

# Gibson Assembly<sup>®</sup> Ultra Kit

## Instructions

**Catalog Numbers** GA1200-S, GA1200-10, GA1200-50,  
GA1200-10MM, GA1200-50MM,  
GA1200-B05, GA1200-B10, GA1200-B20,  
GA1200-B30, GA1200-B40, GA1200-B50

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# Kit Information

## Gibson Assembly® Ultra Kit Contents

Quantity	Component	Cat. GA1200-S	Cat. GA1200-10	Cat. GA1200-50	Storage Temperature
		(5 Reactions)	(10 Reactions)	(50 Reactions)	
Volume					
1 tube (each)	GA Ultra Master Mix A (2X)	25 µL	50 µL	250 µL	-20°C (Store Master Mixes in aliquots)
	GA Ultra Master Mix B (2X)	50 µL	100 µL	500 µL	
	GA Positive Control (2X)	10 µL (2 Control Rxns)	10 µL (2 Control Rxns)	25 µL (5 Control Rxns)	
1 each	GA Ultra Quick Reference Manual				

## GA Ultra Master Mixes (2X)

The GA Ultra Master Mixes (2X) are also available as a standalone product (without a positive control). The following table lists available sizes of the master mixes.

**IMPORTANT:** Aliquot GA Ultra Master Mixes (2X) to reduce the number of freeze-thaw cycles. Properly aliquoted GA Ultra Master Mixes are stable at -20°C for up to 6 months.

Catalog Number	GA Ultra MM A (2X)	GA Ultra MM B (2X)	Number of Reactions	Storage Temperature	Aliquoting Instructions
	Volume				
GA1200-10MM	50 µL	100 µL	10	Aliquot and store at -20°C	<ol style="list-style-type: none"> <li>Upon receipt, place the master mix on ice to thaw.</li> <li>After the master mix has thawed completely, briefly vortex and centrifuge.</li> <li>Aliquot the master mix for future use (maximum volume of 250 µL).</li> </ol>
GA1200-50MM	250 µL	500 µL	50		
GA1200-B05	5 mL	10 mL	1000		
GA1200-B10	10 mL	20 mL	2000		
GA1200-B20	20 mL	40 mL	4000		
GA1200-B30	30 mL	60 mL	6000		
GA1200-B40	40 mL	80 mL	8000		
GA1200-B50	50 mL	100 mL	10,000		

The GA Ultra Assembly reaction is mediated in the following steps by the following kit components:

Step	Ultra Kit Component	Reaction
1	GA Ultra Master Mix A (2X)	<ul style="list-style-type: none"> <li>GA Ultra Master Mix A creates single-strand DNA 5' overhangs by chewing back DNA from the 3' end.</li> </ul>
Intermediate	—	<ul style="list-style-type: none"> <li>The reaction is slowly cooled to allow for annealing of the complementary overlap regions.</li> </ul>
2	GA Ultra Master Mix B (2X)	<ul style="list-style-type: none"> <li>GA Ultra Master Mix B incorporates nucleotides to fill in the gaps in the annealed DNA fragments.</li> <li>GA Ultra Master Mix B covalently joins the annealed complementary DNA fragments, seals any nicks and creates a contiguous DNA fragment.</li> </ul>

## Positive Control

The positive control DNA supplied with this kit is sufficient for 2 reactions (Cat. GA1200-S and GA1200-10) or 5 reactions (Cat. GA1200-50). 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
- Competent cells with a transformation efficiency  $\geq 1 \times 10^9$  CFU/µg pUC19

**Note:** We recommend *E. coli* 10G chemically competent cells (Lucigen Cat. No. 60107) or TransforMax™ EPI300™ Electrocompetent *E. coli* (Lucigen Cat. No. EC300110)

- Minifuge
- Vortex mixer

## Additional Optional Materials

- If you are using electrocompetent cells for transformation, you will also need a Gene Pulser® Xcell™ Microbial System and Gene Pulser®/MicroPulser™ Cuvettes: 0.1-cm Gap Width

The following items are only needed if you are generating DNA fragments with PCR.

- High-fidelity DNA Polymerase for producing fragments for Gibson Assembly®  
**Note:** We recommend Phusion® High Fidelity DNA Polymerase (Thermo Fisher Cat. No. F-530S)
- QIAquick PCR purification Kit (Qiagen Cat. 28106)
- Spectrophotometer

# Overview

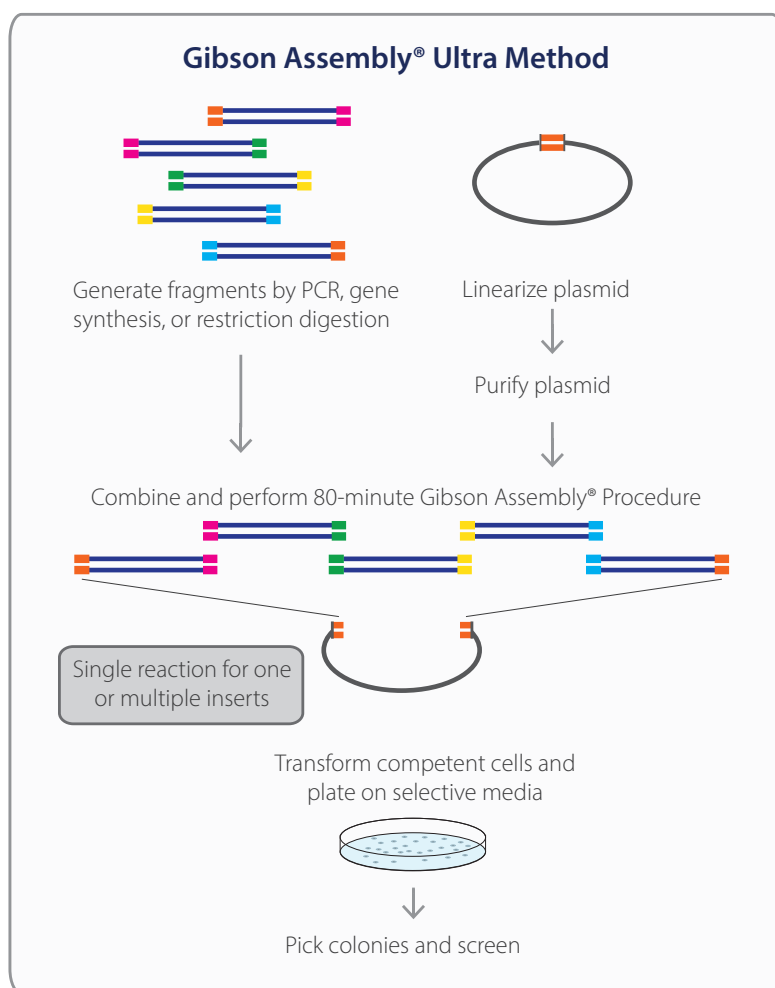
## Introduction

The Gibson Assembly® method is a well-established assembly reaction that can be used to join multiple DNA fragments with homologous overlapping ends. Gibson Assembly® offers an efficient and robust cloning strategy without the limitation of restriction enzyme sites. The Gibson Assembly® Ultra Kit is an ideal choice for complex cloning applications and contains an optimal enzyme mixture the assembly of 2 to 15 DNA fragments of widely varying sizes using only small amounts (nanograms) of DNA.

## Key Features of the Gibson Assembly® Ultra Kit

- Accurate
- Ideal for complex assembly
- Seamless
- Optimal for 1–15 inserts
- Suitable for fragments ranging from 100 bp – 100 kb

## Gibson Assembly® Ultra Synopsis

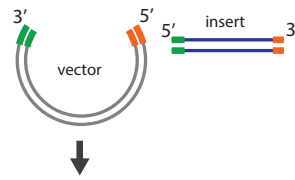


**Figure 1. Synopsis of Gibson Assembly® Ultra Cloning.** The Gibson Assembly® Ultra method allows for the simultaneous assembly of 1 to 15 inserts in a single reaction that takes approximately 80 minutes. This method requires a linearized vector and 20–80 bp sequence overlaps at the ends of the DNA fragments. Overlap sequences are intrinsic to the construct(s) and plasmid, eliminating the need for specific restriction sites. Following the 2-step assembly reaction, constructs are ready for transformation and colony screening.

## Gibson Assembly® Ultra Workflow

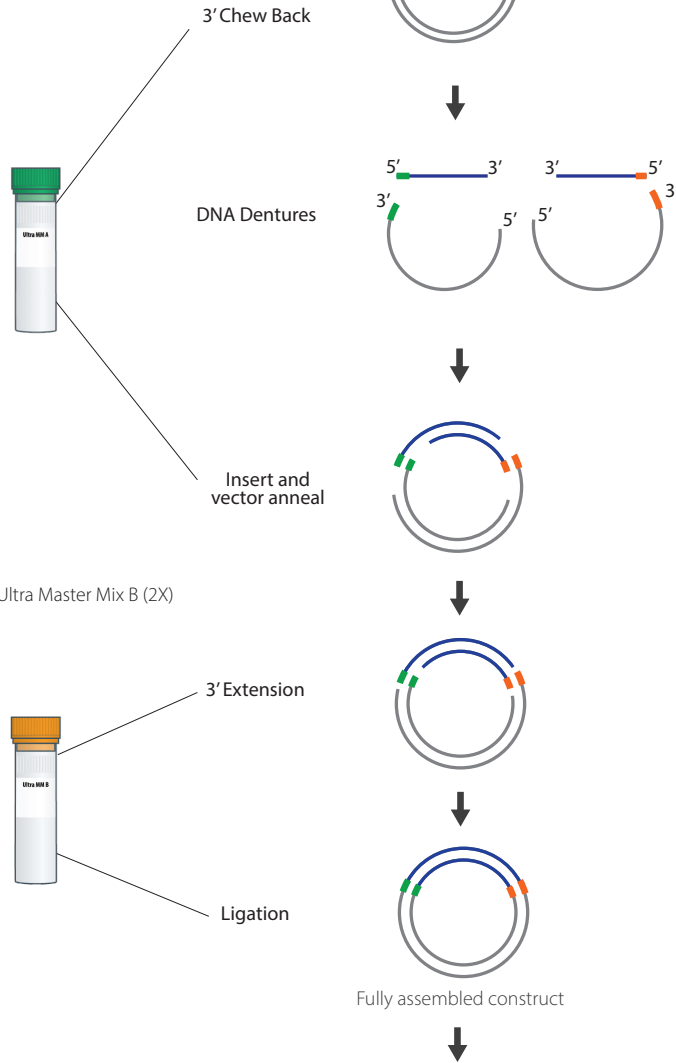
### Before Starting

Generate linear DNA fragments with overlapping ends by PCR, gene synthesis, or restriction digestion

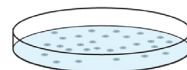


### Gibson Assembly Ultra Overview

Add vector & insert with overlapping ends to GA Ultra Master Mix A (2X)



### Transformation and Downstream Applications



**Figure 2. Overview of the Gibson Assembly® Ultra cloning workflow.** DNA fragments containing homologous overlapping ends are assembled in 80 minutes with the Ultra Kit. The GA Ultra Master Mixes mediate strand chew-back, extension, and ligation to yield a fully assembled construct that is ready for transformation.

## Before Starting

The following sections discuss and illustrate cloning a single insert with vector. The same principles presented in these sections may be applied to the assembly of up to 15 fragments with Gibson Assembly® Ultra method.

### Preparing DNA for the Gibson Assembly® reaction

Preparing DNA for Gibson Assembly® cloning requires adding homologous overlap regions to the ends of fragments and linearizing circular DNA. There are four primary methods for preparing DNA fragments for Gibson Assembly® cloning:

- Using PCR to add homologous overlaps to DNA fragments.
- Linearizing the vector using restriction enzyme digestion (typically used in conjunction with adding homologous overlaps to the insert using PCR). Multiple DNA fragments may be prepared using restriction enzyme digestion when DNA fragments containing the requisite overlap regions are excised from a plasmid before assembly with the Gibson Assembly® method. See “Appendix B: Restriction enzyme scars can be removed with the Gibson Assembly® reaction” on page 21 for more information about removing restriction enzyme digestion scars using the Gibson Assembly® Ultra method.
- Generating DNA fragments on the benchtop BioXp™ 3200 System. (see [sgidna.com/bioxp](http://sgidna.com/bioxp) for more information).
- Ordering synthetic DNA from a custom gene synthesis provider.

In general, you may assemble any combination of synthesized or BioXp-generated or PCR-generated or restriction enzyme-generated fragments. The only exception is that fragments generated by restriction enzyme digestion may not be placed adjacent to synthesized fragments.

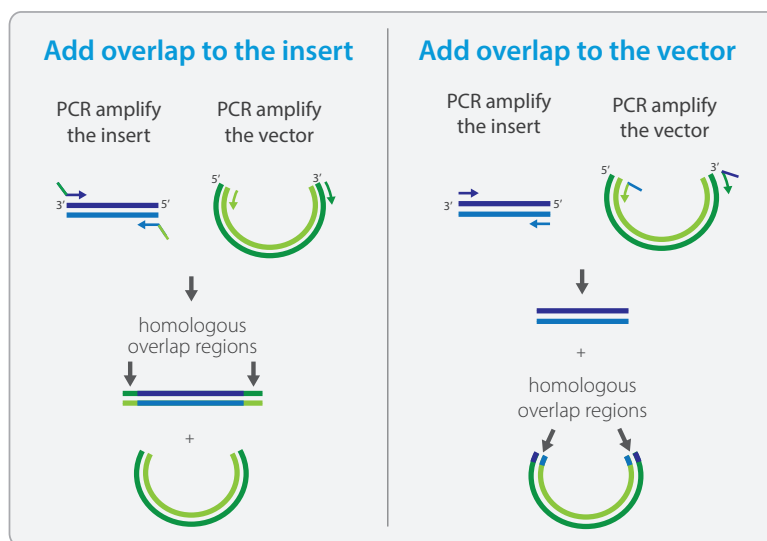


## Using PCR to add homologous overlaps to DNA fragments

Designing optimal homologous overlap regions for Gibson Assembly® cloning is critical to a successful assembly reaction. The free online tool at [sgidna.com/gibson-assembly-primers](http://sgidna.com/gibson-assembly-primers) is available to assist with primer design. For detailed instructions on how to use the tool, refer to the [Gibson Assembly® Guide](#). Additional information about primer design is available in “Appendix A: Adding homologous overlaps to DNA fragments with PCR” on page 18.

Homologous overlap regions can be added to the insert or the vector, as shown in Figure 3. The overlap region can also be split between the insert and the vector. In this case the overlap region is partially insert-derived and partially vector derived.

See “Guidelines: Homologous Overlap Regions” on page 12 for more information about homologous overlap regions, including the recommended length.



**Figure 3. Using PCR to add homologous overlaps to the insert or the vector prior to Gibson Assembly® Cloning.** Homologous overlaps may be added to the insert (shown on the left panel) or to the vector (shown on the right panel) or split between the insert and vector (not shown).

## Protocol for PCR Amplification

After designing the requisite primers, use the following basic amplification conditions to generate DNA fragments with homologous overlapping ends. 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 potential for the introduction of amplification errors. After PCR amplification, analyze the fragments on a gel to verify the presence of fragments of the expected size. If multiple bands are present, consider gel extraction before proceeding to the Gibson Assembly® method. For best results, we suggest performing a clean-up of the PCR reaction to remove dNTPs, enzymes, and buffer components in all cases.

### Polymerase Chain Reaction Setup

Component	Volume
Insert or Vector DNA (100 pg/μL – 1 ng/μL in TE)	5 μL
10 μM Forward Primer	2.5 μL
10 μM Reverse Primer	2.5 μL
10 mM dNTPs	1 μL
5X Phusion HF Buffer	10 μL
Phusion® DNA Polymerase (2 U/ μL)	0.5 μL
Nuclease-free Water	28.5 μL
<b>Total</b>	<b>50 μL</b>

### PCR Conditions

Initial Denaturation	98°C	30 seconds	1 Cycle
Amplification	98°C	10 seconds	25–30 Cycles
	Primer T <sub>m</sub> (60°C to 70°C)	20 seconds	
	72°C	30 seconds/kb	
Final Extension	72°C	5 minutes	1 Cycle
Hold	4°C	as necessary	1 Cycle

## Using restriction enzyme digestion to prepare DNA for the Gibson Assembly® reaction

For restriction enzyme digested vectors, gel extract the linearized vector to minimize vector background and reduce the number of observed background colonies.

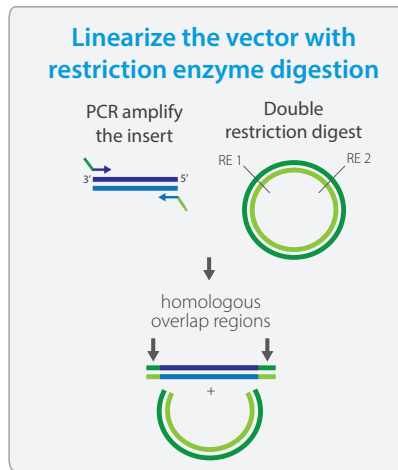


Figure 4. Using restriction enzyme digestion to linearize a vector prior to Gibson Assembly® Cloning.

### 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.
- Vectors may be linearized by PCR or restriction enzyme digestion.
- For PCR-generated vectors, treat the PCR-amplification mixture with DpnI to reduce template carryover.

## Guidelines: Homologous Overlap Regions

- The optimal length of the overlap region depends on the number and length of the fragments in the assembly reaction.
- 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.
- **Hint:** Add a restriction enzyme site to the primers between the overlap region and the sequence-specific segment to enable subsequent release of the insert from the vector. (Be certain that the restriction enzyme site introduced in the primers is not also present within the insert.) See “Adding a restriction enzyme site to vector primers for downstream use” on page 20 for additional details.

### Suggested Length of the Overlap Region: Inserts

Number of Inserts	Insert Size							
	0.1–0.5 kb	0.5–2 kb	2–5 kb	5–8 kb	8–10 kb	10–20 kb	20–32 kb	32–100 kb
1	20 bp	30 bp	30 bp	40 bp	40 bp	80 bp	80 bp	80 bp
2	30 bp	30 bp	40 bp	40 bp	40 bp	80 bp	80 bp	80 bp
3	40 bp	40 bp	40 bp	40 bp	40 bp	80 bp	80 bp	—
4	40 bp	40 bp	40 bp	40 bp	40 bp	80 bp	—	—
5	40 bp	40 bp	40 bp	40 bp	40 bp	—	—	—
6	40 bp	40 bp	40 bp	40 bp	40 bp	—	—	—
7	40 bp	40 bp	40 bp	40 bp	—	—	—	—
8	40 bp	40 bp	40 bp	40 bp	—	—	—	—
9	40 bp	40 bp	40 bp	—	—	—	—	—
10	40 bp	40 bp	40 bp	—	—	—	—	—
11	40 bp	40 bp	40 bp	—	—	—	—	—
12	40 bp	40 bp	40 bp	—	—	—	—	—
13	40 bp	40 bp	—	—	—	—	—	—
14	40 bp	40 bp	—	—	—	—	—	—
15	40 bp	40 bp	—	—	—	—	—	—

#### Key

Gibson Assembly® HiFi 1 Step Kit Recommended

Gibson Assembly® Ultra Kit Recommended

— = Not recommended

### Suggested Length of the Overlap Region: Vectors

Vector Size	Overlap Length	Example
2–5 kb	30 bp	pUC19, pBR322
5–8 kb	40 bp	BAC vector
8–15 kb	40 bp	Lentiviral vector

# Protocols

## Guidelines for the Gibson Assembly® Ultra Procedure

- The total volume for the combined DNA fragments in the assembly reaction is  $\leq 5 \mu\text{L}$ .
- Only use DNA samples with  $A_{260/280} > 1.8$ .
- For the positive control, use  $5 \mu\text{L}$  of the positive control DNA in the assembly reaction.
- Keep GA Ultra Master Mixes A and B (2X) on ice at all times.
- **IMPORTANT:** Vigorously vortex GA Ultra Master Mixes A and B (2X) for 15 seconds immediately before use.
- 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.

## Calculating the amount of DNA to use in a Gibson Assembly® reaction

Use DNA at a concentration  $\geq 40 \text{ ng}/\mu\text{L}$ . If the amount of DNA is limited, the assembly reaction may be performed using DNA at concentrations between  $20\text{--}40 \text{ ng}/\mu\text{L}$  with reduced efficiency. Do not use DNA at concentrations  $< 20 \text{ ng}/\mu\text{L}$ .

For a typical Gibson Assembly® Ultra reaction, combine  $25\text{--}50 \text{ ng}$  of vector with approximately  $10\text{--}300 \text{ ng}$  of insert. For best results, we recommend balancing the molar ratio of the DNA fragments. For fragments  $>1 \text{ kb}$ , use an equimolar ratio. For DNA fragments  $\leq 1 \text{ kb}$ , we recommend using a 5-fold molar excess of insert. To precisely determine the pmol or ng of DNA for a given size fragment, use the following formulas:

$$\text{pmol DNA} = [\text{ng DNA}/(660 \times \# \text{ of bases})] \times 1000$$

$$\text{ng of DNA} = [\text{pmol DNA} \times (660 \times \# \text{ of bases})]/1000$$

Refer to the following table for approximate pmol of DNA for a given fragment size and amount.

Fragment size	ng of DNA	pmol of DNA
0.5 kb	20 ng	0.061
	40 ng	0.121
1 kb	10 ng	0.015
	25 ng	0.038
5 kb	10 ng	0.003
	25 ng	0.008
8 kb	25 ng	0.005
	50 ng	0.009
10 kb	25 ng	0.004
	50 ng	0.008
15 kb	50 ng	0.005
	100 ng	0.010
20 kb	50 ng	0.004
	100 ng	0.008
30 kb	50 ng	0.003
	100 ng	0.005

## Gibson Assembly® Ultra Method

1. Thaw the GA Ultra Master Mix A (2X) on ice.
2. Dilute your DNA fragments with nuclease-free water in PCR tubes to a total volume of 5  $\mu$ L according to the Guidelines on page 13.

### Example:

Insert fragment(s)	10–300 ng
Linear vector	25 ng
Nuclease-free water	to 5 $\mu$ L

3. **Vortex the thawed master mix immediately before use.**
4. In a 0.2 mL PCR tube on ice, combine 5  $\mu$ L of DNA fragments and 5  $\mu$ L of GA Ultra Master Mix A (2X). Mix the reaction by pipetting up and down.
5. (Optional) Set up a positive control reaction by aliquoting 5  $\mu$ L of GA Positive Control (2X) into a 0.2-mL PCR tube on ice. Add 5  $\mu$ L of GA Ultra Master Mix A (2X) and mix the reaction by pipetting up and down.
6. Vortex and spin down all reactions.
7. Transfer assembly reaction tubes to a thermocycler and program the following conditions:

3' end Chew Back	1 cycle	Overlap size:	
		< 80 bp	$\geq$ 80 bp
		37°C for 5 min	37°C for 15 min



Inactivation	1 cycle	75°C for 20 min (for all overlap sizes)
Slowly Cool	1 cycle	0.1°C/sec to 60°C
Anneal	1 cycle	60°C for 30 min
Slowly Cool	1 cycle	0.1°C/sec to 4°C

8. Thaw the GA Ultra Master Mix B (2X) on ice. **Vortex and briefly centrifuge the thawed master mix immediately before use.**
9. While keeping the tubes on ice, add 10  $\mu$ L of GA Ultra Master Mix B (2X) to the reaction from step 7. Mix the reaction by pipetting up and down.
10. Incubate the reaction using the following conditions:

Repair	1 cycle	45°C for 15 min
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11. After the incubation is complete, store reactions at  $-20^{\circ}\text{C}$  or proceed to transformation.
12. (Optional) Analyze the assembly reaction with electrophoresis of 5–10  $\mu$ L of the reaction on an 0.8–2% agarose gel. A high molecular weight smear is indicative of a successful assembly reaction (see the example in “Appendix C: Expected Results” on page 22).

## Transformation with *E. coli* 10G Chemically Competent Cells (Recommended)

### Before starting

We recommend using the following protocol with *E. coli* 10G chemically competent cells (Lucigen Cat. No. 60107). If you are using competent cells other than Lucigen 10G cells, follow the transformation protocol provided with your competent cells. Use competent cells with a transformation efficiency  $\geq 1 \times 10^9$  CFU/ $\mu$ g pUC19.

Because some of the ingredients in the buffer mix can negatively impact the survival of some competent cells, we recommend diluting the assembly reaction before performing the transformation. Dilute Ultra assemblies up to 2-fold. You may need to empirically determine the optimal level of dilution, depending on the type of cells used.

### Procedure

1. Pre-chill 15 mL disposable polypropylene culture tubes (17 x 100 mm, one tube for each transformation reaction).
2. Thaw chemically competent cells on ice for 5–15 minutes.
3. Add 40  $\mu$ L of thawed, chemically competent cells to each cold tube.
4. Add 2  $\mu$ L of the diluted assembly reaction to each cold tube of competent cells. Mix by briefly stirring (do not pipet up and down).
5. Incubate the cells and DNA on ice for 30 minutes without mixing.
6. Heat shock the cell/DNA mixture in a 42°C water bath for 45 seconds.
7. Return tubes to ice for 2 minutes.
8. Add 950  $\mu$ L room temperature recovery media to the cells in the culture tube.
9. Incubate the tubes with shaking at about 250 rpm for 90 minutes at 37°C to allow cells to recover.
10. While cells are shaking, pre-warm two LB plates with appropriate antibiotics in an incubator for at least 30 minutes.
11. At the end of the 90 minute recovery, plate 150  $\mu$ L the transformants on the LB plate with antibiotics. See “Recommended Plating Volume” on page 17 for additional guidance. Use glass beads to swirl and plate evenly. Label plates with the following information:
  - Plate Name
  - Plating volume (150  $\mu$ L)
  - Plating Date
  - Initials

**Note:** If you are using competent cells other than 10G cells, plate all of the cells. You may need to centrifuge tubes for 1 minute and discard more than half of the clear supernatant in order to plate all of the cells.
12. Place the plates in a 37°C incubator upside down, overnight.
13. On the following day, pick well isolated colonies for plasmid preparation. Screen for the presence of insert using colony PCR, if desired.

# Transformation with TransforMax™ EPI300™ Electrocompetent *E. coli*

## Before starting

The following protocol has been optimized for transforming DNA assembled with the Gibson Assembly® Ultra Kit into TransforMax™ EPI300™ Electrocompetent *E. coli*. (Lucigen Cat. No. EC300110). If you are using competent cells other than TransforMax™ EPI300™ Electrocompetent *E. coli*, follow the transformation protocol provided with your competent cells. Use competent cells with a transformation efficiency  $\geq 1 \times 10^9$  CFU/ $\mu$ g pUC19.

Because some of the ingredients in the buffer mix can negatively impact the survival of some competent cells, we recommend diluting the assembly reaction before performing the transformation. Dilute Ultra assemblies up to 2-fold. You may need to empirically determine the optimal level of dilution, depending on the type of cells used.

## Procedure

1. Add 1 mL SOC media to 1.5-mL microcentrifuge tubes (one tube per reaction). Label the tubes and place on ice for 10 minutes.
2. Chill clean electroporation cuvettes on ice.
3. Pipet 30  $\mu$ L of EPI300™ cells directly between the slit of the cuvettes on ice (one cuvette per reaction).
4. Add 2  $\mu$ L of the diluted assembly reaction to the cells in the cuvette. Mix by pipetting up and down gently two times. Place the cuvette back on ice.
5. Incubate cuvette on ice for one minute.
6. Gently tap cuvette on a benchtop two times to make sure all contents are at the bottom of the cuvette in between the slit.
7. Insert the cuvette into a BioRad Electroporator or equivalent, and press PULSE.  
**Note:** Pulse Settings for EPI300™ cells are 1200 V, 25  $\mu$ F, 200  $\Omega$ , 0.1 cm cuvette.
8. While the pulse is taking place ( $\approx$ 2 seconds), remove about 800  $\mu$ L SOC from a pre-chilled 1.5 mL tube (step 1), and immediately add the SOC to the cuvette after the pulse.
9. Mix the cells and SOC by pipetting up and down. Add the cell and SOC mixture back into the tube containing the remaining 200  $\mu$ L SOC.  
**Note:** Work as quickly as possible until the cells are transferred into the 1.5 mL microcentrifuge tube.
10. Incubate the cells and SOC for 1 hour at 37°C with shaking at about 200 rpm.
11. While cells are shaking, pre-warm two LB plates with appropriate antibiotics in an incubator for at least 30 minutes.
12. At the end of the 1-hour incubation, plate 50–100  $\mu$ L of cells on LB plate with appropriate antibiotics. See “Recommended Plating Volume” on page 17 for additional guidance.  
**Note:** Use glass beads to swirl and plate evenly. Label plates with the following information:
  - Plate Name
  - Plating volume (50–100  $\mu$ L)
  - Plating Date
  - Initials
13. Place the plates in a 37°C incubator upside down, overnight.
14. On the following day, pick well isolated colonies for plasmid preparation. Screen for the presence of insert using colony PCR, if desired.



## Recommended Plating Volume

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

Recommended Plating Volume				
Competent cell transformation efficiency	Number of fragments	Plating volume*	For example, we normally plate... †	Expected number of colonies ‡
$>1 \times 10^9$ CFU/ $\mu$ g pUC19	1–2	<b>1/50</b>	2 $\mu$ L and 20 $\mu$ L	> 100
	3–5	<b>1/10</b>	10 $\mu$ L and 100 $\mu$ L	
	>5	<b>1/2</b>	100 $\mu$ L and 500 $\mu$ L‡	> 50
$>1 \times 10^8$ CFU/ $\mu$ g pUC19	1–2	<b>1/10</b>	10 $\mu$ L and 100 $\mu$ L	—
	3–15	<b>1/2</b>	100 $\mu$ L and 500 $\mu$ L‡	

\* The plating volume is the fraction of transformation reaction plated per the total transformation mixture.

† Volumes are based on a 1000  $\mu$ L transformation mixture.

‡ The expected number of colonies is for EPI300™ *E. coli* only.

‡ Spin down the reaction before plating.

## 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:

$$\text{Cloning efficiency (CE, \%)} = \text{Number of white colonies} / \text{Total colonies} \times 100$$

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.

# Appendix

## Appendix A: Adding homologous overlaps to DNA fragments with PCR

Designing the primers for PCR preparation of substrate DNA is critically important for the success of the assembly reaction.

This section covers the basics of Gibson Assembly® primer design and is intended to assist you in creating Gibson Assembly® constructs. We recommend taking advantage of the free online primer design tool at [sgidna.com/gibson-assembly-primers](http://sgidna.com/gibson-assembly-primers), which will design primers for you. For detailed instructions on how to use the tool, refer to the [Gibson Assembly® Guide](#).

### Primer design

If you elect to manually design your primers, use the following guidelines for assistance:

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 following sections.

### PCR Primer Characteristics

- 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. See “Suggested Length of the Overlap Region: Inserts” on page 12 for additional recommendations.
- 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  $T_m$  greater than 30°C is more than 5 bp from the 3' terminus of the primer.

## Strategies for adding the overlap sequence to PCR primers

One advantage of the Gibson Assembly® Ultra Kit is that it allows for flexibility in designing primers for substrate DNA amplification. Guidance for different design scenarios are presented in the following sections.

### Adding overlap to vector primers

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.

### Adding overlap 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).

### Splitting the overlap between the insert and vector primers

Splitting the overlap between primers provides the greatest flexibility in primer design. This strategy 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, consider splitting the overlap between the vector and insert primers.

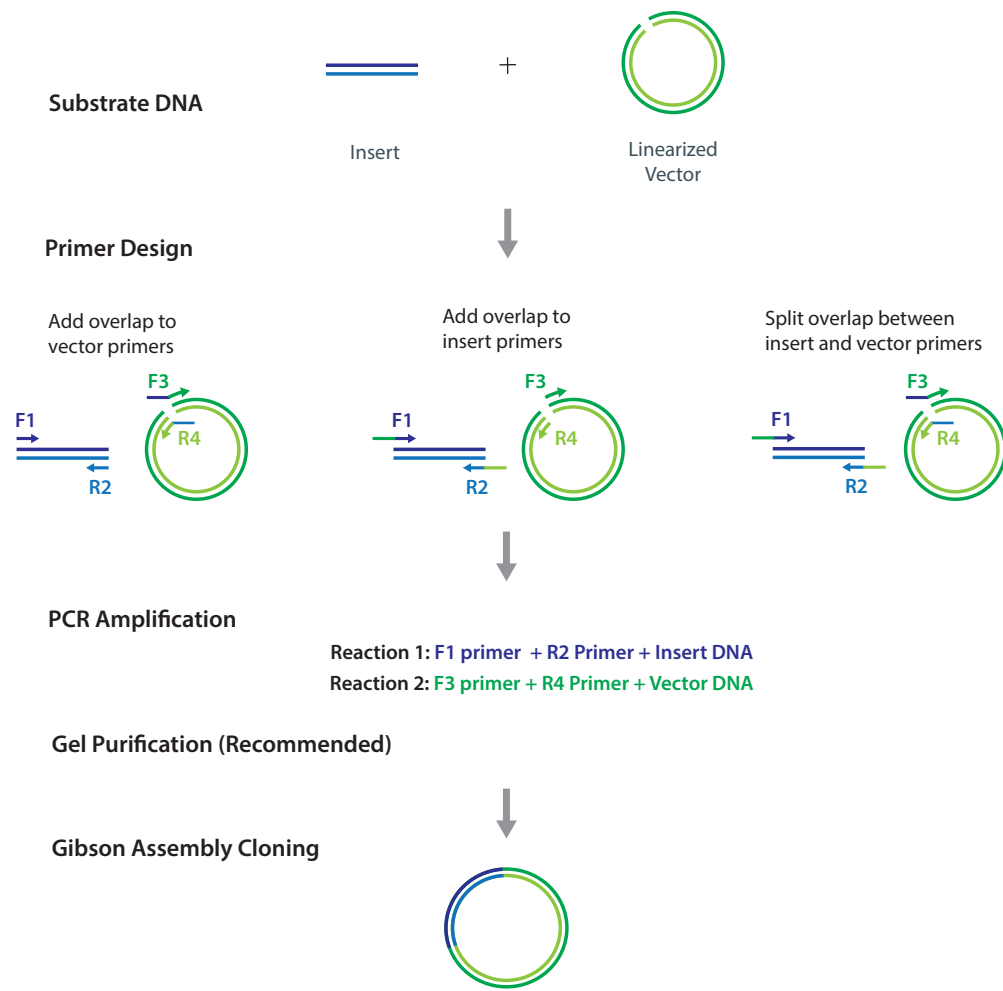


Figure 5. Scenarios for adding homologous overlap sequence to PCR primers before proceeding with the Gibson Assembly® cloning reaction. The final DNA construct is the same in all scenarios.

## Adding a restriction enzyme site to vector primers for downstream use

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 yellow, below). In this scenario, the vector is prepared by amplifying the vector with the Vector\_F3 primer and Vector\_R4 primer.

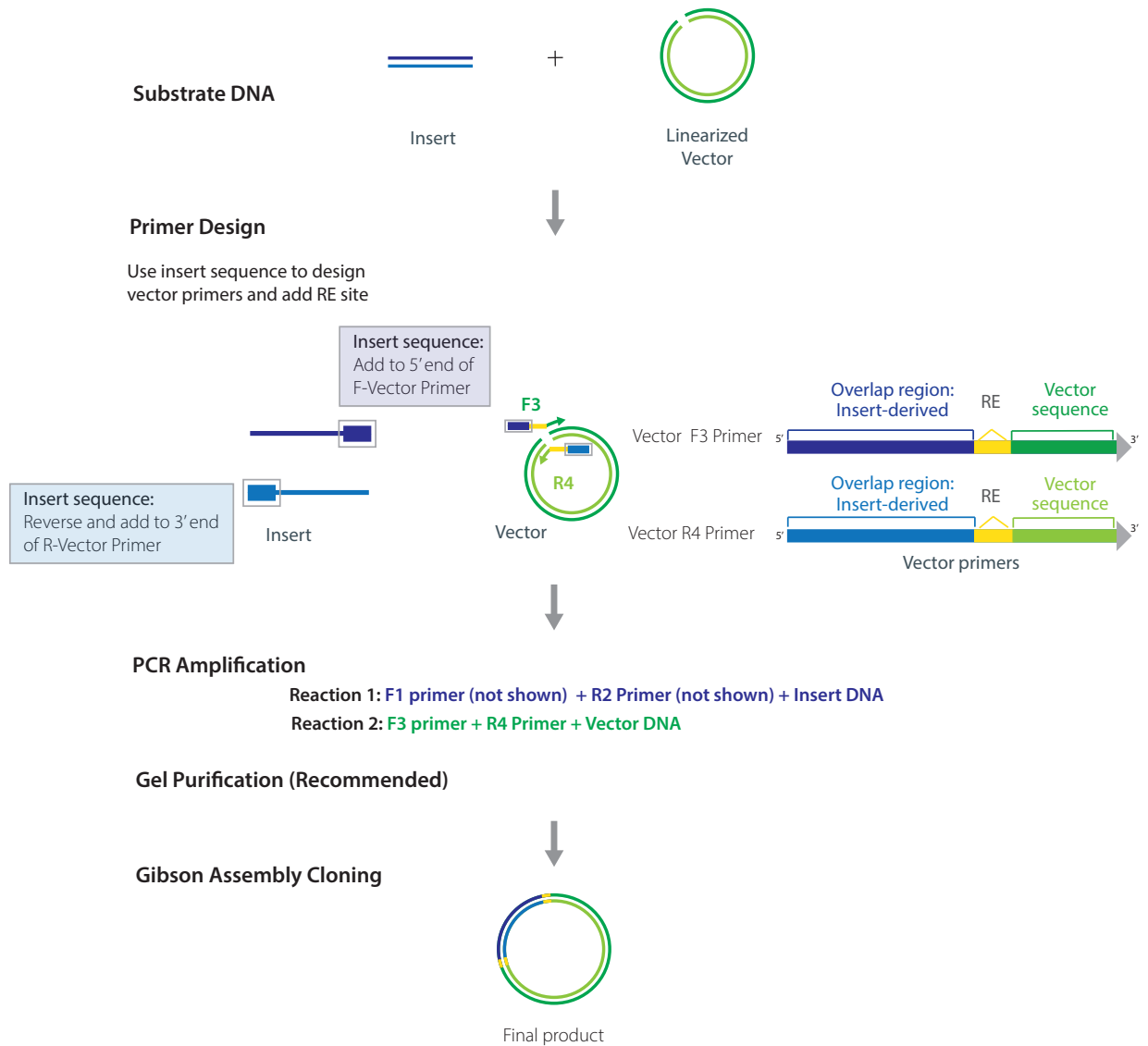
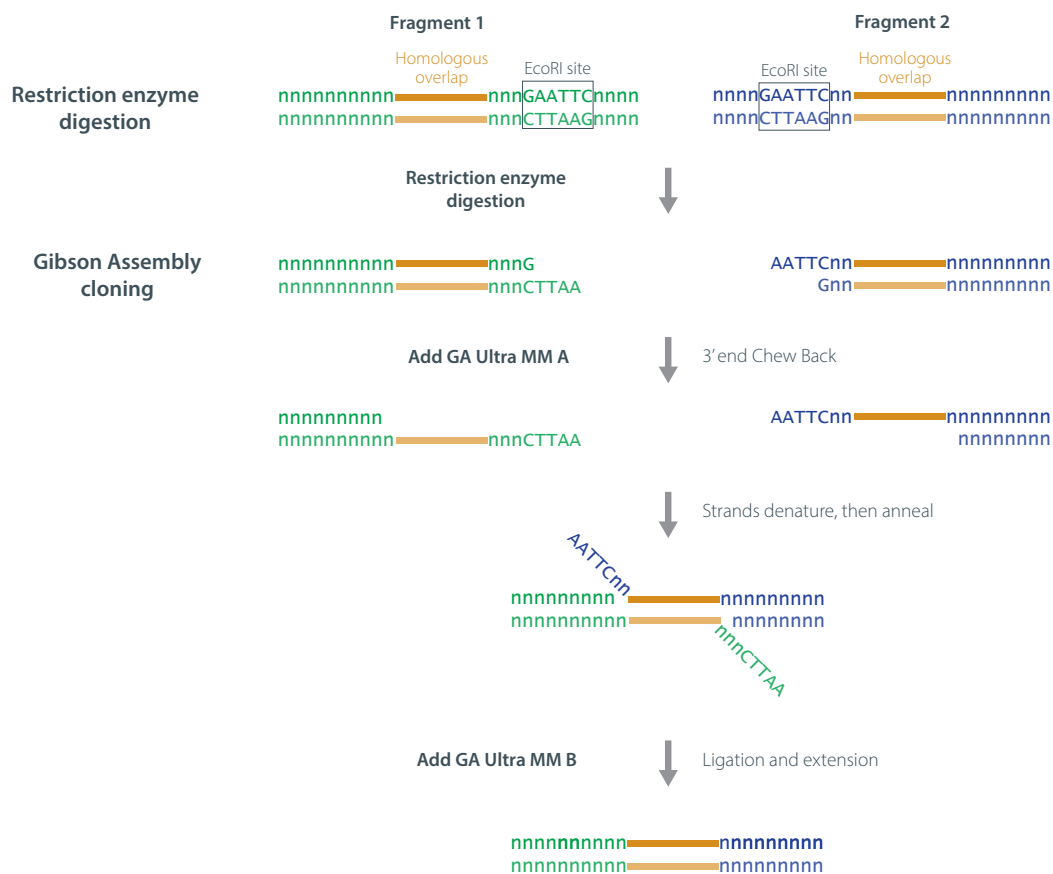


Figure 6. Adding restriction enzyme site to vector PCR primers before proceeding with the Gibson Assembly® cloning reaction.

## Appendix B: Restriction enzyme scars can be removed with the Gibson Assembly<sup>®</sup> reaction

Fragments for Gibson Assembly<sup>®</sup> cloning may be prepared by restriction enzyme digest. In this scenario, 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.

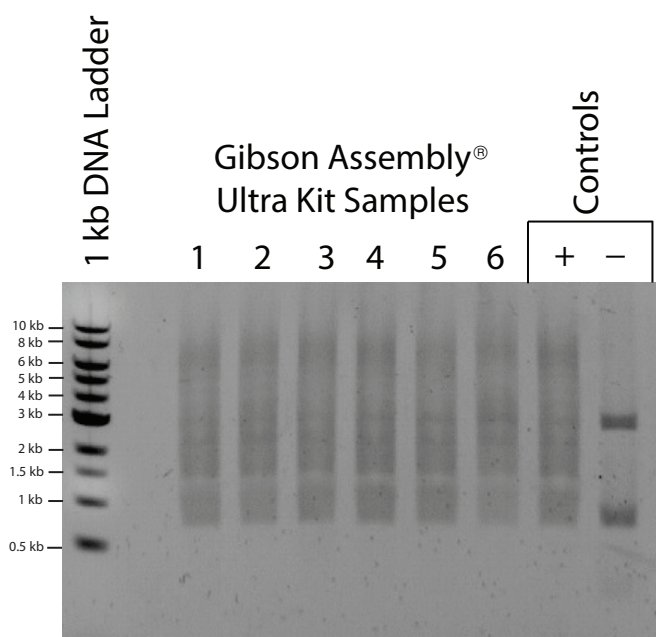
Restriction enzymes creating blunt ends, 3' overhangs, or 5' overhangs may all be used to prepare fragments for assembly. The following illustration depicts the elimination of both ends of the cleavage sequence created from digestion with restriction enzymes such as *EcoRI*, *BamHI*, *HinDIII*, and *NotI* that leave a 5' overhang. As shown below, the 3' remnants of the cleavage site are removed by the chew-back reaction. 5' overhangs are removed during the ligation step of the assembly reaction.



**Figure 7. An example depicting the elimination of an EcoRI scar from overlapping fragments during the Gibson Assembly<sup>®</sup> Ultra reaction.** Restriction enzymes that produce 5' DNA overhangs include *EcoRI*, *BamHI*, *HinDIII*, and *NotI*. The illustration above depicts two fragments with homologous overlap regions (shown in orange) and an external EcoRI site. Following restriction enzyme digestion with *EcoRI*, the fragments may be assembled using the Gibson Assembly<sup>®</sup> Ultra reaction. The restriction enzyme digestion scar is eliminated by the 3' chew back, exonuclease, and ligation activities of the Gibson Assembly<sup>®</sup> Ultra Master Mixes.

## Appendix C: Expected Results

To evaluate the success of the assembly reaction, perform gel electrophoresis with 5–10  $\mu\text{L}$  of the assembly reaction on a 0.8–2% agarose gel. The following image shows successful assembly reactions with the Gibson Assembly<sup>®</sup> Ultra kit.



**Figure 8. Gel electrophoresis of DNA following the Gibson Assembly<sup>®</sup> Ultra reaction.** Four 0.8 kb PCR fragments were assembled into a 2.7 kb vector in 6 replicates. The reaction contained 25 ng of each insert and 20 ng of vector and assembly was performed following the procedure described in this manual. 10  $\mu\text{L}$  from the 20  $\mu\text{L}$  total reaction was loaded onto an 0.8% agarose gel, along with a 1 kb molecular marker (NEB). The (-) control contained the DNA fragment without enzyme mix. Note that the 0.8 kb insert fragments and the 2.7 kb vector are clearly visible in the negative control lane and indistinguishable in the sample and positive control lanes. The successful assembly of the 6 replicates appears as a smear, as shown on the gel image (Lanes 1–6).

## Appendix D: Frequently asked questions

### General Gibson Assembly® Cloning Questions

#### 1. What are the advantages of this method?

- The Gibson Assembly® method enables assembly of small and large DNA constructs, using overlapping oligonucleotides or dsDNA fragments as starting material.
- Because it is not dependent on restriction enzyme sites, this method can be used to insert DNA fragments into any position of a linearized (restriction-digested or PCR-amplified) vector.
- The Gibson Assembly® method is a seamless method leaving no problematic scars at the junctions in your DNA fragment.
- The Gibson Assembly® method is much faster than traditional cloning methods.
- Resulting DNA product may be used immediately for transformation, PCR, or rolling circle amplification.
- The Gibson Assembly® method may be used to construct genes, genetic pathways, as well as genomes, and it has the capability of cloning multiple inserts into a vector simultaneously.
- The Gibson Assembly® method can be used to perform site-directed mutagenesis to make simultaneous DNA sequence changes including insertions, deletions, and substitutions.
- Multiple DNA fragments can be assembled simultaneously in a single reaction.

#### 2. Can I PCR-amplify the assembled product?

Yes. Because a covalently joined DNA molecule is produced, it may be PCR-amplified. In addition, if the final product is circular, it may be used in rolling circle amplification with  $\phi$ 29 polymerase.

#### 3. Can ssDNA oligonucleotides be combined and assembled with dsDNA fragments?

Yes. However, the optimal concentration of each oligonucleotide should be empirically determined. As a starting point, use 45 nM of each oligonucleotide. Keep in mind that oligonucleotides >90 bases may have secondary structures that interfere with assembly.

#### 4. Can I assemble linear fragments without a vector?

Yes, as long as the two extreme ends of the linear construct do not share homology to the ends of any internal fragments. We have not tested assembly of linear fragments for constructs >10 kb.

### Questions about Primers

#### 1. When using PCR to generate dsDNA fragments with Gibson Assembly® overlaps, are PAGE or HPLC purified oligonucleotide primers required? What if I want to anneal two single-stranded oligonucleotides to create my fragments?

While you can use PAGE or HPLC purified oligonucleotides, it is not a requirement. Standard desalted primers can be used in either case.

## Questions about Inserts

### 1. How large of a DNA fragment can I assemble?

The Gibson Assembly® Ultra Master Mix has been used to assemble DNA fragments greater than 1 Mbp with multi-step assemblies. Assembled products as large as 300 kb DNA fragments have been successfully transformed into *E. coli*, which is the approximate upper limit for cloning into *E. coli*.

### 2. How many fragments can I assemble at once?

The number of fragments that may be assembled is dependent on the length and the sequence of the DNA fragments. With the Ultra Kit, we recommend assembling up to 15 inserts into a vector at once in order to reliably produce a clone with the correct insert. The Table “Suggested Length of the Overlap Region: Inserts” on page 12 provides guidance on the numbers and sizes of fragments that can be assembled in a single round using either the HiFi 1-Step method or the Ultra two-step method.

### 3. Will this method work to assemble repetitive sequences?

Yes. Design DNA fragments that incorporate the repetitive sequences internally (not at the overhanging ends). This strategy will ensure that each DNA fragment has a unique overlap and will be assembled in the correct order. The repetitive sequence can also be internalized in the first stage of a two-stage assembly strategy. If having the repetitive sequence at the ends is unavoidable, the correct DNA molecules may still be produced at lower efficiencies. Alternatively, longer overlaps that increase the uniqueness of the fragments being joined can be used.

### 4. Can $\leq 200$ bp dsDNA fragments be assembled by this method?

Yes, the Gibson Assembly® Ultra kit allows for the assembly of inserts as small as 100 bp. For optimal results, use a  $\geq 5$ -fold molar excess of these smaller fragments relative to the vector.

## Questions about Vectors

### 1. Do I have to prepare my vector using PCR?

While vectors can have overlapping regions added by PCR, you are not required to prepare your vector by PCR. Cloning vectors can be linearized by restriction enzyme digest. Blunt ends, 5' overhangs, and 3' overhangs are all compatible with Gibson Assembly® cloning without further modification. If you are using vector prepared with a restriction enzyme digestion, you will need to add homologous overlap sequences to the insert.

## Homologous Overlap Region Questions

### 1. What are the shortest overlaps that can be used with this method?

As a starting point, we recommend using 40 bp overlaps when assembling dsDNA and 20 bp overlaps when assembling ssDNA oligonucleotides. Short overlaps can result in lower efficiency cloning. The ideal overlap length depends on fragment length and the numbers of fragments being assembled. The Table “Suggested Length of the Overlap Region: Inserts” on page 12 provides more specific guidance.

### 2. What are the longest overlaps that can be used with this method?

The kits are optimized for the assembly of DNA molecules with  $\leq 80$  bp overlaps.



## Procedural Questions

### 1. How should I store a Gibson Assembly® kit?

We recommend storing the kits at  $-20^{\circ}\text{C}$ . It is important to store Gibson Assembly® kits at a stable temperature (*i.e.*, not in a frost-free freezer) and in a location where the kit will not be subject to temperature shifts (*e.g.*, do not store the kits in the door of an upright freezer). To have the most consistent and robust performance during the storage interval we suggest that you subject your Gibson Assembly® kit or master mix to no more than 5 freeze-thaw cycles. If you believe that you will require more than 5 freeze-thaw cycles to use your product, we suggest that you aliquot the master mix.

### 2. What is the recommended starting concentration of insert and vector DNA?

For DNA fragments  $<1$  kb, the concentration should be  $>40$  ng/ $\mu\text{L}$ . With 25–50 ng of vector, the recommended range is 10–300 ng of each DNA fragment in equimolar amounts or in vector: insert ratios of 1:5. Generally, 1:5 is recommended for smaller fragments.

### 3. I have a limited amount of DNA. What is the lowest amount of DNA I can use?

One of the advantages of the Ultra kit is that small amounts of DNA may still be assembled efficiently. For fragments  $<1$  kb, 20–40 ng of DNA can be used.

### 4. Can longer or shorter incubation times be used?

Incubation times for the Ultra kit have been optimized for use with a wide number and range of fragment sizes, and we recommend using the incubation periods included in the protocol.

### 5. Do I need to inactivate restriction enzymes following vector digestion?

Restriction enzyme inactivation is only necessary if the insert contains the restriction site recognized by the restriction enzyme used for vector linearization.

### 6. Is it necessary to gel-purify restriction fragments or PCR products?

While gel purification helps to remove extraneous bands and helps you more accurately quantify the amount of DNA present, generally, this is not necessary. A cleanup kit or a standard phenol-chloroform extraction followed by ethanol precipitation is sufficient.

### 7. To ensure that my clone is error-free, what are your sequencing recommendations?

SGL-DNA Gibson Assembly® kits and master mixes include a proofreading DNA polymerase to minimize the potential of an incorrect base being inserted during the extension reaction.

To ensure that your insert is error-free, we suggest sequencing the entire insert and about 500 bp of the vector at the insert/vector junction. If PCR was used to prepare the vector, you may want to consider sequencing the entire vector.

## Troubleshooting and Optimization

### 1. How can I reduce the number of vector-only background colonies?

To significantly reduce the background of unwanted vector-only colonies, use a PCR-derived vector rather than a vector produced using a restriction enzyme digest. If the number of background colonies continues to be a problem, purify the PCR-amplified vector from an agarose gel following electrophoresis. A vector prepared by a double digestion or phosphatase treatment may also reduce the number of background colonies if a vector produced by restriction enzyme digestion is used.

### 2. What should I do if my assembly reaction yields no colonies, a small number of colonies, or clones with the incorrect insert size, following transformation into *E. coli*?

- Ensure that you are plating multiple volumes of the assembly reaction onto multiple LB plates with appropriate antibiotics.
- Assemble and transform the positive control provided in the kit. The successful cloning of the positive control will demonstrate that the assembly mixture is functional and the transformation conditions are suitable.
- Analyze the reaction on an agarose gel. See “Appendix C: Expected Results” on page 22.
- Check the design of the overlapping DNA fragments.
- If the amount of vector is increased, be sure to use appropriately balanced molar amounts of input fragments. See “Calculating the amount of DNA to use in a Gibson Assembly® reaction” on page 13 for further details.
- Consider whether the cloned insert may be toxic to *E. coli* and whether a low-copy vector, such as a BAC should be used.

### 3. I want to clone a large continuous piece of genomic DNA. It is larger than the suggested sizes for your kit. What suggestions do you have for cloning a large piece of genomic DNA?

For large DNA fragments, we often break them into ~10 kb sections. Fragments of this size can be generated using appropriate restriction enzymes or Cas9 endonuclease with an appropriately designed guide RNA. After obtaining the set of ~10 kb fragments, ~80 bp overlaps are added using PCR. For the amplification of large fragments, we suggest a highly processive, proof-reading RNA polymerase such as Phusion®, Q5®, or KOD Extreme™. The fragments can then be joined by the Gibson Assembly® method. If multiple large fragments need to be joined (e.g. more than four 10 kb fragments), we suggest performing multistage assemblies.

### 4. The fragments I would like to assemble have some repeated elements in them. What guidance do you have to improve the cloning efficiency using fragments that have a high percentage of similarity?

There are a number of strategies you can consider:

- Design the overlaps such that the repetitive sequences are buried within each fragment as much as possible.
- Use the Ultra kit with a 1–2 minute chew-back at 37°C instead of the recommended 5-minute chew-back.
- To generate a full-length fragment, use the assembly mixture as a template for PCR with primers corresponding to the ends of the insert. If the amplification is successful, the insert can be assembled with the vector using the Gibson Assembly® method.
- If none of these work, run the assembly reaction on a gel to see if laddering is occurring. To determine which region(s) might not be assembling, join pairs of adjacent regions with Gibson Assembly® and analyze the reaction on a gel. This can help identify if regions are not being effectively joined.

## Appendix E: Troubleshooting

Issue	Possible cause	Inquiries and recommendations
No colonies from positive control or experimental samples	Competent cell issue	<ul style="list-style-type: none"> <li>Be certain to use high efficiency competent cells with a transformation efficiency <math>\geq 1 \times 10^9</math> CFU/<math>\mu</math>g pUC19.</li> <li>Competent cells are fragile. Be certain to follow competent cell handling guidelines and do not vortex or pipet up and down.</li> <li>Using cells other than <i>E. coli</i> 10G or TransforMax™ EPI300™ Electrocompetent <i>E. coli</i> may require protocol optimization.</li> </ul>
	Improperly handling GA Ultra Master Mix A or B (2X)	<ul style="list-style-type: none"> <li>Aliquot GA Ultra Master Mix A and B (2X) to reduce the number of freeze-thaw cycles.</li> <li>Always thaw the master mix on ice.</li> <li><b>IMPORTANT! Vigorously vortex the master mix for 15 seconds immediately before use.</b></li> </ul>
	Selection issue	<ul style="list-style-type: none"> <li>Confirm that you are using the appropriate antibiotic at the correct concentration for selection.</li> </ul>
No colonies from experimental samples, but visible colonies from the positive control	Primer design issue	<ul style="list-style-type: none"> <li>Evaluate your primers, if applicable. Confirm that you followed overlap length guidelines as well as primer design guidelines.</li> </ul>
	Low efficiency assembly	<ul style="list-style-type: none"> <li>Purify the PCR amplification reaction assembly.</li> <li>Perform PCR amplification of the assembly reaction to confirm successful assembly.</li> </ul>
The presence of many clones without the insert (vector background)	Improper vector linearization	<ul style="list-style-type: none"> <li>If you linearize your vector using restriction enzyme digestion, always use a <i>double</i> restriction enzyme digest.</li> <li>If you prepare your vector by PCR amplification, treat the reaction with DpnI before use in the Gibson Assembly® reaction</li> </ul>
The presence of many clones with incorrect inserts	Mixed population of DNA fragments used in the assembly reaction	<ul style="list-style-type: none"> <li>Gel purify fragments prepared using PCR prior to Gibson Assembly® cloning</li> </ul>

## Ordering Information

Gibson Assembly® product ordering information			
Product	Product type	Number of reactions	Catalog number
HiFi 1-Step (2X)	Kit	5	GA1100-S
		10	GA1100-10
		50	GA1100-50
	Master Mix only	10	GA1100-10MM
		50	GA1100-50MM
	Bulk Master Mix	1000	GA1100-B05
		2000	GA1100-B10
		4000	GA1100-B20
		6000	GA1100-B30
		8000	GA1100-B40
10,000		GA1100-B50	
HiFi HC 1-Step (4X)	Kit	5	GA1100-4XS
		10	GA1100-4X10
		50	GA1100-4X50
	Master Mix only	10	GA1100-4X10M
		50	GA1100-4X50M
	Bulk Master Mix	1200	GA1100-4XB03
		2000	GA1100-4XB05
		4000	GA1100-4XB10
		6000	GA1100-4XB15
		8000	GA1100-4XB20
10,000		GA1100-4XB25	
Ultra	Kit	5	GA1200-S
		10	GA1200-10
		50	GA1200-50
	Master Mix only	10	GA1200-10MM
		50	GA1200-50MM
	Bulk Master Mix	1000	GA1200-B05
		2000	GA1200-B10
		4000	GA1200-B20
		6000	GA1200-B30
		8000	GA1200-B40
10,000		GA1200-B50	
Positive Control	Control	—	GA1000-05C
Site-Directed Mutagenesis	Kit	5	GA2100-S
		10	GA2100-10

Learn more and order Gibson Assembly® kits at [sgidna.com/gibson-assembly](https://sgidna.com/gibson-assembly)



**sgidna.com**

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