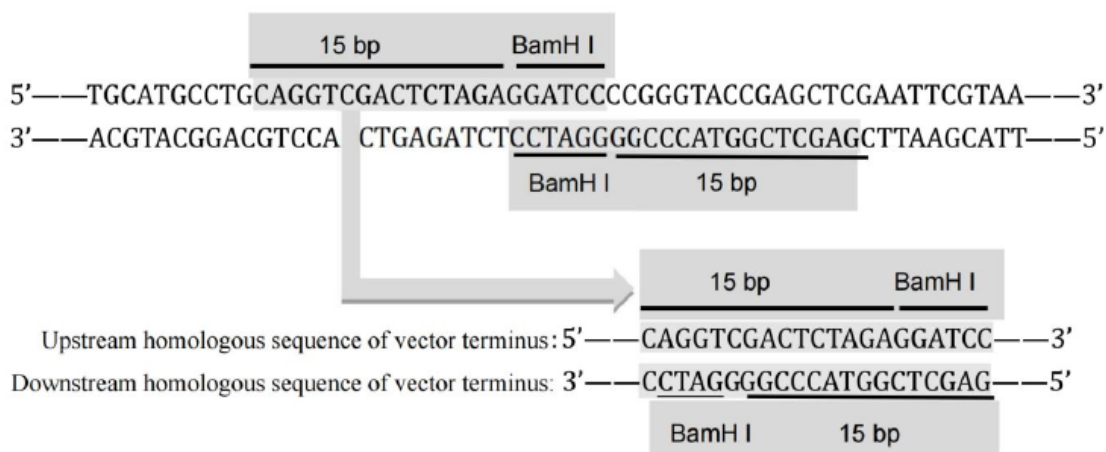


5.2.1.1. If the vector is linearized using double restriction digestion (Hind III + EcoR I):



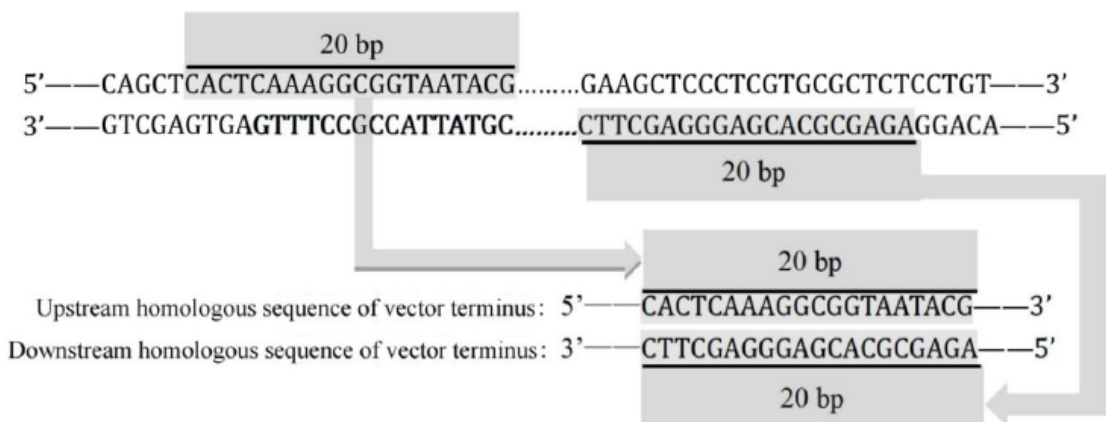
(Note: After cloning, the Hind III and EcoR I sites will remain intact.)

5.2.1.2. If the vector is linearized using single restriction digestion (BamH I):



(Note: After cloning, both ends of the insert fragment will retain complete BamH I restriction sites.)

5.2.1.3. If the vector is prepared using reverse PCR amplification:



(Note: After cloning, the insert fragment will be seamlessly ligated with the vector.)

Figure 2. Insert Fragment Primer Design Strategy

Note: If the final primer length exceeds 40 bp, it is recommended to use PAGE purification for primer synthesis. When calculating the annealing temperature of the amplification primers, only the T_m value of the gene-specific amplification sequence should be considered; the homologous sequence from the vector termini should not be included in the calculation. To achieve high cloning efficiency, a T_m of ≥48°C is recommended.

5.2.2. PCR Amplification of the Insert Fragment

The insert fragment can be amplified using any PCR enzyme, without considering the presence or absence of an A-tail at the ends of the product (as this will be removed during recombination and will not appear in the final vector). However, to minimize the introduction of amplification mutations, it is recommended to use a high-fidelity polymerase for amplification (e.g., 2× mRNex® Plus PCR Master Mix (With Dye), Cat. LM201002-2).

After PCR amplification, a small amount of the product should be analyzed by agarose gel electrophoresis to assess amplification yield and specificity. The mRNex® recombination reaction system is compatible with conventional PCR reaction systems. Therefore, if the amplification template is not a circular plasmid with the same antibiotic resistance as the vector and the electrophoresis shows a single band, the PCR product can be used directly for recombination without purification.

However, if the PCR product has low purity, it is recommended to purify the amplification product using a high-quality gel extraction kit. This increases product purity and is beneficial for enhancing recombination efficiency.

5.3. Quantification of Linearized Vector and Insert Fragment Concentration

It is recommended to use agarose gel electrophoresis and compare band intensity for DNA quantification. Prepare a series of equal-volume dilution gradients for both the linearized vector and the amplified insert fragment. Load 1 µL of both the original and diluted products onto an agarose gel for electrophoresis. The band intensity should then be compared to a DNA molecular weight standard (DNA marker) to determine the approximate concentration.

5.4. Recombination Reaction

5.4.1. Calculation of Linearized Vector and Insert Fragment Usage

The optimal amount of vector for the mRNex® recombination reaction system is 0.03 pmol, with an optimal molar ratio of vector to insert fragment of 1:2 to 1:3, meaning the optimal amount of the insert fragment should be 0.06–0.09 pmol. The DNA mass corresponding to these molar amounts can be approximately calculated using the following formulas:

- Optimal vector amount = $[0.02 \times \text{number of vector base pairs}] \text{ ng}$ (0.03 pmol)
- Optimal insert fragment amount = $[0.04 \times \text{number of insert fragment base pairs}] \text{ ng}$ (0.06 pmol)
or = $[0.06 \times \text{number of insert fragment base pairs}] \text{ ng}$ (0.09 pmol)

Example:

When cloning an insert fragment of 2 kb into a vector of 5 kb in length:

- The optimal amount of vector should be: $0.02 \times 5000 = 100 \text{ ng}$
- The optimal amount of the insert fragment should be: $0.04 \times 2000 = 80 \text{ ng}$ or $0.06 \times 2000 = 120 \text{ ng}$

Note:

- 1) If the insert fragment is longer than the vector, the calculation method for the optimal amounts should be reversed—that is, treat the insert fragment as the vector and the vector as the insert fragment for the calculation.
- 2) The amount of linearized vector should be between 50–200 ng, and the amount of the amplified insert fragment should be between 20–200 ng. If the amounts calculated using the above formula exceed these ranges, select the minimum or maximum allowed amount accordingly.
- 3) When using unpurified linearized vector and insert fragment amplification products directly, the total volume added should not exceed one-fifth of the total reaction volume. For example, in a 20 µL reaction system, the total input volume should not exceed 4 µL.

5.4.2. Recombination Reaction System

(It is recommended to prepare the reaction mixture on ice, ensuring all components are thoroughly mixed before use.)

Component	Recombination Reaction	Negative Control-1	Negative Control-2	Positive Control
ddH ₂ O	Up to 20 µL	Up to 20µL	Up to 20µL	Up to 20 µL
2×mRNex® Enzyme Premix	10µL	0µL	0µL	10µL
Linearized Vector	XµL	XµL	0µL	1µL
Insert Fragment	YµL	0µL	YµL	1µL

5.4.3. Recombination Reaction Conditions

5.4.3.1. After preparing the reaction mixture, gently mix the components using a pipette by aspirating and dispensing. Briefly centrifuge to collect the reaction mixture at the bottom of the tube.

5.4.3.2. Incubate at 50°C for 20 minutes. If the insert fragment is larger than 5 kb, extend the incubation time to 25 minutes.

[Note]: It is recommended to perform the reaction using an instrument with precise temperature control, such as a PCR machine or a water bath.

5.4.3.3. After the reaction is complete, place the reaction tube on ice for 5 minutes to cool, which helps prevent reduced transformation efficiency caused by high temperatures.

5.4.3.4. The reaction product can be used directly for transformation or stored at -20°C for future use. Thaw before transformation when needed.

5.5. Transformation and Plating of Recombinant Products

5.5.1. Thaw competent cloning cells (e.g., DH5α Chemically Competent Cells, Cat: LM304002) on ice.

5.5.2. Add 10 µL of cooled recombinant product to 100 µL of competent cells. Gently flick the tube wall a few times to mix and incubate on ice for 25 minutes.

5.5.3. Heat shock the cells at 42°C for 45 seconds, then immediately incubate on ice for 1–2 minutes.

5.5.4. Add 750 µL of SOC or LB medium and incubate at 37°C for 2 minutes to allow cell recovery. Then, shake the culture at 37°C, 200 rpm for 60 minutes.

5.5.5. Centrifuge at 5000 rpm for 1 minute, discard 750 µL of the supernatant, and resuspend the bacterial pellet in the remaining medium. Use a sterile spreader to gently spread the suspension onto an agar plate containing the appropriate antibiotic. Once the liquid is absorbed, invert the plate and incubate overnight at 37°C.

5.6. Clone Verification

The most convenient and rapid method for clone verification is colony PCR. Use a sterile pipette tip or toothpick to pick a single colony and mix it into 20–50 µL of LB medium. Use 1 µL of this mixture as the PCR template. It is recommended to use at least one universal sequencing primer for colony PCR to avoid false-positive results. Further verification can be performed using restriction enzyme digestion or sequencing.

6. Application Example

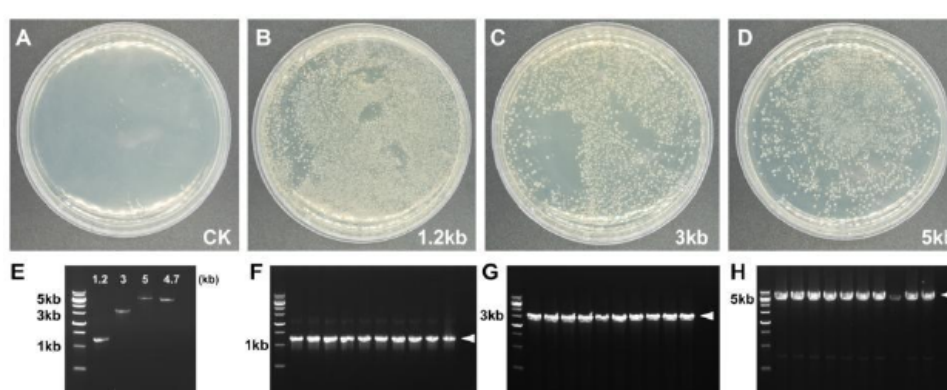


Figure 3: The mRNex® Plus One Step Cloning Kit effectively clones single-fragment genes of various lengths.

A–D: Recombinant transformation plates. E: Electrophoresis gel showing concentration detection of the insert fragment and vector. F–H: Electrophoresis gel of PCR verification for the insert fragments. Arrows indicate the target bands.

Vector: pFastBac1 (4.7 kb); Insert fragment sizes: 1.2 kb, 3 kb, and 5 kb. Molar ratio of vector to insert fragment: 1:3. Recombination reaction conditions: 50°C for 20 minutes.

7. Frequently Asked Questions (FAQ)

7.1. How to select the optimal cloning site?

When selecting a cloning site, avoid regions where there are repetitive sequences within 50 bp upstream or downstream of the insertion point. Maximum recombination efficiency is achieved when the GC content within 25 bp upstream and downstream of the cloning site is maintained between 40% and 60%. Recombination efficiency may be significantly reduced if the GC content exceeds 70% or drops below 30%.

7.2. Why are there no colonies or very few colonies?

If this issue occurs, it is recommended to use a positive control to eliminate the possibility of reagent-related issues. Further evaluation should consider the following factors:

7.2.1. Improper primer design: It is recommended to design primers using the format:

[Homologous sequence (15–25 bp) + Complete restriction site + Gene-specific amplification sequence], with a GC content of 40%–60%.

7.2.2. Low efficiency of competent cells: Use freshly prepared or properly frozen competent cells with a transformation efficiency of $>10^7$ cfu/ μ g. Set up a control transformation with a plasmid to check the efficiency of the competent cells. The volume of the ligation product should not exceed 1/10 of the competent cell volume, as exceeding this may reduce transformation efficiency.

7.2.3. Incorrect amounts or ratios of vector and insert fragment: Prepare the recombination reaction according to the recommended amounts and ratios.

7.2.4. Impure linearized vector or insert fragment inhibits the reaction: When using unpurified linearized vector and insert fragment amplification products directly, the total volume should not exceed 1/5 of the total reaction system (e.g., no more than 4 μ L in a 20 μ L system). It is recommended to purify both the linearized vector and insert fragment by gel extraction and dissolve the purified products in ddH₂O.

7.3. Why are there many false positives?

The following factors may contribute to false positives:

7.3.1. Incomplete linearization of the vector: Even trace amounts of undigested circular plasmid can result in a high transformation background. Use a negative control to verify complete linearization of the vector. Optimizing the digestion system, increasing the amount of restriction enzyme, extending digestion time, and purifying digestion products by gel extraction can effectively reduce the presence of residual circular plasmids.

7.3.2. Non-specific amplification products in the insert fragment:

- A. Optimize the PCR system to improve specificity.
- B. Purify PCR products through gel extraction.
- C. Screen more colonies for verification.

7.3.3. Contamination of the reaction system with plasmids carrying the same antibiotic resistance:

If circular plasmid templates (vector or insert fragment) are used directly in the recombination reaction, residual plasmids can cause a high transformation background. It is recommended to use pre-linearized plasmids as templates, perform DpnI digestion of the PCR product, or purify PCR products using gel extraction.

7.4. Why is there no band in colony PCR?

This issue may arise due to the following reasons:

7.4.1. Incorrect primers: It is recommended to use universal primers targeting the vector or at least one universal primer for colony PCR detection.

7.4.2. Inappropriate PCR system or program: If no bands appear for either the target or empty plasmid, optimize the PCR system or program. Alternatively, extract the plasmid and use it as a template for PCR verification or perform a restriction enzyme digestion test.

7.4.3. Recombination failure: If only bands corresponding to the empty plasmid appear without the target band, this suggests unsuccessful recombination, possibly due to incomplete vector linearization. It is recommended to optimize the digestion system