codex

Gibson Assembly® HiFi kit — User guide

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Patents

Gibson Assembly® US patent numbers 7,776,532, 8,435,736, and 8,968,999.

Regulatory statement

For research use only.

Kit information

Gibson Assembly® (GA) HiFi kit

Component	Quantity	Cat. GA1100-S (5 reactions)	Cat. GA1100-10 (10 reactions)	Cat. GA1100-50 (50 reactions)	Storage temperature
		Volume			
GA HiFi master mix (2X)	1 each	25 µL	50 µL	250 µL	–20 °C (Store master mix in aliquots)
GA positive control (2X)	1 each	10 µL (2 control rxns)	10 µL (2 control rxns)	25 µL (5 control rxns)	
GA HiFi – Quick reference manual	1 each				

Gibson Assembly® HiFi master mix (2X)

The Gibson Assembly® HiFi master mix (2X) is also available as a standalone product (without a positive control). The following table lists available sizes of the master mix. The Gibson Assembly® HiFi master mix is also available in a 4X version for more dilute DNA samples. IMPORTANT: Aliquot Gibson Assembly® master mix (2X) to reduce the number of freeze-thaw cycles. Properly aliquoted Gibson Assembly® master mix is stable at –20 °C for up to six months.

Catalog number	Volume	Number of reactions	Storage temperature	Aliquoting instructions
GA1100-10MM	50 µL	10	Aliquot and store at –20 °C	Upon receipt, place the master mix on ice to thaw. After the master mix has thawed completely, briefly vortex and centrifuge. Aliquot the master mix for future use (maximum volume of 250 µL).
GA1100-50MM	250 µL	50		
GA1100-B05	5 mL	1,000		
GA1100-B10	10 mL	2,000		
GA1100-B20	20 mL	4,000		
GA1100-B30	30 mL	6,000		
GA1100-B40	40 mL	8,000		
GA1100-B50	50 mL	10,000		

Gibson Assembly® positive control

The positive control DNA supplied with this kit is sufficient for two reactions (GA1100-S and GA1100-10) or five reactions (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
- Competent cells with a transformation efficiency ≥ 1 × 109 CFU/µg pUC19
  Note: We recommend *E. cloni*® 10G chemically competent cells (Lucigen cat. no. 60107) or TransforMax™ EPI300™ electrocompetent *E. coli* (Lucigen cat. no. EC300110)
- 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
- Microcentrifuge, such as the Bio360™ microcentrifuge
- Vortex mixer

Additional optional materials

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

- High-fidelity DNA polymerase for producing fragments to be assembled with Gibson Assembly® method
  Note: We recommend Phusion® High Fidelity DNA Polymerase (Thermo Fisher cat. no. F-530S)
- QIAquick PCR purification
- Spectrophotometer

Overview

Introduction

The Gibson Assembly® method can be used to rapidly clone multiple DNA fragments into any vector in one hour or less without the use of restriction enzymes. Instead of relying on the presence of restriction sites, user-defined overlapping ends are incorporated into the fragments to allow the seamless joining of adjacent fragments. By designing DNA fragments with homologous overlapping ends, users of the Gibson Assembly® method can create DNA constructs in a single round of cloning.

The method is initiated by combining DNA fragments with the Gibson Assembly® master mix. The master mix enzyme cocktail mediates strand chew back, exposing a single strand which allows for annealing of the terminal homologous overlap sequences. Annealing of the homologous overlap sequences is followed by extension and ligation to yield an assembled product. Seamless assembly can be readily applied to both routine cloning and large and complex cloning projects.

Key features of the Gibson Assembly® HiFi kit

- Accurate
- Seamless
- Optimal for one to five inserts
  Note: To assemble more than five inserts, use the Gibson Assembly® Ultra kit (Codex cat. no. GA1200)
- Suitable for fragments ranging from 500 bp to 32 kb
  Note: Fragments larger than 32 kb (up to 100 kb) may be assembled using multi-stage reactions
- The Gibson Assembly® Ultra kit (Codex cat. no. GA1200), can be used to assemble larger fragments (100 bp to 100 kb)

Gibson Assembly® synopsis

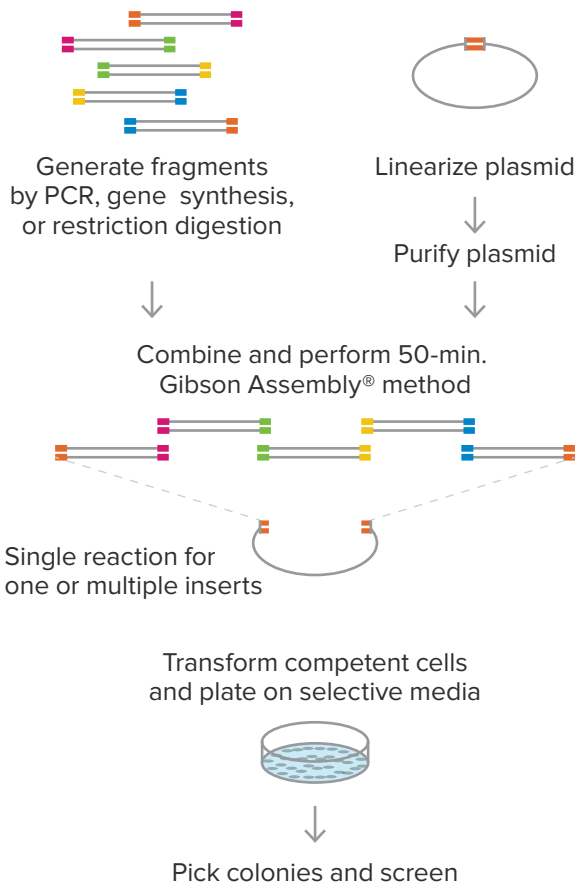
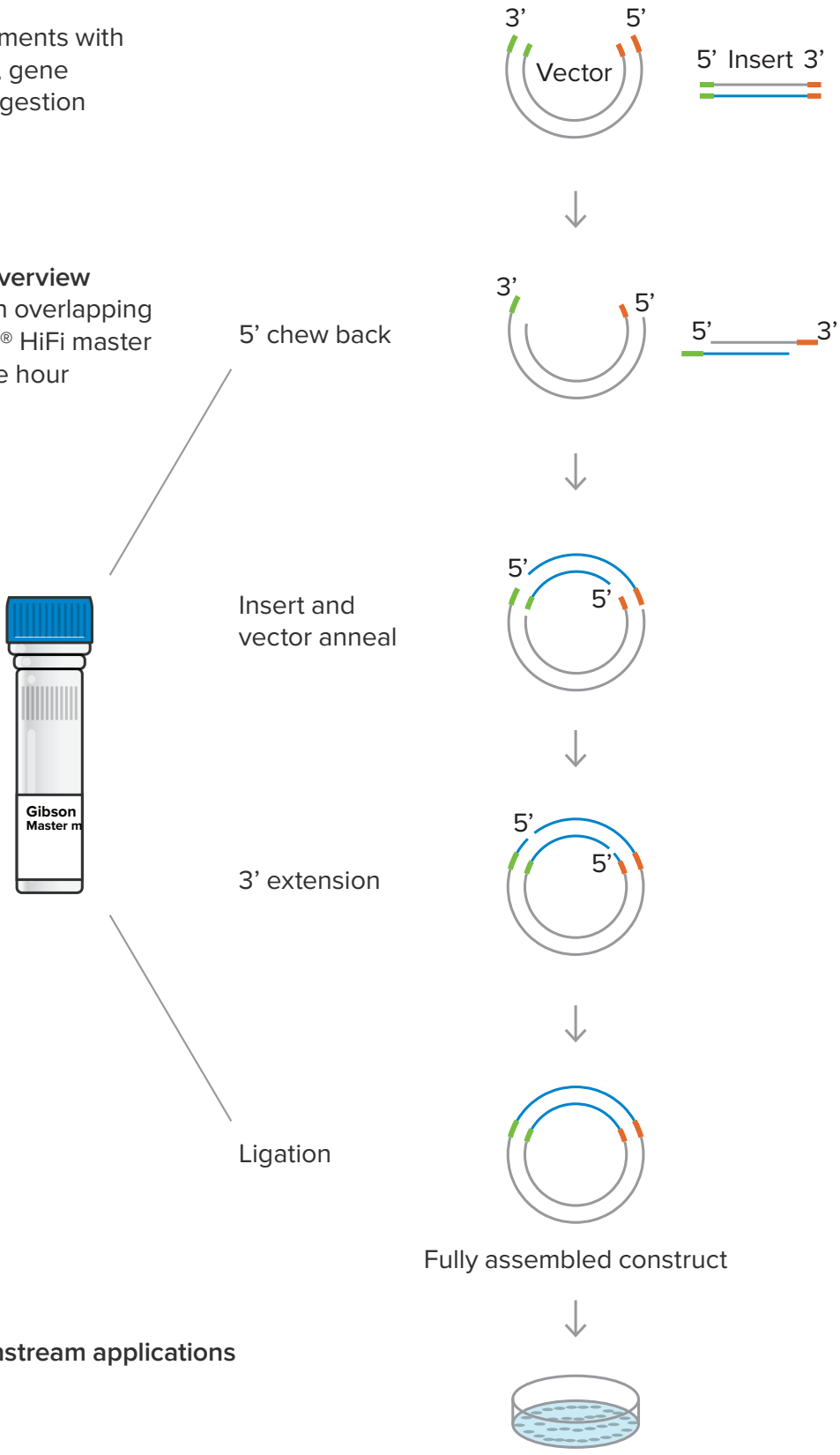


Figure 1. Synopsis of Gibson Assembly® HiFi cloning. The Gibson Assembly® method allows for the simultaneous assembly of one to five inserts in a single reaction that takes approximately one hour. This method requires a linearized vector and 20–80 bp sequence overlaps at the ends of the DNA fragments. Overlap sequences are intrinsic to the construct(s) and plasmid, eliminating the need for specific restriction sites. Following the 50-minute assembly reaction, constructs are ready for transformation and colony screening.

Gibson Assembly® HiFi workflow

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

Gibson Assembly® HiFi overview
Add vector and insert with overlapping ends to Gibson Assembly® HiFi master mix A (2X) at 50 °C for one hour



Transformation and downstream applications

Figure 2. Overview of the Gibson Assembly® HiFi cloning workflow. DNA fragments containing homologous overlapping ends are assembled in 60 minutes with the Gibson Assembly® HiFi kit. The Gibson Assembly® HiFi master mix mediates 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 five fragments with the Gibson Assembly® HiFi 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 seams can be removed with the Gibson Assembly® reaction for more information about removing restriction enzyme digestion seams using the Gibson Assembly® method.
- Generating DNA fragments on the benchtop BioXp™ 3200 system (see codexdna.com for more information).
- Ordering synthetic DNA from a custom gene synthesis provider.

In general, you may assemble any combination of synthesized, BioXp™ system-generated, 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. A free online tool is available at codexdna.com to assist with primer design. For detailed instructions on how to use the tool, refer to the Gibson Assembly® cloning guide. Additional information about primer design is available in Appendix A: Adding homologous overlaps to DNA fragments with PCR. 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 for more information about homologous overlap regions, including the recommended length.

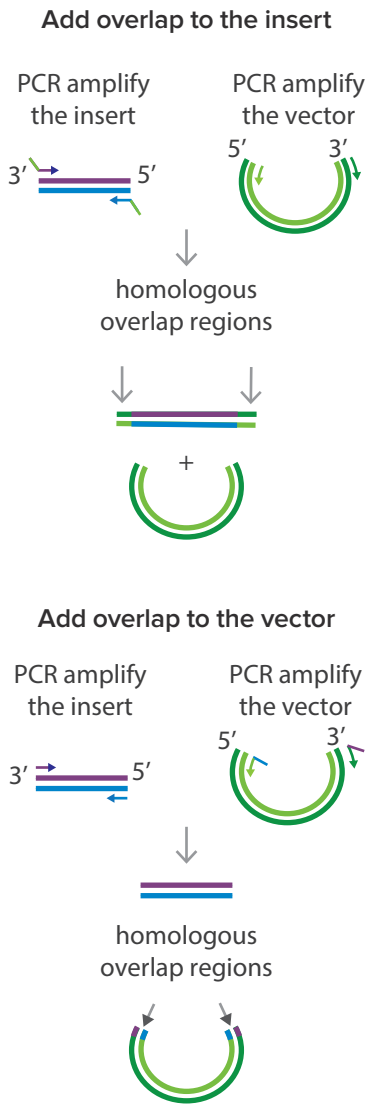


Figure 3. Using PCR to add homologous overlaps to the insert or the vector prior to Gibson Assembly® cloning. Homologous overlaps may be added to the insert (shown on the top panel) or to the vector (shown on the bottom panel) or split between the insert and vector (not shown).

Protocol for PCR Amplification

After designing the requisite primers, you can 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
Total	50 µL

PCR conditions

Initial denaturation	98 °C	30 sec	1 cycle
Amplification	98 °C	10 sec	25–30 cycles
	Primer T_m 60–70 °C	20 sec	
	72 °C	30 sec/kb	
Final extension	72 °C	5 min	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.

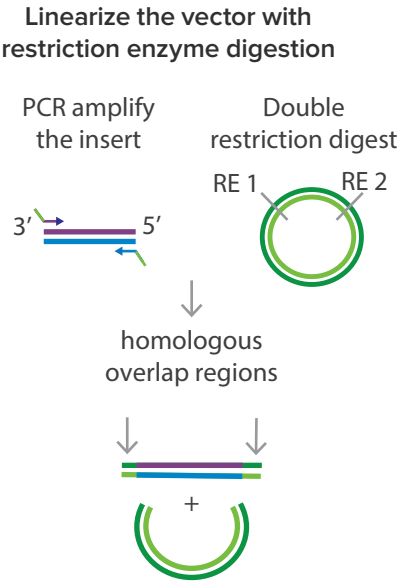


Figure 4. Using restriction enzyme digestion to linearize a vector prior to Gibson Assembly® cloning.

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 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	—	—	—	—	—	—

● Gibson Assembly® HiFi 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® HiFi procedure

- The total volume for the combined DNA fragments in the assembly reaction is ≤ 5 µL.
- Only use DNA samples with A260/280 > 1.8.
- For the positive control, use 5 µL of the positive control DNA in the assembly reaction.
- Keep Gibson Assembly® master mix (2X) on ice at all times.
- IMPORTANT: Vigorously vortex Gibson Assembly® master mix (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 > 40 ng/µL. 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.

For a typical Gibson Assembly® HiFi or Ultra kit reaction, combine 25–50 ng of vector with approximately 10–300 ng of insert. For best results, we recommend balancing the molar ratio of the DNA fragments. For fragments > 1 kb, use an equimolar ratio. For DNA fragments ≤ 1 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:

- pmol DNA = [ng DNA/(660 x # of bases)] x 1000
- ng of DNA = [pmol DNA x (660 x # 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® HiFi procedure

1. Thaw Gibson Assembly® HiFi master mix (2X) on ice.

2. In PCR tubes, prepare DNA fragments in nuclease-free water according to the guidelines outlined. Example:

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

3. **Vigorously vortex the master mix for 15 seconds immediately before use**, after it is thawed.

4. In a tube on ice, combine 5 µL of DNA fragments and 5 µL of Gibson Assembly® 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 Gibson Assembly® 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 one 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 by performing gel electrophoresis with 5–10 µL of the reaction on an 0.8–2% agarose gel.

Transformation with *E. cloni*® 10G chemically competent cells (recommended)

Before starting

We recommend using the following protocol with *E. cloni*® 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 ≥ 1×10^9 CFU/µg pUC19.

Because some 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 HiFi assemblies up to 5-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 µL of thawed, chemically competent cells to each cold tube.

4. Add 2 µL 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 two minutes.

8. Add 950 µL 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 µL the transformants on the LB plate with antibiotics. Use glass beads to swirl and plate evenly. Label plates with the following information:
    - Plate name
    - Plating volume (150 µL)
    - 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 one 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® HiFi 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 ≥ 1 × 109 CFU/µg pUC19.

Because some 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 HiFi assemblies up to 5-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 ten minutes.

2. Chill clean electroporation cuvettes on ice.

3. Pipet 30 µL of EPI300™ cells directly between the slit of the cuvettes on ice (one cuvette per reaction).

4. Add 2 µL 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 uF, 200 Ω, 0.1 cm cuvette.

8. While the pulse is taking place (~2 seconds), remove about 800 µL 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 µL 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 one 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 one-hour incubation, plate 50–100 µL of cells on LB plate with appropriate antibiotics.

Note: Use glass beads to swirl and plate evenly. Label plates with the following information:
    - Plate name
    - Plating volume (50–100 µL)
    - 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)

Competent cell transformation efficiency	# of fragments	Plating volume*	E.g., we normally plate...†	Expected # of colonies‡
> 1 × 10^9 CFU/µg pUC19	1–2	**1/50**	2 µL and 20 µL	> 100
	3–5	**1/10**	10 µL and 100 µL	
> 1 × 10^8 CFU/µg pUC19	1–2	**1/10**	10 µL and 100 µL	–
	3–5	**1/2**	100 µL and 500 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.
§ 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:
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.

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 create your Gibson Assembly® constructs. We recommend taking advantage of the free tool at codexdna.com, which will design primers for you. For detailed instructions on how to use the tool, refer to the Gibson Assembly® cloning 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 the Gibson Assembly® method 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 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® HiFi 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.

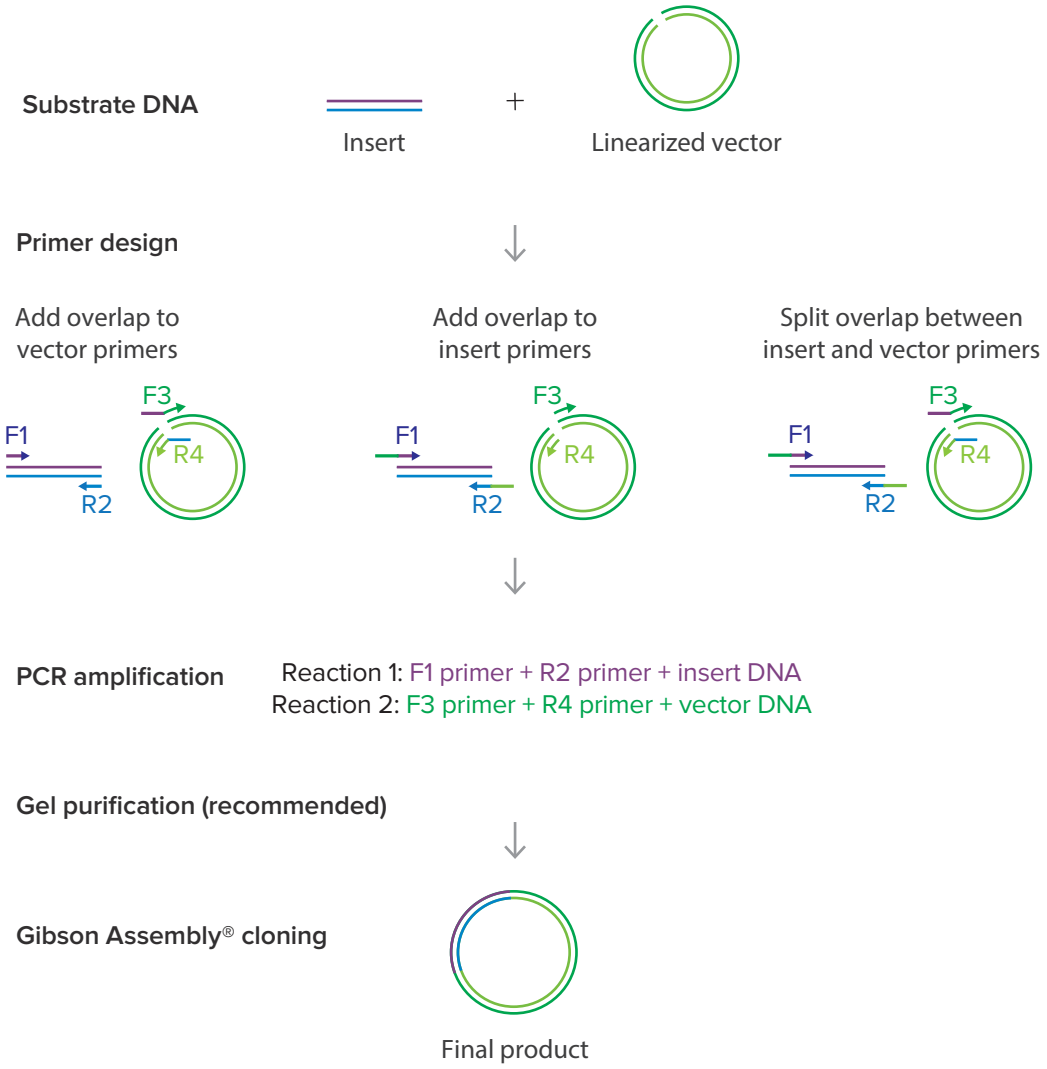


Figure 5. Scenarios for adding homologous overlap sequence to PCR primers before proceeding with the Gibson Assembly® cloning reaction. The final DNA construct is the same in all scenarios.

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.

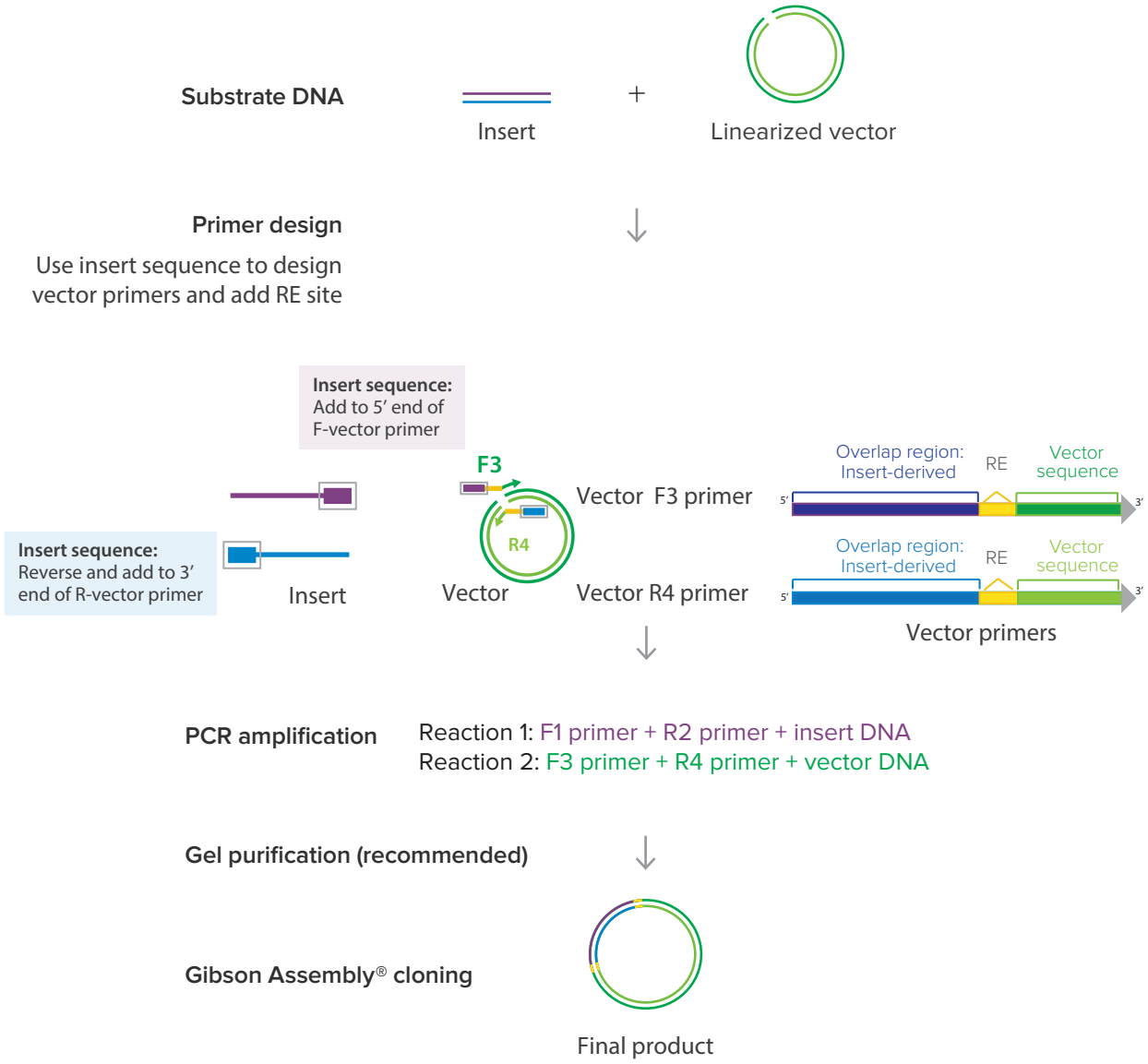


Figure 6. Adding restriction enzyme site to vector PCR primers before proceeding with the Gibson Assembly® cloning reaction.

Appendix B: Restriction enzyme seams can be removed with the Gibson Assembly® reaction

Fragments for Gibson Assembly® 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 5' remnants of the cleavage site are removed by the chew back reaction. 3' overhangs are removed during the ligation step of the assembly reaction.

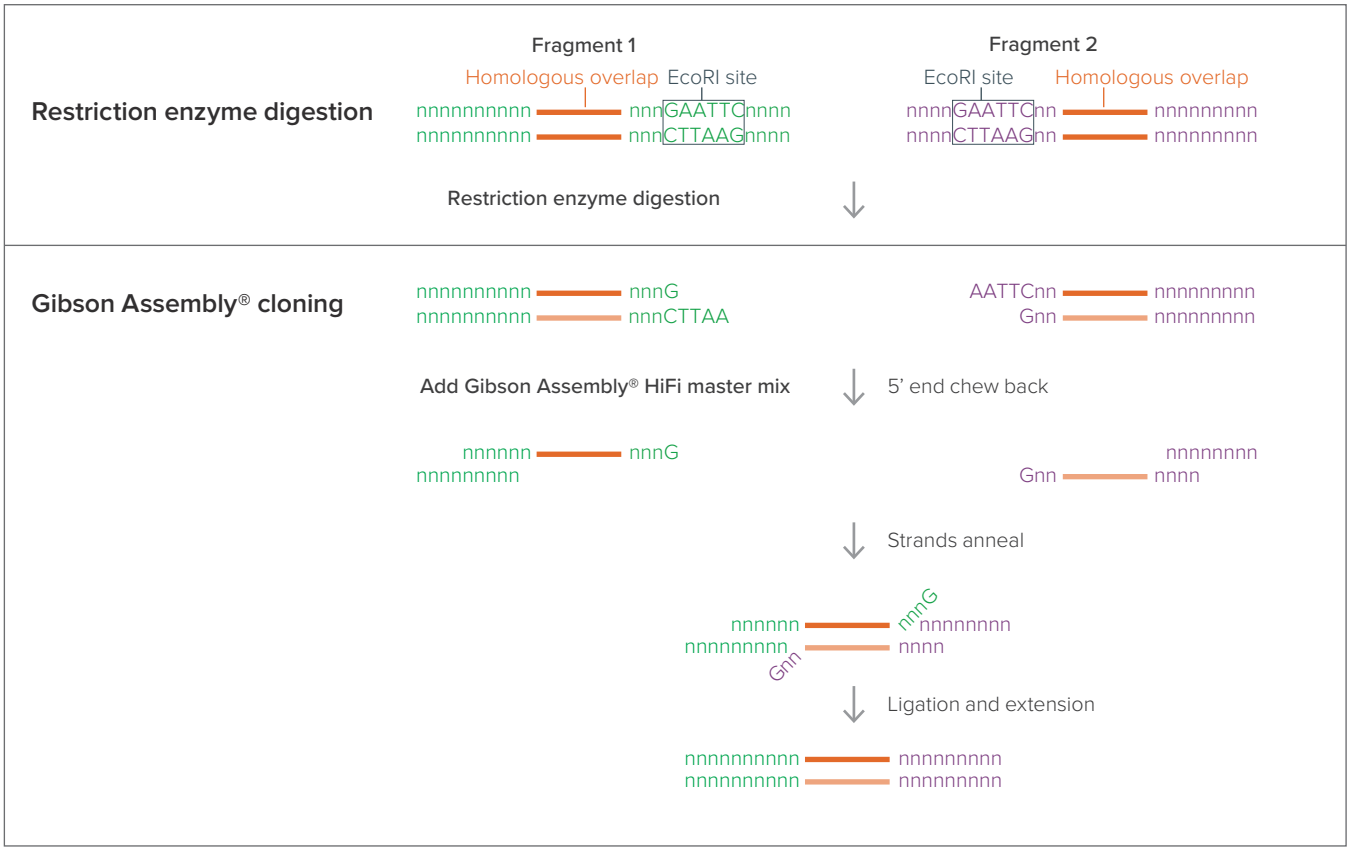


Figure 7. An example depicting the elimination of an EcoRI seam from overlapping fragments during the Gibson Assembly® HiFi reaction. Restriction enzymes that produce 5' DNA overhangs include EcoRI, BamHI, HinDIII, and NotI. The illustration above depicts two fragments with homologous overlap regions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

Appendix C: Expected results

To evaluate the success of the assembly reaction, perform gel electrophoresis with 5–10 µL of the assembly reaction on a 0.8–2% agarose gel. The following images show successful assembly reactions with the Gibson Assembly® HiFi kit.

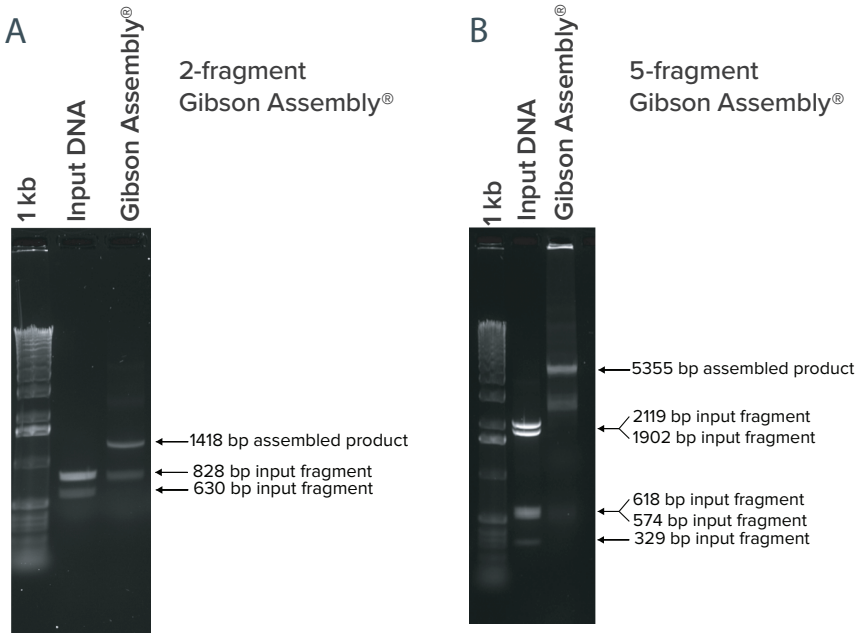


Figure 8. Gel electrophoresis of DNA following the Gibson Assembly® cloning reaction.

Data courtesy of Dr. Manoj Rajaure, Laboratory of Sankar Adhya, Center for Cancer Research, National Cancer Institute, Bethesda, MD

Appendix D: Frequently asked questions

General Gibson Assembly® cloning questions

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

   - The Gibson Assembly® method enables one-step 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 seams 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 φ29 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® HiFi 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 Gibson Assembly® HiFi kit, we recommend assembling five or fewer inserts into a vector at once in order to reliably produce a clone with the correct insert. For assembly using more than five fragments, we recommend using the Gibson Assembly® Ultra kit. The table Suggested length of the overlap region: inserts provides guidance on the numbers and sizes of fragments that can be assembled in a single round using either the HiFi method or the Ultra 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 ≤ 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 ≥ 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* 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 ≤ 80 bp overlaps.

Procedural questions

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

   We recommend storing the kits at –20 °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/µL. 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 Gibson Assembly® HiFi 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?**

   Yes, for the Gibson Assembly® HiFi kit, the assembly reaction has been optimized for a 1-hour, 50 °C incubation. However, extended incubation times (e.g. 2–16 hours) have been shown to improve cloning efficiencies in some cases. Alternatively, for the assembly of ≤ 3 fragments, 15 minutes has been shown to be sufficient. Reaction times less than 15 minutes are not recommended. Incubation times for the Gibson Assembly® 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?**

   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. An efficient assembly reaction will show assembled products of the correct size and the absence or reduction of the starting substrate DNA fragments (see Appendix C: Expected results).
   - 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 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 the 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 the Gibson Assembly® method 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	• Be certain to use high efficiency competent cells with a transformation efficiency ≥ 1 × 109 CFU/µg pUC19. • Competent cells are fragile. Be certain to follow competent cell handling guidelines and do not vortex or pipet up and down. • Using cells other than *E. cloni* 10G or TransforMax™ EPI300™ electrocompetent *E. coli* may require protocol optimization. • Components of Gibson Assembly® HiFi master mix (2X) can negatively impact the survival of some competent cells. We recommend diluting the assembly reaction before performing the transformation up to 5-fold.
	Improperly handling Gibson Assembly® HiFi master mix (2X)	• Aliquot Gibson Assembly® HiFi master mix (2X) to reduce the number of freeze-thaw cycles. • Always thaw the master mix on ice. • IMPORTANT! Vigorously vortex the master mix for 15 seconds immediately before use.
	Selection issue	Confirm that you are using the appropriate antibiotic at the correct concentration for selection.
No colonies from experimental samples, but visible colonies from the positive control	Primer design issue	Evaluate your primers, if applicable. Confirm that you followed overlap length guidelines as well as primer design guidelines.
	Low efficiency assembly	• Purify the PCR amplification reaction assembly. • Perform PCR amplification of the assembly reaction to confirm successful assembly.
The presence of many clones without the insert (vector background)	Improper vector linearization	• If you linearize your vector using restriction enzyme digestion, always use a double restriction enzyme digest. • If you prepare your vector by PCR amplification, treat the reaction with DpnI before use in the Gibson Assembly® reaction.
The presence of many clones with incorrect inserts	Mixed population of DNA fragments used in the assembly reaction	Gel purify fragments prepared using PCR prior to Gibson Assembly® cloning

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