SYNTHETIC GENOMICS®

Gibson Assembly® HiFi 1-Step Kit Instructions

Catalog Numbers GA1100-10, GA1100-50, GA1100-S,
GA1100-10MM, GA1100-50MM

REV. 2.3 04.28.15
Part number 99011

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Gibson Assembly®: US Patent Nos. 7,776,532 and 8,435,736.

Table of Contents

Kit Information

Products

Component	Catalog Number	Number of Reactions	Positive Control Reactions	Storage Temperature
GA HiFi 1-Step Kit	GA1100-S	5 reactions	2 reactions	−20°C
	GA1100-10	10 reactions	2 reactions	
	GA1100-50	50 reactions	5 reactions	
GA 1-Step Master Mix (2X)	GA1100-10MM	10 reactions	—	
	GA1100-50MM	50 reactions		

Gibson Assembly® HiFi 1-Step Kit Contents

Component	Quantity	Cat. GA1100-S (5 Reactions)	Cat. GA1100-10 (10 Reactions)	Cat. GA1100-50 (50 Reactions)
		Volume		
GA 1-Step Master Mix (2X)	1 each	25 µl	50 µl	250 µl
GA Positive Control (2X)	1 each	10 µl (2 Control Reactions)	10 µl (2 Control Reactions)	25 µl (5 Control Reactions)
GA 1-Step Quick Reference Manual	1 each			

GA 1-Step Master Mix (2X)

GA 1-Step Master Mix (2X) contains a proprietary mixture of enzymes and reagents optimized to facilitate one-step assembly[1]. One of the components of the master mix, Phusion® DNA Polymerase, mediates junction repair. As a result, products assembled with GA 1-Step Master Mix (2X) demonstrate low rates of junction error and high sequence fidelity.

Positive Control

The positive control DNA supplied with this kit is sufficient for 2 reactions (Cat. GA1100-S and GA1100-10) or 5 reactions (Cat. GA1100-50). The positive control consists of a mixture of 10 ng of a 1.5 kb insert and 30 ng of a 2.7 kb vector containing an ampicillin resistance gene. Select for the 4.2 kb assembled construct on LB agar plates with 100 µg/ml ampicillin, 0.1 mM IPTG, and 40 µg/ml X-Gal.

Additional Required Materials

- DNA fragments for the Gibson Assembly® reaction
- Thermocycler
- Luria-Bertani (LB) plates with appropriate antibiotic
- SOC Outgrowth Medium
- High efficiency electrocompetent cells

  Recommended: TransforMax™ EPI300™ Electrocompetent *E. coli*
- Gene Pulser®/MicroPulser™ Cuvettes: 0.1 cm Gap Width
- Gene Pulser® Xcell Microbial System

Additional Optional Materials

- High Fidelity DNA Polymerase for producing fragments to be assembled with Gibson Assembly®

  Recommended: Thermo Fisher High Fidelity Phusion® DNA Polymerase (Cat. F-530S)
- QIAquick PCR purification Kit
- Spectrophotometer

Overview

Introduction

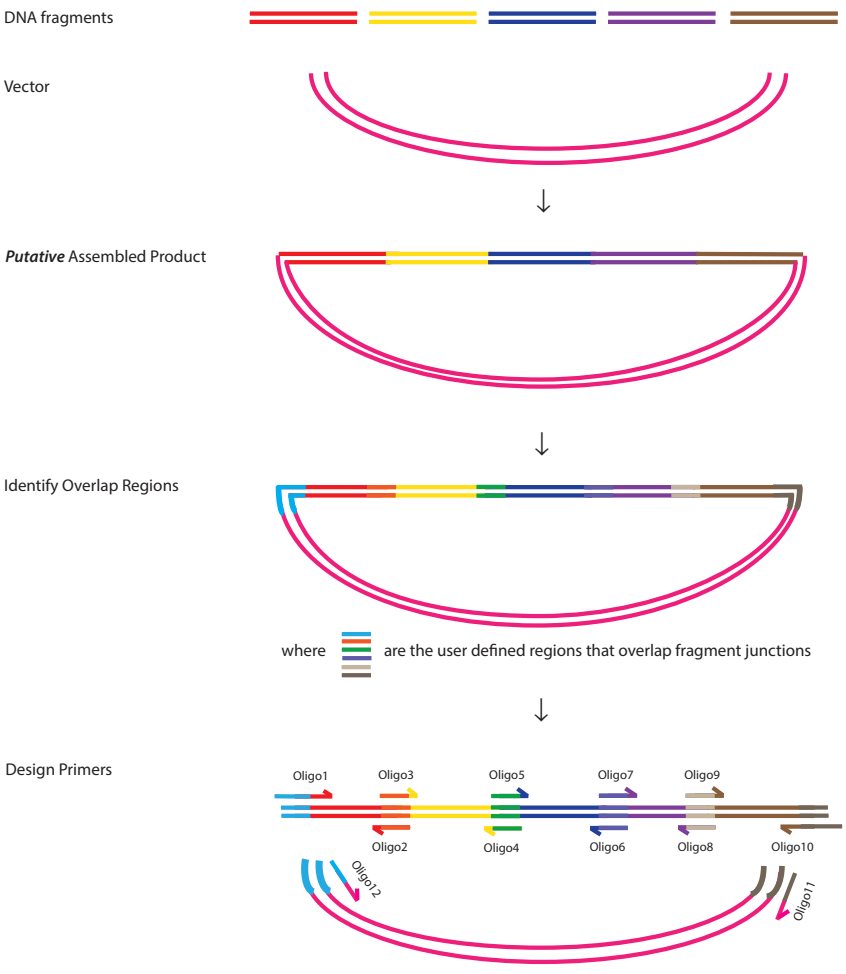
Developed by Dr. Daniel Gibson and his team at the J. Craig Venter™ Institute in 2009, the Gibson Assembly® Method is a well-established assembly reaction that can be used to join multiple, overlapping DNA fragments in a one-step, single-tube, isothermal reaction. DNA fragments of various lengths are uniformly assembled using complementary overlaps between fragments. The inherent flexibility of this approach lends itself to small and large constructs and encompasses both single and multiple insert assemblies. The resulting products may be used for a variety of downstream applications including transformation, PCR, and rolling-circle amplification (RCA).

Key Features

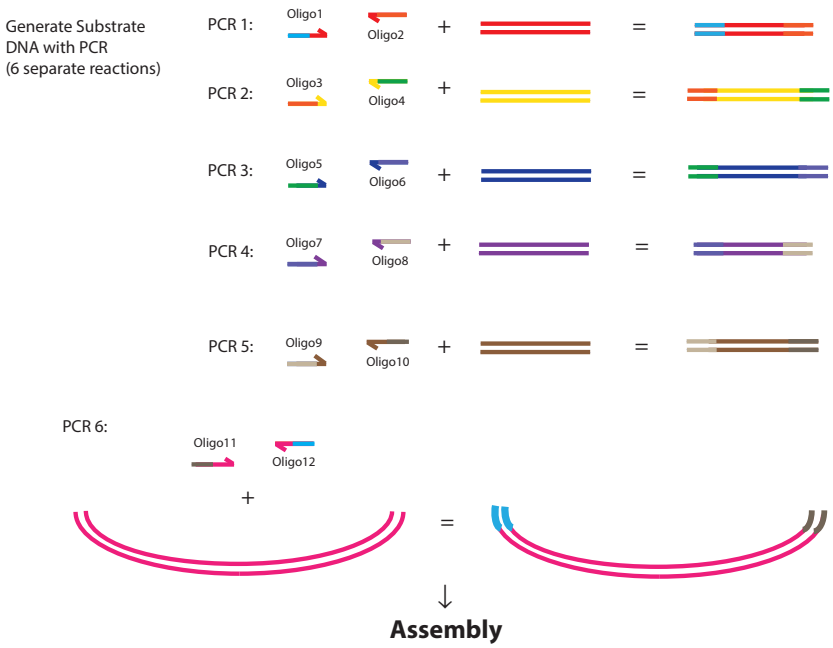
Key Features of the Gibson Assembly® HiFi 1-Step Kit
• Accurate
• Seamless
• Optimal for 1–5 inserts
• Suitable for fragments ranging from 500 bp – 32 kb

Gibson Assembly® Synopsis

Before Starting: Identify Overlap Regions and Design Primers



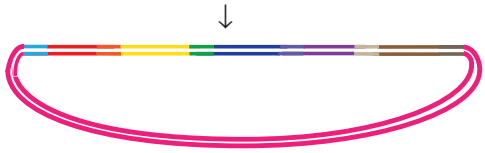
Produce DNA Fragments



Step 1A:
Combine Substrate DNA and GA 1-Step Master Mix:

5′ Chew back

Step 1B:
Annealing



Step 1C:
Repair and Ligatation

Final Assembled Product
(Overlap regions not shown)

Planning your PCR experiment

For optimal manual design of primers for substrate DNA preparation:

1. Identify the junctions of the DNA fragments.
2. Create a file containing the putative final product by cutting and pasting the source DNA sequences into your new file. Annotate the sequence to identify junctions and the source of each DNA fragment.

   Note: At this stage, you can make custom changes to the junction sequence (for example, add a restriction enzyme site allowing you to release an insert from a vector).
3. Select optimal primer sequences, taking into consideration typical PCR-primer properties, such as T_m° values, G/C ratio, and GC anchors/clamps, in addition to the features outlined in the next sections.

   Note: Confirm that the termini of your substrate DNA fragments do not contain stable single stranded DNA secondary structure, such as a hairpin, stem loops, or repeated sequences, which would directly compete and interfere with the single-stranded annealing and priming of neighboring assembly fragments. Most primers will contain some hairpin secondary structure, but in general, make certain that any hairpin with a calculated T_m° > 30°C is > 5 bp from the 3′ terminus of the primer.

Primer Design

Gibson Assembly® Reaction Substrate DNA: Considerations

Double stranded DNA fragments (as well as substrate vectors) to be joined in the Gibson Assembly® Reaction are generated by PCR or by restriction enzyme digest. For optimal results:

- Only use DNA samples with $A_{260/280}$ > 1.8.
- Use DNA at a concentration ≥ 40 ng/µl.

  Note: If the amount of DNA is limited, the assembly reaction may be performed using DNA at concentrations between 20–40 ng/µl with reduced efficiency. Do not use DNA at concentrations < 20 ng/µl.
- Use a high fidelity PCR polymerase when amplifying your DNA.

  We recommend Phusion® DNA Polymerase.

PCR Primer Characteristics

- Designing the primers for PCR preparation of substrate DNA is critically important for the success of the assembly reaction.
- PCR primers used to amplify DNA fragments for Gibson® Assembly contain:
  - **A 5' homologous overlap sequence**: homologous to the terminus of the fragment it will join. This sequence is required for the alignment and assembly of adjacent fragments.
  - **A 3' gene-specific sequence:** required for template priming during PCR amplification
- Each primer should be at least 30–60 nucleotides (nt) long, with overlap regions that are at least 20–40 bp long.
- The length of the homologous overlap sequence is dependent on the GC content at the junction and the length of fragment. Overlapped sequences can be created by extended PCR primers used to amplify inserts and/or vector. See the following sections for detailed instructions.

Homologous Overlap Regions

- The optimal length of the overlap region depends on the number and length of the fragments in the assembly reaction.

Suggested Length of the Overlap Region

Number of fragments	Fragment size	Length of overlap regions
1–2	≤ 8 kb	20–40 bp
	8–32 kb	25–40 bp
3–5	≤ 8 kb	40 bp
	8–32 kb	40–100 bp

- For higher order assembly, longer overlap regions will result in higher efficiency.
- You may need to optimize PCR amplification reactions when using PCR primers with long homologous overlap regions.
- You may add a restriction enzyme site to the primers between the overlap region and the sequence-specific segment enabling subsequent release of the insert from the vector.

  Note: Be certain that the restriction enzyme introduced in the primers is not also present within the insert.

Producing DNA Fragments for Cloning into a Vector

To clone an insert into a vector:

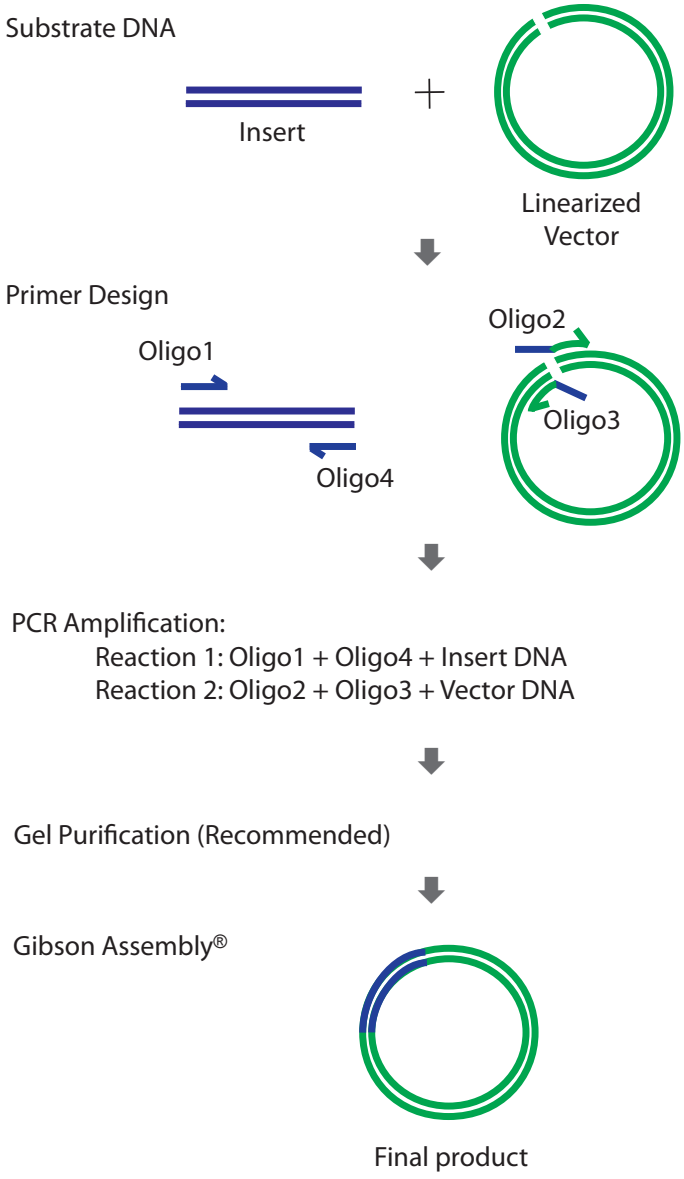
1. Design two sets of primers spanning the terminal ends of the planned insert and vector junctions as shown in the following examples on the following pages.
2. Generate the fragments with PCR amplification of the insert and the vector using a high-fidelity polymerase in two separate PCR reactions: Oligo1 and Oligo4 in one reaction and Oligo2 and Oligo3 in another reaction (as shown in Examples 1–3).

PCR products may be used directly in assembly reactions without additional purification, although results may be improved by gel purifying or column-purifying the PCR products (*e.g.,* using a QIAquick PCR purification Kit) prior to proceeding to assembly (as noted in Examples 1–3).

Primer Design: The Overlap Region

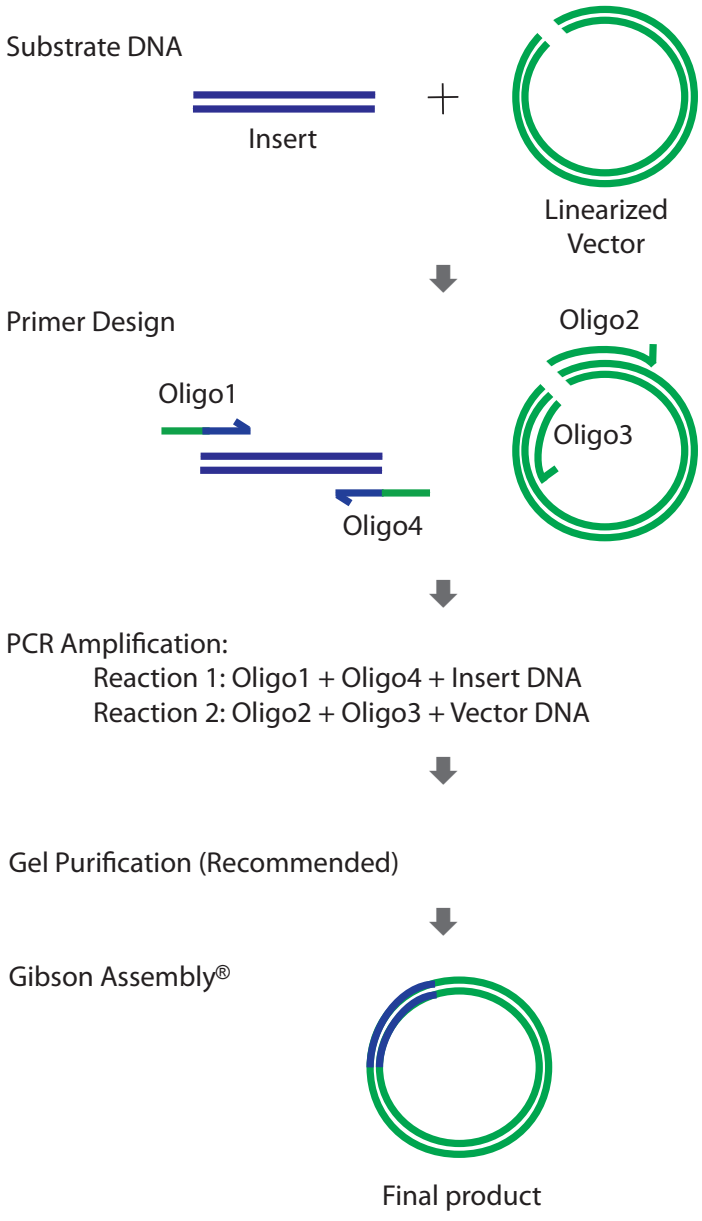
Example 1: Overlap added to vector primers

One advantage of the Gibson Assembly® HiFi 1-Step Kit is that it allows for flexibility in designing primers for substrate DNA amplification. If you intend to clone an insert into multiple vectors or if you intend to shuttle the insert(s) between different vectors, we recommend adding the overlap region to the vector primer only. In this scenario, insert DNA amplified from a single PCR reaction may be used in multiple assembly reactions with a number of different vectors.



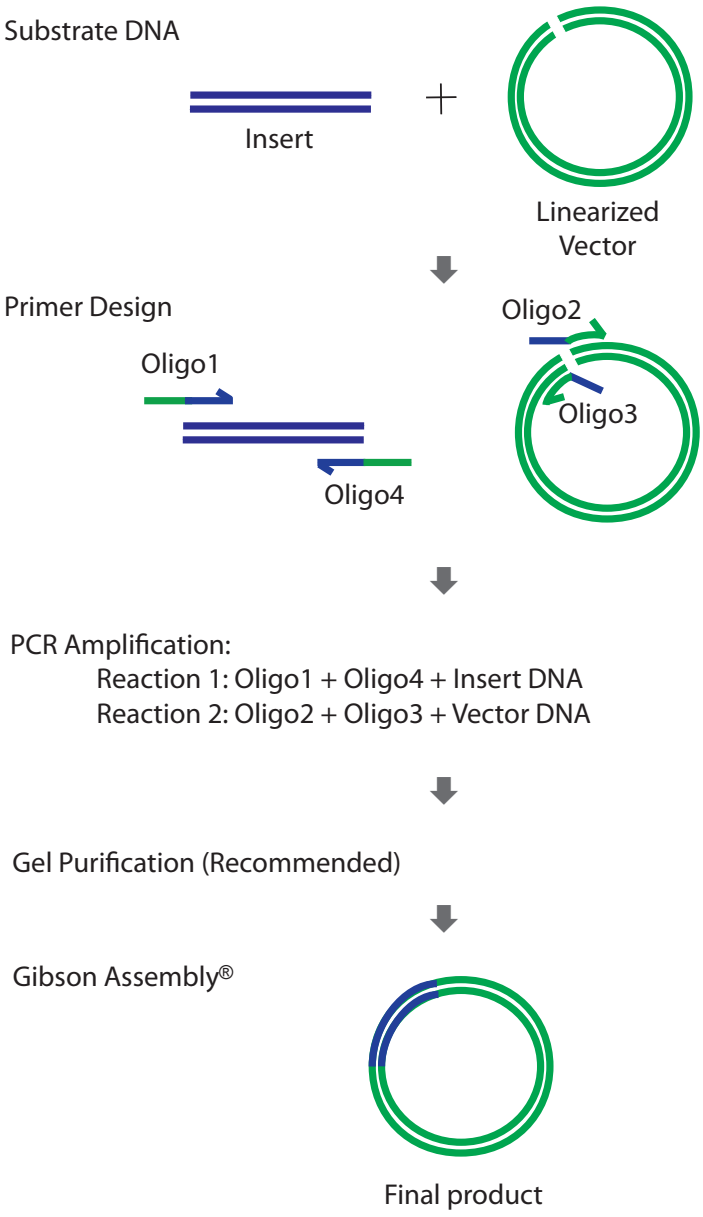
Example 2: Overlap added to insert primers

It may be advantageous to add the overlap region to the insert in situations where amplification of a large vector is problematic (amplification of a vector without overlap tails is more efficient than amplification using primers with overlap tails).



Example 3: Overlap split between vector and insert primers

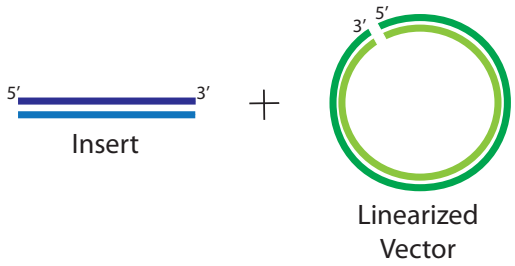
The scenario shown in the following illustration provides the greatest flexibility in primer design. Splitting the overlap may allow for the highest combined efficiency of the PCR amplification reactions of both the insert(s) and vector substrates since the overlap region will be split between all primers. If you experience problems with amplification when adding the overlap regions exclusively to the insert or the vector primers (as described and shown in Examples 1 and 2), consider splitting the overlap between the vector and insert primers as shown below.



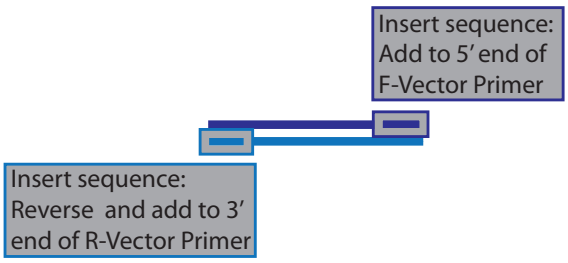
Example 4: Modification- Overlap added to vector primers, with addition of restriction enzyme sequence

Similar to the illustration of “Example 1: Overlap added to vector primers” on page 10, the following example shows overlap sequence added to the vector primers. Additionally, a restriction enzyme site, which may be used to subsequently release the assembled insert from the vector, is added to the vector primers (between the overlap region and the vector-specific sequence, depicted in orange, below). In this scenario, the vector is prepared by amplifying the vector with the F-Vector Primer and R-Vector Primer.

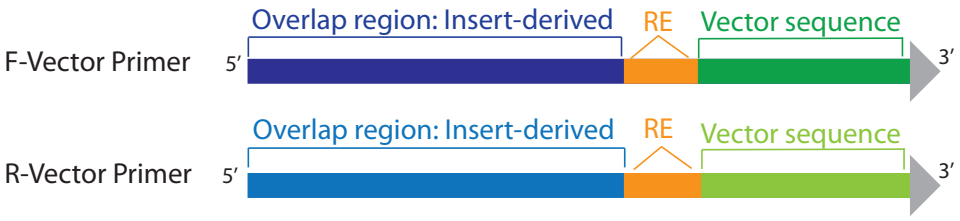
Substrate DNA



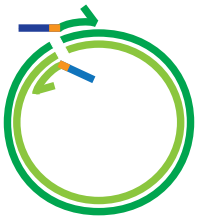
Primer Design



Vector Primers Sequences



Vector Primers aligned for PCR



DNA Preparation

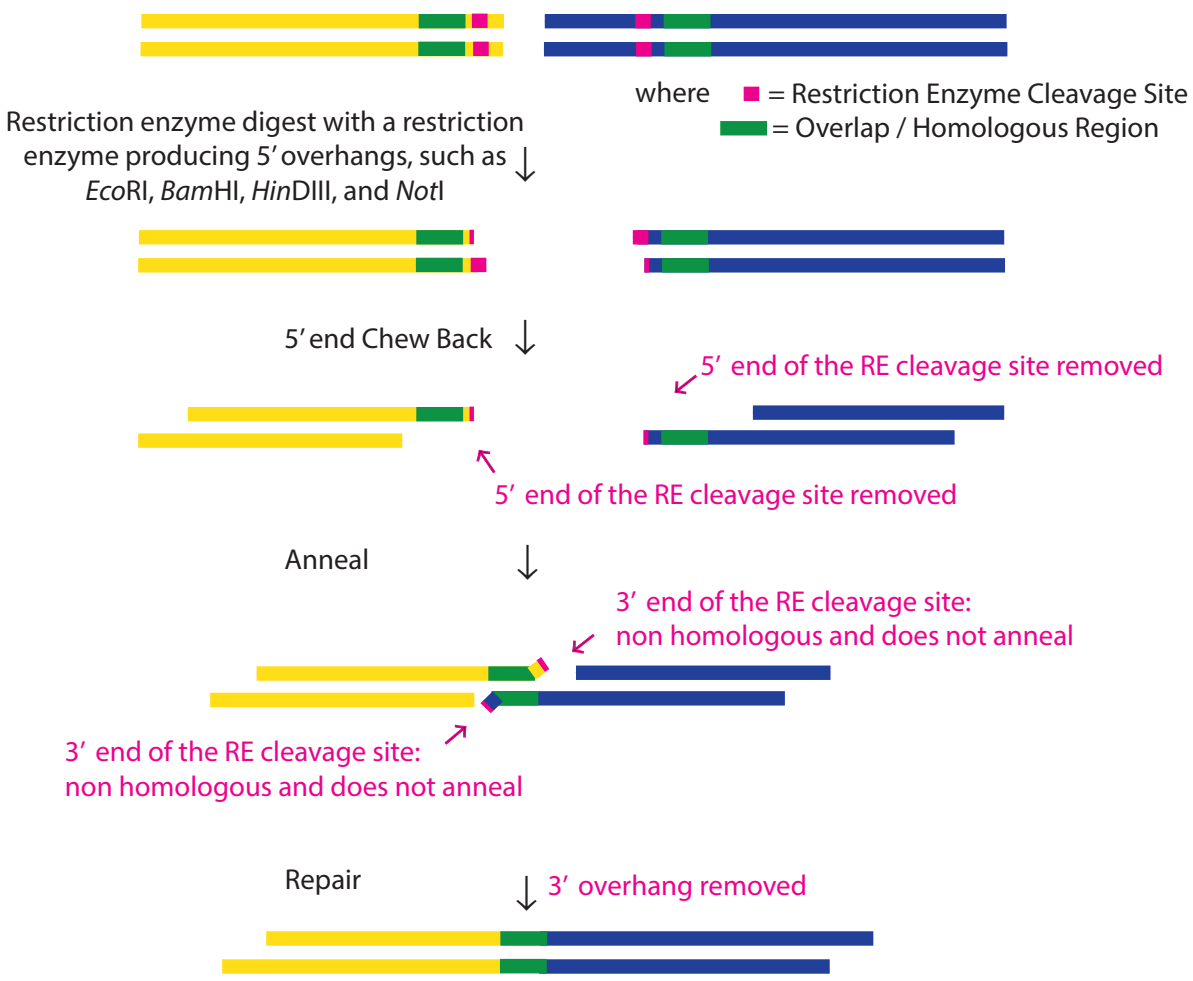
PCR Amplification of the Substrate DNA

We recommend using a high-fidelity polymerase, such as Phusion® DNA polymerase, and reducing the number of PCR cycles used during amplification, to minimize the introduction of errors.

Restriction Enzyme Generated Fragments

Fragments for assembly may also be prepared by restriction enzyme digest. Select restriction enzyme sites that are external to the overlap sequence since the partial restriction sites that remain in the fragment or vector following the digestion will be eliminated during the assembly process.

Restriction enzymes creating blunt ends, 3' overhangs, or 5' overhangs may all be used to prepare fragments for assembly. The following illustration depicts the elimination of both ends of the cleavage sequence created from digestion with restriction enzymes such as *Eco*RI, *Bam*HI, *Hin*DIII, and *Not*I that leave a 5′ overhang. As shown below, the 5' remnants of the cleavage site are removed by the chew-back reaction and the 3′ overhangs are removed during the repair.



Vector Considerations

- For small insert(s) (< 10 kb) or non-toxic gene(s), use a high copy number vector such as pUC19.
- For large insert(s) or toxic gene(s), use a low copy number or inductive vector such as a bacterial artificial cloning (BAC) cloning vector.
- As indicated, assembly vectors may be generated either with PCR or restriction enzyme digestion.
- For PCR-generated vectors, treat the PCR-amplification mixture with DpnI to reduce template carryover.

Purifying Vector and Insert DNA

- Purify DNA inserts by column or ethanol purification to remove restriction or PCR enzymes that could interfere with the Gibson Assembly® method.
- If your PCR reaction or synthetic assembly yields non-specific fragments, we recommend purifying the fragment of interest using gel extraction or another size fractionation method.
- For restriction enzyme digested vectors, gel extract the linearized vector to minimize vector background and reduce the number of observed background colonies.

Protocols

Guidelines for the Gibson Assembly® HiFi 1-Step Procedure

- Use approximately 10–100 ng of each DNA fragment (including the cloning vector) in equimolar amounts, according to the following guidelines:

  Note: For DNA fragments ≤1 kb, we recommend using a 5-fold molar excess.

Fragment size	Amount	pmols*
≤1 kb	20–40 ng	0.04
1–5 kb	10–25 ng	0.008–0.04
5–8 kb	25 ng	0.005–0.008
8–20 kb	25–100 ng	
20–32 kb	100 ng	0.005

  *Use the following formula for calculations:

$$\frac{\text{pmol}}{\mu\text{l}} \cong 1.55 \times \frac{\text{ng}/\mu\text{l}}{\text{bp}}$$

- The total volume for the combined DNA fragments in the assembly reaction is ≤ 5 µl.
- For the positive control, use 5 µl positive control DNA in the assembly procedure.
- Keep GA 1-Step Master Mix (2X) on ice at all times.
- To assemble multiple fragments, create a master mix of fragments in the proper ratios to minimize pipetting error.
- For best results, follow the procedure precisely as described on the following pages.

Gibson Assembly® HiFi 1-Step Procedure

1. Thaw GA 1-Step Master Mix (2X) on ice.
2. In PCR tubes, prepare DNA fragments in nuclease-free water according to the guidelines outlined above.

   Example:

Insert fragment(s)	10–100 ng
Linear vector	25 ng
Nuclease-free water	to 5 µl

3. **Vortex the master mix immediately before use** after it is thawed.
4. In a tube on ice, combine 5 µl of DNA fragments and 5 µl of GA 1-Step Master Mix (2X). Mix the reaction by pipetting.
5. (Optional) For the positive control, combine 5 µl of the Positive Control (2X) and 5 µl of GA 1-Step Master Mix (2X) in a tube on ice. Mix the reaction by pipetting.
6. Vortex and spin down all reactions.
7. Incubate the reactions at 50°C for 1 hour.
8. After the incubation is complete, store the reactions at –20°C or dilute reactions for downstream applications such as PCR or electrocompetent *E. coli* transformation (see the protocols on the following pages).
9. (Optional) Analyze the assembly reaction with electrophoresis of 5–10 µl of the reaction on an 0.8–2% agarose gel. A high molecular weight ladder is indicative of a successful assembly reaction (see the example in “Expected Results” on page 21).

Guidelines and Recommendations for Transformation

- We recommend using Epicentre TransforMax™ EPI300™ electrocompetent cells with the Bio Rad Gene Pulser Xcell electroporator.

  Note: For transformation with electrocompetent cells other than EPI300™ cells, we recommend:

  - Selecting electrocompetent cells with a transformation efficiency ≥ 1×10^9 CFU/µg pUC19.
  - Using 2% (v/v) of the assembly reaction per transformation (e.g. use 1 µl of the assembly reaction per 50 µl of high efficiency competent cells). Keep in mind that increasing the volume of assembly reaction used for transformation does not necessarily lead to increased colony output.
  - Following the protocol and electroporation parameters supplied with your electrocompetent cells.

- For transformation using chemically competent cells, follow the procedure “Transformation using Chemically Competent Cells” on page 19.
- Observe the value of the TC (Time Constant) for each transformation, which conveys efficiency (4.5–5 is ideal, ≤4 is not acceptable).
- Before starting, prepare ice buckets, tubes, and pipettors so that the transformation steps can be completed quickly and efficiently.

Transformation Procedure for Electrocompetent EPI300™ Cells (Recommended Procedure)

1. Prepare 15 ml snap cap tubes with 1 ml SOC per tube for each transformation reaction.
2. Chill electroporation cuvettes on ice.
3. Dilute the assembly reaction (from step 8 of the"Gibson Assembly® HiFi 1-Step Procedure" on page 16). We typically prepare a 1:5 dilution by adding 40 µl of nuclease-free water to 10 µl of Gibson Assembly® Reaction. Keep diluted reactions on ice.
4. Pipet 2.5 µl of the diluted assembly reaction into a clean 1.5 ml microfuge tube.
5. Thaw TransforMax™ EPI300™ Electrocompetent *E. coli* (Epicentre® EC300110) on ice. Mix gently.
6. Add 30 µl of thawed, electrocompetent cells to each cold tube containing the diluted reaction (from step 3, above). Mix gently with the end of a pipette tip and return the tube to ice.
7. Incubate the cells and DNA on ice for one minute.
8. After the incubation, pipet the cell/DNA mixture into a chilled cuvette. Tap the cuvette gently onto the benchtop 2–3 times, insert the cuvette into the electroporator, close the lid, and press the pulse button.

   Note: The pulse settings for electrocompetent TransforMax™ EPI300™ cells are 1200 V, 25 µF, 200 Ω, 0.1 cm cuvette.
9. During the pulse (≈2 seconds), remove about 800 µl SOC from the snap cap tube (step 1). Add the SOC to the cuvette immediately after the end of the pulse.
10. Thoroughly pipet the mixture up and down. Add the mixture back to the snap cap tube containing about 200 µl SOC, and repeat steps 5–8 for the remaining tubes. Work as quickly as possible until the cells are transferred into the snap cap tube.
11. Incubate the tubes with shaking at about 200 RPM for 1 hour at 37°C to allow the cells to recover.
12. Pre-warm LB plates in an incubator upside down for 10–15 minutes.
13. After the 1 hour incubation, plate 1/10–1/100 of the transformation reaction (10–100 µl out of 1 ml) onto LB agar plates with appropriate antibiotics.

    Note: See "Recommended Plating Volume" on page 20 for more information.
14. (Optional) For the positive control, plate 1/100 volume of the transformed reaction onto LB plates containing 100 µg/ml ampicillin or carbenicillin with 40 µg/ml X-Gal and 0.1 mM IPTG.
15. Incubate plates at 37°C upside down, overnight.
16. Pick colonies for screening. We typically pick 6–10 colonies and screen the clones by colony PCR or by plasmid DNA purification followed by restriction enzyme digestion.

Transformation using Chemically Competent Cells

Note: Use the following procedure as a starting point. For optimal efficiency, you may need to adjust the amount of the assembly reaction used for transformation, as well as transformation conditions and parameters according to the competent cell manufacturer's recommendations.

1. While keeping tubes on ice, pipet 1 µl of the assembly reaction (from step 8 of the "Gibson Assembly® HiFi 1-Step Procedure" on page 16) into a clean 1.5 ml microfuge tube.
2. Thaw chemically competent cells on ice. Mix gently.
3. Add 50 µl of thawed, chemically competent cells to each cold tube containing the assembly reaction (from step 1, above). Mix gently with the end of a pipette tip.
4. Incubate the cells and DNA on ice for 30 minutes without mixing.
5. Heat shock the cell/DNA mixture according to the instructions provided with your competent cells.

   Note: For most competent cells, the heat shock parameters are 42°C for 30 seconds.
6. Return tubes to ice for 2 minutes
7. Add 950 µl of room temperature SOC media to the tubes.
8. Incubate the tubes with shaking at about 200 RPM for 1 hour at 37°C to allow the cells to recover.
9. Pre-warm LB plates in an incubator upside down for 10–15 minutes.
10. After the 1 hour incubation, plate 1/10–1/100 of the transformation reaction (10–100 µl out of 1 ml) onto LB agar plates with appropriate antibiotics.

    Note: See "Recommended Plating Volume" below for more information.
11. (Optional) For the positive control, plate 1/100 volume of the transformed reaction onto LB plates containing 100 µg/ml ampicillin or carbenicillin with 40 µg/ml X-Gal and 0.1 mM IPTG.
12. Incubate plates at 37°C upside down, overnight.
13. Pick colonies for screening. We typically pick 6–10 colonies and screen the clones by colony PCR or by plasmid DNA purification followed by restriction enzyme digestion.

Recommended Plating Volume

Always plate two plates (one low and one high volume)

Plating Volume for EPI300™ *E. coli* and Competent Cells with Transformation Efficiencies > 1×10^9 CFU/µg pUC19			
Number of fragments	Plating volume*	For example, we normally plate... †	Expected number of colonies ‡
1–2	1/50	2 µl and 20 µl	> 100
3–5	1/10	10 µl and 100 µl	

* The plating volume is the fraction of transformation reaction plated per the total transformation mixture.
† Volumes are based on a 1000 µl transformation mixture.
‡ The Expected number of colonies is for EPI300™ E. coli only.

Plating Volume for Competent Cells with Transformation Efficiencies ≥ 1×10^8 CFU/µg pUC19		
Number of fragments	Plating volume*	For example, we normally plate... †
1–2	1/10	10 µl and 100 µl
3–5	1/2	100 µl and 500 µl Note: Spin down the reaction before plating

* The plating volume is the fraction of transformation reaction plated per the total transformation mixture.
† Volumes are based on a 1000 µl transformation mixture.

Transformation Results and Analysis

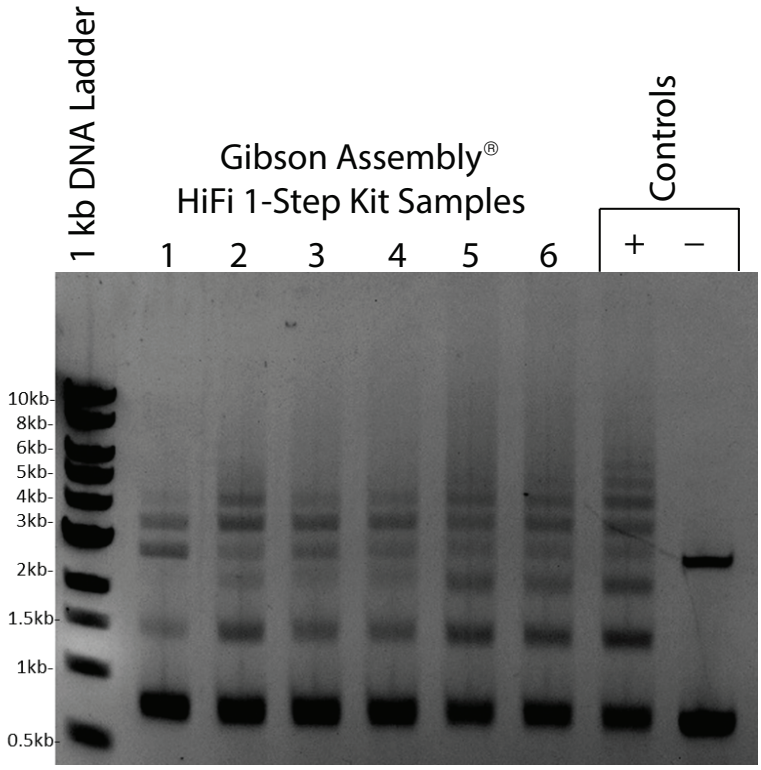
For the positive control, white colonies indicate successful assembly with insert; blue colonies indicate the absence of insert:vector assembly. Calculate cloning efficiency using the following formula:

Cloning efficiency (CE, %) = Number of white colonies/ Total colonies x 100

We typically observe positive control cloning efficiencies > 90%. Colony output is dependent on several factors, including transformation efficiency. Note that low colony output is not necessarily indicative of low cloning efficiency.

Expected Results

Completed Assembly Reaction: Gel Electrophoresis



Four 0.8 kb PCR fragments were assembled into a 2.7 kb vector in 6 replicates. Each reaction contained 25 ng of each insert and 20 ng of vector and assembly was performed following the procedure described in this manual. 10 µl from the 20 µl total reaction was loaded onto an 0.8% agarose gel, using a 1 kb molecular marker from NEB. The (–) control contained the DNA fragment without enzyme mix. The 0.8 kb insert fragments and the 2.7 kb vector are clearly visible in the negative control lane. Successful assembly of the 6 replicates appears as a ladder, as shown on the gel image (Lanes 1–6).

References

1. Gibson, D.G., *et al.* (2009) Nat. Methods 6, 343-345.
2. Gibson, D.G., *et al.* (2010) Science 329, 52-56.