GenBuilder™ Cloning Kit User Manual

Cat.no L00701 **Version 11242017**

I. Introduction

I.1 Product Information

The GenBuilder Cloning Kit enables seamless and one-step cloning of multiple DNA fragments into a linearized vector. The proprietary enzyme mix fuses together DNA fragments efficiently and precisely by recognizing 15-40 bp overlaps at the end of each fragment. The system allows cloning of DNA fragments into any linearized vector, requires no pre-existing recombination sites or helper sequences, and eliminates the need for a complicated restriction and ligation process. The GenBuilder Cloning kit is recommended for simultaneous cloning of up to five DNA fragments. For a greater number of fragments, or for assembling ssDNA oligos with dsDNA fragments, the GenBuilder Plus Cloning kit (L00744) is recommended.

I.2 Kit Contents and Storage

The kit contains GenBuilder 2X Master Mix and Positive Control.

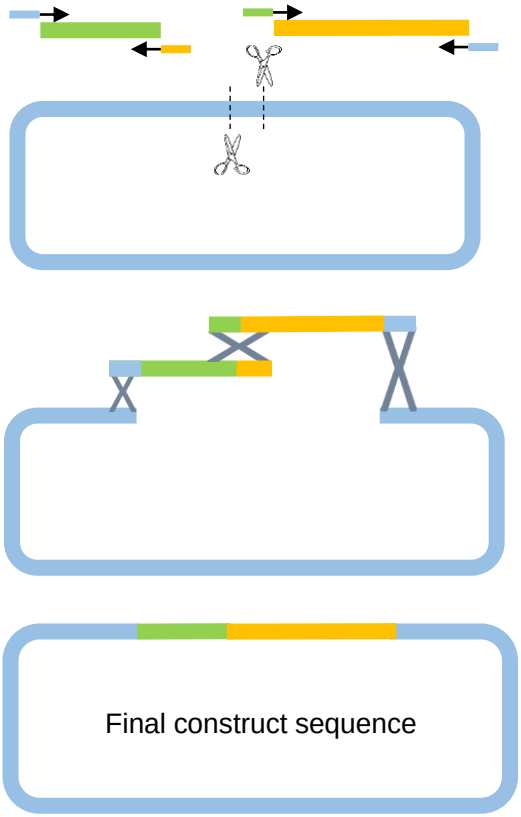
Keep all components at –20°C for long term storage. Thaw the master mix on ice prior to use. For the best performance, please aliquot the master mix into smaller volumes and avoid unnecessary freeze-thaw cycles. The GenBuilder 2X Master Mix is stable at 4°C for at least two weeks

I.3 GenBuilder Cloning Kit Workflow

1. Prepare the DNA fragments with overlaps and the linearized vector for assembly.

2. Add DNA fragments and linearized vectors in the GenBuilder master mix. Mix well and incubate at 50° C for 15 mins.

3. Transform assembly product into competent cells and plate on selective medium. Obtain colonies containing the assembled DNA.



Ⅱ. DNA Preparation for GenBuilder Cloning Kit

II.1 Prepare Linearized Vector

A successful and efficient GenBuilder cloning kit reaction starts with the preparation of a linearized cloning vector. A linearized vector can be prepared using restriction endonuclease digestion or PCR amplification.

Follow the guidelines below to prepare your linearized vectors:

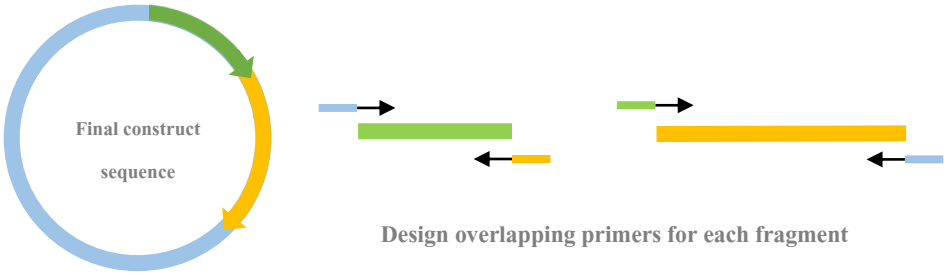
- When generating the linearized vector by restriction endonuclease digestion, it is recommended to use two enzymes to perform a double-digest. This significantly reduces the chance of carrying over the uncut vector into the DNA assembly reaction, and therefore reduces the amount of background after cloning.
- A complete digestion of the target vector is very important for successful cloning. To ensure complete digestion, we recommend increasing the digestion time to 2 hours with appropriate amount of enzymes.
- When using PCR to amplify the vector, it is best to use linearized plasmid rather than circular plasmid DNA as a template. PCR amplification from double digested plasmids would further minimize the number of empty (carryover) clones.
- When circular plasmid DNA has to be used as a template to amplify the linearized vector by PCR, it is recommended to use a dam+ strain plasmid and digest the PCR product with *Dpn*I restriction endonuclease to minimize then number of carryover plasmids. It is noted that *Dpn*I cleaves only when its recognition site is methylated.

II.2 Primer Design

GenBuilder assembly reaction requires that each DNA fragment (including the cloning vector) share a 15-40 bp homology sequences at each terminus. Therefore, PCR primers should be designed in a way that they generate PCR products containing ends that are homologous to adjacent fragments or vectors.

Follow the guidelines below when designing your PCR primers:

- PCR primers used to amplify DNA fragments must have two sequence components: a 5’ overlap sequence that is homologous to the terminus of the adjacent fragment and a 3’ gene-specific sequence for template priming in PCR amplification.
- To prevent errors in primer design, it is highly recommended to first perform DNA fragment assembly *in silico* and create a final sequence file displaying both DNA strands.



- For efficient cloning of PCR fragments into a vector, we recommend using a 15-40 bp overlap with a T_m equal to or greater than 50° C. For cloning one or two fragments, 15 bp overlap is sufficient for successful DNA assembly. When performing complex assembly reactions (cloning more than 3 fragments simultaneously), increasing the overlap region to 40 bp is recommended.
- If you are cloning the insert into an enzymatically linearized vector, all 15 nucleotides providing the requisite homology must be on the 5' end of the primer. To recombine two adjacent inserts, you may split the 15 bp homology between the fragments (e.g., 7 bp on the reverse primer of fragment 1 and 8 bp on the forward primer of fragment 2).
- For higher efficiency and specificity, it is recommend to avoid structured sequences in overlap regions. It is also important to carefully design the overlap regions so that they are distinct from each other to avoid incorrect assembly.
- The rules of thumb above also apply to one-piece DNA cloning with a target vector.
- GenScript provides high quality and economical GenParts™ DNA fragments up to 2,000 bp of your choice which can be used directly for GenBuilder assembly.

II.3 Primer Design for Cloning Two Adjacent Fragments into One Fragment

The overlap sequence could either come from one fragment (Fig. 1) or both adjacent fragments, e.g., half and half (Fig. 2). You may adjust the overlap region for optimal T_m and length.

Figure 1: The overlap sequence is split between the two adjacent fragments.

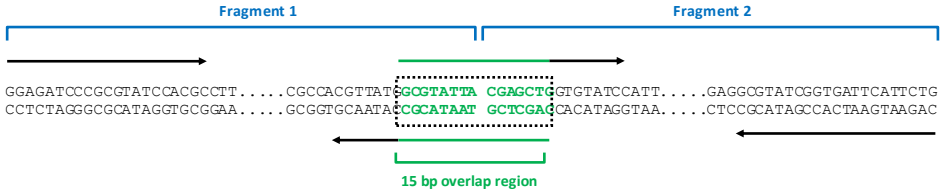
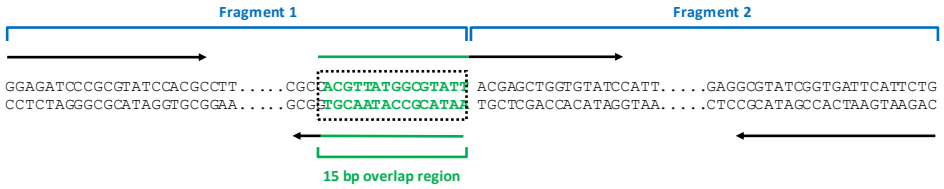


Figure 2: The overlap sequence comes from only one fragment.



II.4 Primer Design for Cloning One DNA Fragment into a Linearized Vector

When vector is linearized by restriction digestion, the entire overlap sequence must originate from the vector sequence and must be added to primers that will be used to amplify the insert. Restriction enzyme digested vectors can have 5´ overhangs, 3´ overhangs, or blunt ends. To determine the overlap sequence between the vector and the insert, start at the 3' end of each DNA strand in the linearized vector.

Figures 3a and 3b shows two examples of PCR primer design for assembly of a DNA fragment with a sticky vector backbone (i.e., with either 5' or 3' protruding ssDNA end), while Figure 3c represents a typical design of the primer sequences for assembly with a blunt end vector.

Figure 3a. Cloning insert into linearized vectors with 5' overhangs

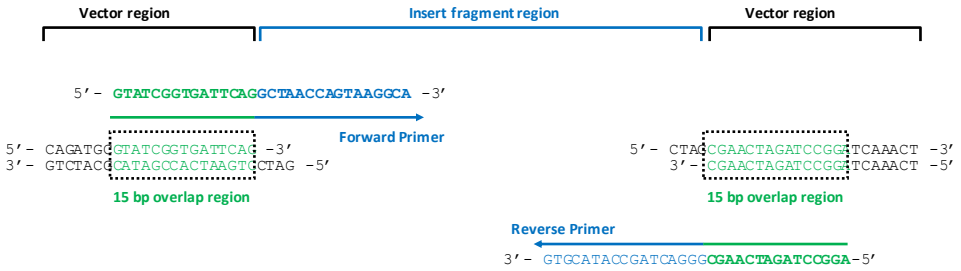


Figure 3b. Cloning insert into linearized vectors with 3' overhangs

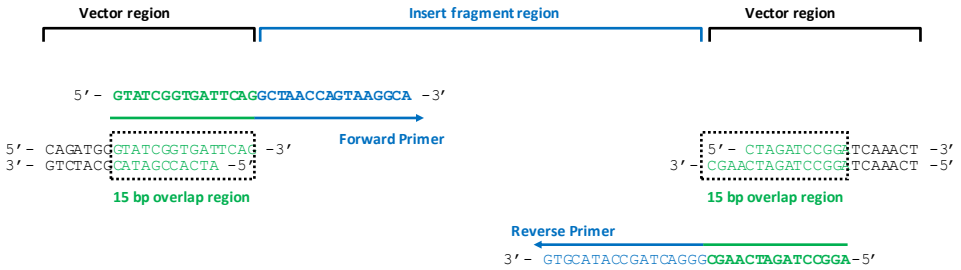
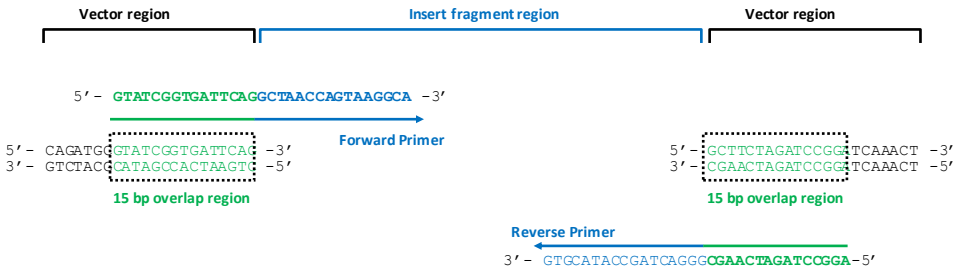


Figure 3c. Cloning insert into linearized vectors with blunt end



II.5 Recommendations for PCR

High quality DNA fragments are essential for a successful GenBuilder assembly reaction. Follow the guidelines below to prepare high quality DNA fragments by PCR:

- We recommend the use of a high fidelity DNA polymerase to amplify your DNA fragments or your linearized vector.
- When plasmid DNA is used as a template for PCR, 100 pg ~ 1 ng of plasmid DNA is generally sufficient.
- If the template plasmid contains the same selectable marker as the destination vector, we recommend digestion of the PCR product with *DpnI* before the DNA assembly reaction.
- Verify your PCR products by gel electrophoresis. If multiple bands are obtained for certain product, it is essential to gel purify the DNA fragments to avoid assembling with undesired inserts.
- GenBuilder cloning kit reaction allows cloning of unpurified PCR products. If your PCR product has a single and clear band, you can add unpurified PCR product directly in the assembly reaction. Do not add more than 10% of crude PCR product in the reaction since an excess volume of unpurified PCR amplicons may significantly reduce assembly efficiency. For the best assembly efficiency, however, we recommend using gel purified DNA fragments for complex assembly.

III. GenBuilder Cloning Kit Reaction

III.1 Calculate Input DNA Quantity

Option 1. We recommend adding 0.1 pmol of each DNA fragment in an assembly reaction.

- Use 2:1 insert to vector molar ratio for cloning one or two fragments.
- Use 1:1 insert to vector molar ratio for cloning more than two fragments.
- Use 5 times more inserts when the insert is less than 200 bp.

Determine the concentration of your DNA fragment solutions by UV or by fluorescence. For 0.1 pmol of each DNA fragment, use the concentration (ng/µl) and length (bp) of the DNA fragment to calculate the volume required for the GenBuilder reaction:

$$\mu l\ of\ DNA\ fragment = \frac{0.65 \times pmol \times bp}{ng/\mu l}$$

For example, to clone a 1,500 kb fragment dissolved in 50 ng/µl solution into a linearized vector, use the following formula to calculate the volume that should be added in GenBuilder assembly reaction:

$$\frac{0.65 \times 0.1\ (pmol) \times 1500\ (bp)}{50\ (ng/ul)} = 1.95\ \mu l$$

Option 2. You can use the following table for a quick estimation of input DNA concentration in GenBuilder reactions (example: 0.1 pmol fragment in the reaction):

Fragment length	Input DNA
0.5 kb	33 ng
1 kb	67 ng
1.5 kb	100 ng
2 kb	133 ng
3 kb	200 ng
5 kb	330 ng

In cases when unable to acquire sufficient fragment or vector DNA, GenBuilder cloning kit reaction allows to reduce DNA concentration to 0.05 pmol in each reaction, albeit with reduced colony number.

III.2 GenBuilder Assembly Reaction Protocol

Note: When ssDNA is used to bridge two adjacent fragments, it is recommended to purchase our ssDNA enhancer (Cat.no M00684) or use the GenBuilder Plus cloning kit (L00744).
We recommend always performing the control reactions in parallel with your assembly reaction. This helps to verify that the kit components are performing optimally. The positive control fragments provided in the GenBuilder cloning kit are designed to clone two 500 bp fragments into a linearized pUC57 vector. The final construct of the assembly contains sequences and elements for expressing red fluorescent protein. Thus, a successful positive control reaction should reconstitute red fluorescent protein (RFP) and produce hundreds of red colonies on selective plates containing Amp and IPTG (depending on the efficiency of competent cells) after overnight incubation of the transformants. The positive rate, which can be calculated as the percentage of red colonies, should be >90%.

1. Set up the following reaction on ice:

	Assembly reaction	Positive control
Each DNA fragment for assembly	0.1 pmol * X μl	10 μl
Linearized vector	0.1 pmol X μl	N/A
GenBuilder 2x Master Mix	10 μl	10 μl
Deionized H_2O	to 20 μl	-

* If unpurified PCR product is used for assembly, the total volume of PCR product should not exceed 10% of the total reaction volume.

* When ssDNA is used to bridge two adjacent fragments, it is recommended to add 5X of ssDNA in the reaction (i.e., 0.5 pmol in this case) and 1 μl of ssDNA enhancer.

2. Gently mix the reactions by pipetting.
3. Incubate the reaction in a thermocycler at 50°C for 15 minutes. For DNA assembly reaction involving more than six fragments, the incubation time may be increased to 60 minutes.
4. Transform 2 µl of the assembly product into competent *E. coli* cells. For electroporation, dilute the reaction product 5-fold and use 1 µl for transformation. It is recommended to use competent cells with high efficiency (>2×10^8 transformants per µg pUC19 plasmid).
5. Spread 1/10 volume of the recovered cells onto selection plates. For a complex assembly (cloning more than 3 fragments), we recommend that you concentrate the cells before spreading on selection plates. For a positive control reaction, spread 1/10 volume of the cells on LB Agar plates containing 100 mg/ml ampicillin and 0.1 mM IPTG.
6. Incubate the plates overnight at 37°C.

Ⅳ. GenBuilder Assembly Transformation

Ⅳ.1 Chemical Transformation Protocol

1. Thaw chemically competent cells on ice.
2. Add 2 µl of the assembly product into the competent cells and mix gently. Do not vortex.
3. Incubate the mixture on ice for 30 minutes.
4. Heat shock the cells at 42°C for 30 seconds without shaking.
5. Immediately transfer the tubes to ice and incubate for 2 minutes.
6. Add 950 µl of SOC media to the tube.
7. Shake the tube at 37°C for 60 minutes
8. Warm selection plates to 37°C.
9. Spread 100 µl of the cells onto the selection plates. Incubate the plate overnight at 37°C.

Note: If you perform more than 4 part assembly, we suggest that you concentrate the cells before spreading.

Ⅳ.2 Electrocompetent Cells Transformation Protocol

1. Chill electroporation cuvettes on ice.
2. Thaw electrocompetent cells on ice.
3. Dilute the assembly reaction 5-fold and add 1 µl to 50 µl electrocompetent cells.
4. Mix gently with the end of a pipette tip
5. Transfer the cell/DNA mixture into a chilled cuvette, wipe off moisture and water from outside of cuvette using a kimwipe.

6. Insert the cuvette into the electroporator, and press the pulse button.
7. Immediately add SOC medium to the cuvette after the end of the pulse.
8. Shake the tube at 37°C for 60 minutes
9. Spread 1/10–1/100 of the transformation reaction of the cells onto the plates.
10. Incubate overnight at 37°C.

V. Troubleshooting

Symptom	Cause	Solution
No colonies following transformation for positive control	Low transformation efficiency	Test transformation efficiency using a circular plasmid. Expect at least 10^8 colonies per µg DNA after overnight incubation. Do not vortex the competent cells. Re-suspend the competent cells by pipetting up and down gently. Competent cells may be thawed only once. Freeze-thaw cycles of the cells significantly reduces competency.
	Transformation handled incorrectly	Perform the transformation procedure exactly as described in the user manual.
	Transformants spread on plates containing the wrong antibiotic	Cells with positive control assembly should be plated on LB/Amp plates with 0.1 mM IPTG.
No colonies following transformation with DNA inserts, but transformation with positive control is successful	DNA fragments do not share end terminal homology	Create the sequence file of the final construct and check the primer design to amplify the overlapping DNA fragments to ensure that there is sufficient and correct overlap to facilitate assembly. Avoid hairpin structures in the overlap region.
	Incorrect amounts of DNA fragments and/or vector were used	Determine the concentration of each of the DNA fragments and the linearized vector(s), and use the formula given in section III.1 to determine input DNA concentration in assembly reactions.

	Low quality DNA fragments	Verify your DNA fragments and linearized vectors by gel electrophoresis. Make sure they have single bands of the correct size. When using unpurified PCR product directly for assembly, make sure the total volume of PCR products does not exceed 10% of the reaction.
Large number of transformants containing no insert	Cloning vector incompletely linearized	Use two different restriction endonucleases to linearize the vector or use PCR to amplify the linearized vector and use *Dpn*I to destroy plasmid template.
	Plates too old or contained incorrect antibiotic	Makes sure to use freshly prepared plates containing the correct selection antibiotic.
Large number of transformants containing incorrect insert	PCR products not pure enough	Verify your DNA fragments and linearized vectors by gel electrophoresis. Make sure that they are single band of the correct size. If the PCR product is contaminated with non-specific bands, it is essential to gel purify the product.
	Incorrect design of overlap	Check overlapping sequences and make sure they are distinct from each other.

GenScript US
860 Centennial Ave., Piscataway, NJ 08854
Tel: 732-885-9188, 732-885-9688
Fax: 732-210-0262, 732-885-5878
Email: product@genscript.com
Web: http://www.genscript.com
For Research Use Only.