DNA MODIFYING ENZYMES

Gibson Assembly® Master Mix

Instruction Manual

NEB #E2611S/L
10/50 reactions
Version 5.0 8/18

be INSPIRED
drive DISCOVERY
stay GENUINE

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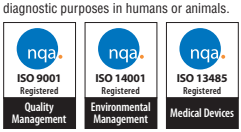

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Components:

Store at –20°C. Thaw, vortex thoroughly before use and keep on ice.

Gibson Assembly Master Mix (2X)

Positive Control

2 overlapping dsDNA fragments for control assembly.

Required Materials Not Included:

DNA Polymerases (for generating PCR products):
We recommend Q5® High-Fidelity DNA Polymerase (NEB #M0491) or related products, such as Q5 Hot Start High-Fidelity DNA Polymerase (NEB #M0493), Q5 Hot Start High-Fidelity 2X Master Mix (NEB #M0494).

LB (Luria-Bertani) plates with appropriate antibiotic.

SOC Outgrowth Medium (NEB #B9020).

Competent Cells:
We recommend NEB 5-alpha Competent *E. coli* (High Efficiency, NEB #C2987).
For assembled products greater than 10 kb, NEB recommends using NEB 10-beta Competent *E. coli* (High Efficiency, NEB #C3019) or NEB 10-beta Electrocompetent *E. coli* (NEB #C3020).

Introduction:

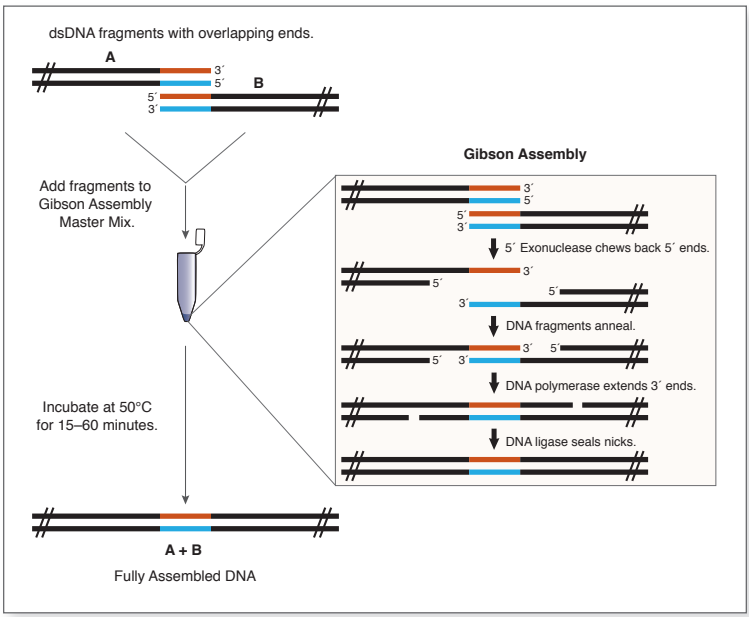
Gibson Assembly was developed by Dr. Daniel Gibson and his colleagues at the J. Craig Venter Institute and licensed to NEB by Synthetic Genomics, Inc. It allows for successful assembly of multiple DNA fragments, regardless of fragment length or end compatibility. It has been rapidly adopted by the synthetic biology community due to its ease-of-use, flexibility and suitability for large DNA constructs.

Gibson Assembly efficiently joins multiple overlapping DNA fragments in a single-tube isothermal reaction (1,2). The Gibson Assembly Master Mix includes three different enzymatic activities that perform in a single buffer:

- The exonuclease creates single-stranded 3´ overhangs that facilitate the annealing of fragments that share complementarity at one end (overlap region).
- The proprietary DNA polymerase fills in gaps within each annealed fragment.
- The DNA ligase seals nicks in the assembled DNA.

The end result is a double-stranded fully sealed DNA molecule that can serve as template for PCR, RCA or a variety of other molecular biology applications, including direct transformation. The method has been successfully used by Gibson's group and others to assemble oligonucleotides, DNA with varied overlaps (15–80 bp) and fragments hundreds of kilobases long (1–2).

Figure 1: Overview of the Gibson Assembly Method



Specification:

10 µl of 2X Gibson Assembly Master Mix was incubated with 6 fragments (5 fragments of 400 bp and one of 2,780 bp, with 40 bp overlap, 0.05 pmol each) in a final volume of 20 µl at 50°C for 60 minutes. NEB 5-alpha Competent *E. coli* (NEB #C2987) were transformed with 2 µl of the master mix/fragment mixture using the transformation protocol on page 12. Greater than 100 white colonies were observed when 1/10 of the outgrowth was spread on an ampicillin plate with IPTG/Xgal and incubated overnight.

Overview of Gibson Assembly Master Mix Protocol:

- Design primers to amplify fragments (and/or vector) with appropriate overlaps (see pages 3–10).
- PCR amplify fragments using a high-fidelity DNA polymerase.
- Prepare linearized vector by PCR amplification using a high-fidelity DNA polymerase or by restriction digestion.
- Confirm and determine concentration of fragments using agarose gel electrophoresis, a Nanodrop™ instrument or other method.
- Add DNAs to Gibson Assembly Master Mix and incubate at 50°C for 15 minutes to 1 hour, depending on number of fragments being assembled.
- Transform into *E. coli* or use directly in other applications.

Design and PCR Amplification of Fragments for Gibson Assembly:

Note: We highly recommend using our web tool, NEBuilder®, available at www.NEBGibson.com, to design PCR primers with overlapping sequences between the adjacent DNA fragments and for their assembly into a cloning vector.

NEBuilder®
Assembly Tool

NEBuilder is the fastest and easiest approach to obtain ready-to-use sequences for overlapping primers. However, it does not give details about the primer design workflow. In some cases, it might be appropriate to further manually alter primer sequences in order to adapt them for the use in more complex assemblies, such as those that include site-specific mutagenesis. For this purpose, it is absolutely necessary to understand the general requirements and rules that apply to PCR primers used in conjunction with Gibson Assembly. The sections below offer step-by-step directions and recommendations for the manual design of primers for the assembly of two or more PCR fragments, as well as primer design for assembly of PCR fragments into a cloning vector prepared either by PCR or by restriction digestion.

Structure of the Overlapping Primers

PCR primers for use in Gibson Assembly must have two sequence components:

- an overlap sequence, required for the assembly of adjacent fragments;
- a gene-specific sequence, required for template priming during PCR amplification;

The non-priming overlap sequence is added at the 5´-end of the primer. This sequence is homologous to the 5´-terminal sequence of the adjacent fragment. The length of overlap sequence is dependent on the GC content of the sequences.

The priming gene-specific sequence is added at the 3´-end of the primer after the overlap sequence. The priming sequence should meet the criteria required for template annealing during PCR amplification.

The Tm of the 3´ gene-specific sequence of the primer can be calculated using the Tm calculator found on the NEB website at http://www.neb.com/TmCalculator.

General Recommendations for Design of Overlapping Primers

To achieve efficient assembly of PCR fragments into a vector, we suggest using a 15–25 nt overlap with a Tm equal to or greater than 48°C (assuming A-T pair = 2°C and G-C pair = 4°C). To prevent errors in primer design it is highly recommended to first perform DNA fragment assembly *in silico* and create a final sequence file displaying both DNA strands, Fig. 2A, Step I). This virtual sequence may then be used as a template to design overlapping primers. Figure 2A shows the workflow for overlapping primer design by using an *in silico*-created DNA sequence file. First, mark the junctions between the adjacent fragments 1, 2 and 3 (Fig. 2A, step II). Next, at or near each junction choose 15–25 nucleotide sequences to serve as the overlap region between the two adjacent fragments (Fig. 2A, step III). For the best fit, in terms of length and Tm, the overlap sequence can be composed of nucleotides which belong to only one fragment (overlap shown in blue) or it can be split between the two adjacent fragments in any combination (overlap shown in orange). Mark the first 5´ and the last 3´ nucleotide of the overlap sequence on both DNA strands (boxed sequence). Finally, starting from the first 5´ nucleotide, copy the entire overlap sequence in the 5´ to 3´ direction and, if necessary, continue to add nucleotides to the 3´ end until the gene-specific priming sequence length is reached (Fig. 2A, step IV). The reverse overlapping primer is designed following the same steps as described above but copying the sequence from the complementary DNA strand in the 5´ to 3´ direction. Keep in mind that the two primers sharing the same overlap sequence are always used in separate PCR reactions, each in combination with the primer which primes the complementary sequence on the opposite end of the respective DNA fragment (Fig. 2A, step V).

Figure 2A: Primer design using *in silico* created final DNA sequence file.

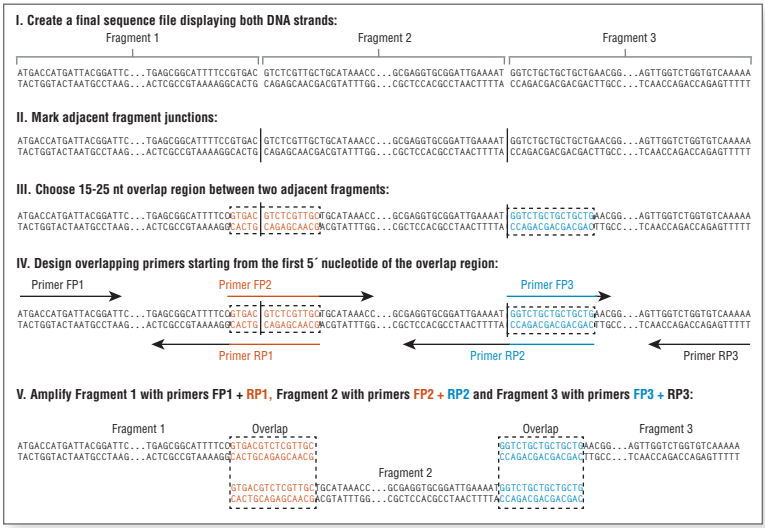
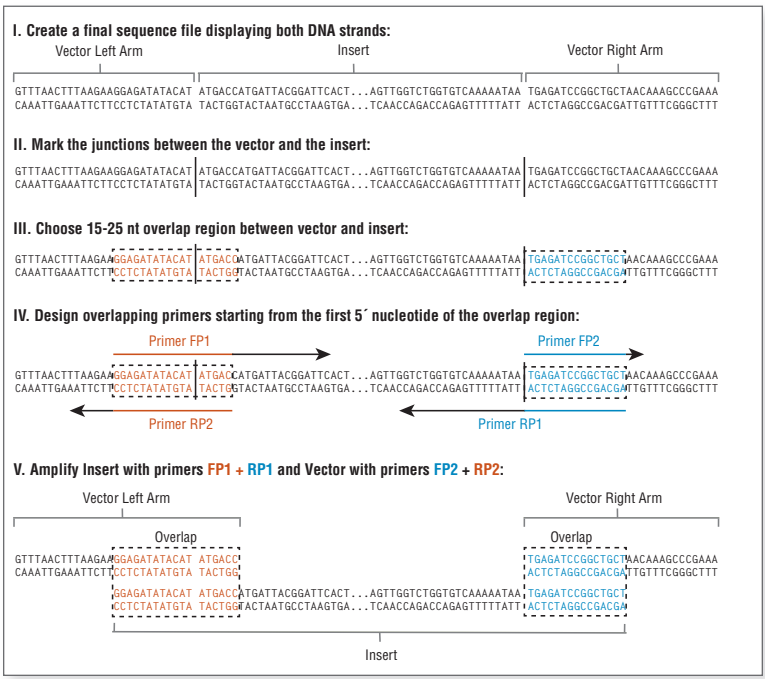


Figure 2B: Primer design for PCR-generated vector and insert using *in silico* created final DNA sequence file.



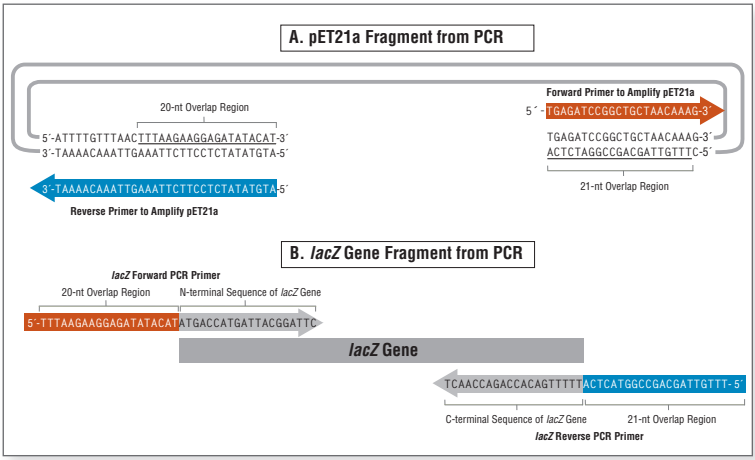
Primer Design for PCR-Generated Vector and Insert

For the purposes of primer design, the vector and the insert may be viewed as two PCR fragments that have to be assembled into a circular DNA molecule. This means that the primer design rules described above may also be applied for generation of the vector fragment and the insert fragment sharing overlapping ends. Use the *in silico*-created final sequence file as a template to design overlapping primers between the vector and the insert by accomplishing the same steps as described above, as shown in Figure 2B.

If you intend to use PCR-generated vector for one specific insertion, then the overlap sequence may be split between the vector and the insert in any combination to make shorter primers (Figure 2B, step III, overlap shown in orange). However, if the same PCR-generated vector will be used for assembly of various inserts, then the entire overlap sequence must originate from the vector sequence and must be added to primers that will be used to amplify the insert (Figure 2B, step III, overlap shown in blue). The latter case is also illustrated in Figure 3 for assembly of the *lacZ* gene into a pET21a vector. The pET21a forward primer (orange arrow) and the reverse primer (blue arrow) start at the position where the *lacZ* gene must be inserted. Both vector-specific primers completely match the vector sequence on the respective strands marked in orange and blue. This inverse PCR strategy yields a linear vector fragment. Generally, 10–100 pg of a vector is recommended as a template in the inverse PCR reaction.

To amplify the *lacZ* gene, both forward and reverse *lacZ*-specific priming sequences (gray) at their 5´ end are fused with 20-nt vector sequences to be used as overlap sequences in assembly with the vector. Within the *lacZ* Forward PCR primer, the overlap sequence (orange) is identical to the 20-nt terminal sequence on the top strand (orange) of the vector's left-arm (in the 5´→3´ direction). Within the *lacZ* Reverse PCR primer, the overlap sequence (blue) is identical to the 21-nt terminal sequence on the bottom strand (blue) of the vector's right-arm (in the 5´→3´ direction). The length of the overlap sequence is determined by the number of nucleotides needed to reach a Tm ≥ 48°C. If necessary, one may add additional nucleotides between the overlap sequence and the *lacZ*-specific sequence, for example, to introduce a unique restriction site.

Figure 3: Primer Design for Vector pET21a and *lacZ* Gene Assembly.



Primer Design for Assembly of Restriction Enzyme Digested Vector and PCR-Generated Insert.

Restriction enzyme-treated vectors can have 5´-overhangs, 3´-overhangs or blunt ends. When vector is linearized by restriction digestion, the entire overlap sequence must originate from the vector sequence and must be added to primers that will be used to amplify the insert. The overlap region of the forward primer for the gene of interest (orange) should line up with the 3´ end of the overhang on the vector's left arm and extend back until the Tm ≥ 48°C (Fig.4A, Left side). This primer also includes gene-specific sequence at the 3´-end (gray). Keep in mind that the restriction site, which was used to digest the vector, will be lost in the assembled product. However, additional nucleotides may be added between the overlap region and gene-specific sequence region to restore the pre-existing restriction site, or to introduce a new, unique restriction site. A similar principle is applied to the design of the reverse primer for the gene of interest (Fig. 4A, Right side).

Figure 4A: Assembly of Restriction Enzyme-Digested Vector and PCR-derived Insert

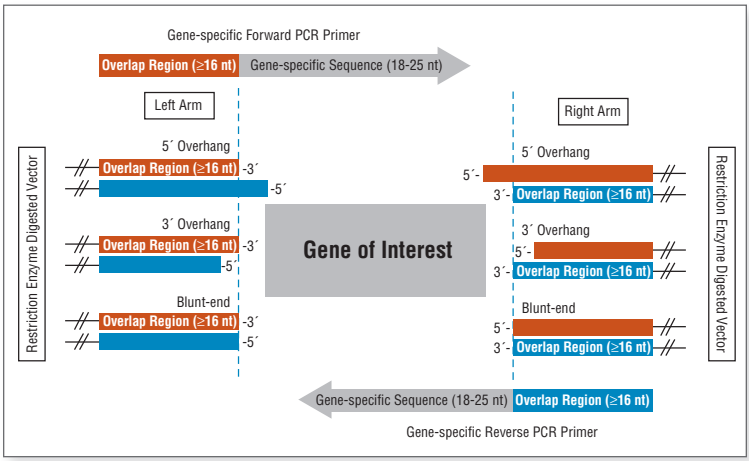
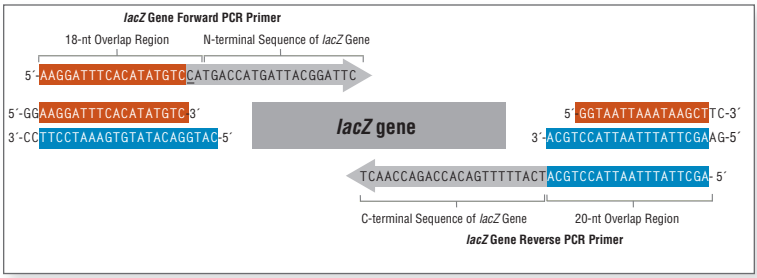


Figure 4B shows primer design for assembly of the *lacZ* gene and pMAL-c5X, digested with NcoI and SbfI. In this example, the forward primer of the gene has a "C" nucleotide (underlined) inserted between the 18-nt overlap and the N-terminal sequence of the *lacZ* gene to ensure the *lacZ* protein is in frame with the maltose binding protein.

Figure 4B: Primer Design for *lacZ* Gene and NcoI/SbfI-cut pMAL-c5X Assembly



Useful Recommendations for Vector Digestion with Restriction Enzymes

In general, the cloning vector can be linearized by any restriction endonuclease or by any combination of two restriction endonucleases displaying unique site(s) at the desired locations within the vector sequence.

Note: Double-digestion of vector DNA with two restriction endonucleases is the best approach to reduce the uncut vector background.

- Some restriction endonucleases cannot efficiently digest supercoiled DNA and thus may leave behind different amounts of uncut vector DNA. If not gel-purified, the uncut vector is transformable and will show up after transformation of the Gibson Assembly reaction, thereby, reducing the overall fraction of recombinant clones. A table "Cleavage of Supercoiled DNA" found at www.neb.com/nebecomm/tech_reference/restriction_enzymes/ may be used as a reference for choosing the most suitable restriction endonucleases and the number of activity units required for complete digestion of plasmid vector.
- Restriction endonucleases might have a reduced activity on plasmid DNA purified using various plasmid purification kits. In such cases, the extended restriction time or increased enzyme concentration may be necessary to digest plasmid vector to completion (or as nearly as possible to completion). When applicable, NEB highly recommends using High Fidelity (HF) restriction endonucleases to avoid star activity which may occur when digesting DNA with elevated amounts of regular restriction endonuclease for extended periods of time.
- Purification of restriction endonuclease-digested vector is not necessary unless the same restriction site is present in insert DNA. In such cases, either heat-inactivate restriction endonuclease before Gibson Assembly reaction or purify the linearized vector, either by phenol-chloroform extraction/alcohol precipitation or by electrophoresis on an agarose gel.

Useful Recommendations for PCR Amplification

NEB recommends using Q5 High-Fidelity DNA polymerase (NEB #M0491) or related products (NEB #M0493 or NEB #M0494) to amplify fragments of interest prior to assembly. The use of this high-fidelity DNA polymerase yields PCR products with blunt ends, thereby reducing the error rates at the fragment junctions.

- When using circular plasmid DNA as a template, it is important to use a minimal amount of DNA (usually recommended 0.1–0.5 ng of plasmid template per 50 µl PCR reaction) in order to reduce the template background after transformation. If higher amounts of plasmid template must be used in PCR reaction or higher amounts of PCR product must be used in the Gibson Assembly reaction, it is recommended to digest the PCR product with DpnI restriction endonuclease in order to destroy plasmid template before setting up the Gibson Assembly reaction (for protocol see below).
- Verify PCR product purity and yield by gel electrophoresis. If non-specific DNA fragments are obtained, you will need to purify the target fragment from the agarose gel to ensure the correct product assembly during the Gibson Assembly reaction.
- PCR product purification is not necessary as long as the product is > 90% pure. You can add unpurified PCR product directly from the PCR reaction into the Gibson Assembly for up to 20% of the total Gibson Assembly reaction volume (i.e. PCR products should account for 4 µl, or less, in a 20 µl Gibson Assembly reaction). Larger volumes of unpurified PCR products could significantly inhibit both the Gibson Assembly and the transformation. In such cases, it is recommended to column-purify PCR products and, if necessary, to concentrate DNA by ethanol precipitation.

(Optional) DpnI Digestion Protocol

When higher amounts of plasmid template must be used in the PCR reaction, it is recommended to digest the PCR product with DpnI (NEB #R0176) restriction endonuclease in order to destroy plasmid template before setting up the Gibson Assembly reaction. DpnI cleaves only *E. coli* Dam methylase-methylated plasmid DNA, but does not cleave the PCR product since it is not methylated.

DpnI Digestion Protocol:

1. In a total 10 µl reaction, mix 5–8 µl of PCR product with 1 µl of 10X Cutsmart and 1 µl (20 units) of DpnI.
2. Incubate at 37°C for 30 minutes.
3. Heat-inactivate DpnI by incubating at 80°C for 20 minutes.
4. Proceed with the Gibson Assembly Cloning procedure, described on page 11.

Gibson Assembly Reaction:

Optimal Quantities

NEB recommends a total of 0.02–0.5 pmols of DNA fragments when 1 or 2 fragments are being assembled into a vector and 0.2–1.0 pmoles of DNA fragments when 4–6 fragments are being assembled. Efficiency of assembly decreases as the number or length of fragments increases. To calculate the number of pmols of each fragment for optimal assembly, based on fragment length and weight, we recommend the following formula:

pmols = (weight in ng) x 1,000 / (base pairs x 650 daltons)

50 ng of 5000 bp dsDNA is about 0.015 pmols.

50 ng of 500 bp dsDNA is about 0.15 pmols.

The mass of each fragment can be measured using the NanoDrop instrument, absorbance at 260 nm or estimated from agarose gel electrophoresis followed by ethidium bromide staining.

Assembly Protocol:

1. Set up the following reaction on ice:

	Recommended Amount of Fragments Used for Assembly		
	2–3 Fragment Assembly	4–6 Fragment Assembly	Positive Control**
Total Amount of Fragments	0.02–0.5 pmols* X µl	0.2–1 pmols X µl	10 µl
Gibson Assembly Master Mix (2X)	10 µl	10 µl	10 µl
Deionized H_2O	10-X µl	10-X µl	0
Total Volume	20 µl***	20 µl***	20 µl

* Optimized cloning efficiency is 50–100 ng of vectors with 2–3 fold of excess inserts. Use 5 times more of inserts if size is less than 200 bps. Total volume of unpurified PCR fragments in Gibson Assembly reaction should not exceed 20%.

** Control reagents are provided for 5 experiments.

*** If greater numbers of fragments are assembled, additional Gibson Assembly Master Mix may be required.

2. Incubate samples in a thermocycler at 50°C for 15 minutes when 2 or 3 fragments are being assembled or 60 minutes when 4-6 fragments are being assembled. Following incubation, store samples on ice or at –20°C for subsequent transformation.

Note: Extended incubation up to 60 minutes may help to improve assembly efficiency in some cases (for further details see FAQ section on page 15).

Chemically Competent Cells Transformation Protocol:

1. Thaw chemically competent cells on ice.
2. Transfer 50 µl of competent cells to a 1.5 ml microcentrifuge tube (if necessary).
3. If the chemically competent cells are from New England Biolabs, add 2 µl of assembled product to NEB competent cells and go to step 4 directly. If competent cells are purchased from other manufacture, dilute assembled products 4-fold with H_2O prior transformation. This can be achieved by mixing 5 µl of assembled products with 15 µl of H_2O. Add 2 µl of the diluted assembled product to competent cells.
4. Mix gently by pipetting up and down or flicking the tube 4–5 times. Do not vortex. Place the mixture on ice for 30 minutes. Do not mix.
5. Heat shock at 42°C for 30 seconds.* Do not mix.
6. Transfer tubes on ice for 2 minutes.
7. Add 950 µl of room temperature SOC media* to tubes.
8. Place the tube at 37°C for 60 minutes. Shake vigorously (250 rpm) or rotate.
9. Warm selection plates to 37°C.
10. Spread 100 µl of the cells onto the plates with appropriate antibiotics. Use Amp plates for positive control sample.
11. Incubate plates overnight at 37°C.

* Please note: Follow the manufacturer's protocols for the duration and temperature of the heat shock step, as well as the optimal medium for recovery. Typically, transformation of our positive control assembly product will yield more than 100 colonies on an Amp plate with greater than 80% colonies containing inserts.

NEB recommends NEB 5-alpha Competent *E. coli* (NEB #C2987) for transformation of Gibson Assembly products. It is also possible to use other NEB competent *E. coli* strains, with the exception of BL21, BL21(DE3), Lemo21(DE3) and Nico21(DE3). For example, Shuffle T7 Express Competent *E. coli* can be used for the expression of a difficult to express protein. When using competent *E. coli* from a vendor other than NEB, we have seen decreased robustness of transformation with the Gibson Assembly reaction.

Usage Notes:

To ensure the successful assembly and subsequent transformation of assembled DNAs, NEB recommends the following:

- *Cells*: Transformation efficiency of competent cells can vary by several logs. Perceived assembly efficiency directly correlates to the competence of the cells used for transformation.

- *Electroporation*: Electroporation can increase transformation efficiency by several logs. When using the Gibson Assembly Master Mix product for electroporation, it is necessary to dilute the reaction 3-fold and use 1 µl for transformation.

  Electrocompetent Cells Transformation Protocol:

  1. Thaw electrocompetent cells on ice.
  2. Transfer 50 µl of electrocompetent cells to a pre-chilled electroporation cuvette with 1 mM gap.
  3. Dilute assembled products 3-fold with H_2O prior electroporation. This can be achieved by mixing 5 µl of assembled products with 10 µl of H_2O. Add 1 µl of the diluted assembly product to electrocompetent cells.
  4. Mix gently by pipetting up.
  5. Once DNA is added to the cells, electroporation can be carried out immediately. It is not necessary to incubate DNA with cells.
  6. Add 950 µl of room temperature SOC media to the cuvette immediately after electroporation.
  7. Place the tube at 37°C for 60 minutes. Shake vigorously (250 rpm) or rotate.
  8. Warm selection plates to 37°C.
  9. Spread 100 µl of the cells onto the plates.
  10. Incubate overnight at 37°C.

- *DNA*: PCR product purification is not necessary if the total volume of all PCR products in the Gibson Assembly reaction is 20% or less of the Gibson Assembly reaction volume. Higher volumes of PCR products may reduce the efficiency of Gibson Assembly and transformation due to the elevated carryover amounts of PCR reaction buffer and unused primers present in the PCR product. Column purification of PCR products may increase the efficiency of both Gibson Assembly and transformation by 2–10 fold and is highly recommended when performing assemblies of three or more PCR fragments or assembling longer than 5 kb fragments. Purified DNA for assembly can be dissolved in ddH_2O (Milli-Q® water or equivalent is preferable), TE or other dilution buffers.

- *Insert*: When directly assembling fragments into a cloning vector, the concentration of assembly fragments should be 2–3 times higher than the concentration of vector. **For assembly of 3 or more fragments, we recommend using equilmolar ratio of fragments.**

- *Biology*: Some DNA structures, including inverted and tandem repeats, are selected against by *E. coli*. Some recombinant proteins are not well tolerated by *E. coli* and can result in poor transformation or small colonies.

Frequently Asked Questions (FAQs):

What are the advantages of this method compared to traditional cloning methods?

Gibson Assembly allows insertion of one or more DNA fragments into virtually any position of the linearized vector and does not rely on the presence of restriction sites within a particular sequence to be synthesized or cloned. Therefore, the user has complete control over what is assembled and insertion of unwanted additional sequence, often used to facilitate the manipulation of multiple DNA sequences, can be avoided. Furthermore, the Gibson Assembly method is fast relative to standard restriction enzyme-based cloning. Lastly, a greater number of DNA fragments can be joined in a single reaction with greater efficiency than conventional methods.

How large of a DNA fragment can I assemble?

Gibson Assembly Master Mix has been used to clone a 15 kb DNA fragment into a 5.4 kb plasmid in *E. coli*, totaling up to 20.4 kb in length. For assembled products greater than 15 kb, NEB recommends NEB 10-beta Competent *E. coli* (High Efficiency, NEB #C3019) or NEB 10-beta Electrocompetent *E. coli* (NEB #C3020).

How many fragments of DNA can be assembled in one reaction?

The number of DNA segments that can be assembled in one reaction is dependent on the length and sequence of the fragments. Gibson Assembly has been used to efficiently assemble up to twelve 0.4 kb inserts into a vector at one time. However, we recommend the assembly of five or fewer inserts into a vector in one reaction in order to produce a clone with the correct insert. A strategy involving sequential assembly can be used if all of the fragments cannot be assembled in a single reaction.

Is this method applicable to the assembly of repetitive sequences?

Yes. However, one must ensure that each DNA fragment includes a unique overlap so that the sequences may anneal and are properly assembled. The repetitive sequence can also be internalized in the first stage of a two-stage assembly strategy. If having repetitive sequences at the ends of each fragment is unavoidable, the correct DNA assembly may be produced, albeit at lower efficiency than other, unintended assemblies.

What are the shortest overlaps that can be used with this assembly method?

Productive assembly has been shown for DNA fragments with as little as 12 bp overlap. However, it depends on the GC content of the overlap. We recommend using 16 bp overlaps or more for dsDNA assembly with a T_m > 48°C (AT pair = 2°C and GC pair = 4°C).

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

The quantity of exonuclease in the Gibson Assembly Master Mix has been optimized for the assembly of DNA molecules with ≤ 100-bp overlaps.

Can ≤ 200 bp dsDNA fragments be assembled by this method?

Yes. For optimal results, use these fragments in ≥ 5-fold excess.

Can ssDNA oligonucleotides be combined and assembled with dsDNA fragments?

Yes. However, the optimal concentration of each oligonucleotide should be determined. As a starting point, we recommend using 45 nM of each oligonucleotide that is less than or equal to twelve 60-base oligonucleotides containing 30-base overlaps.

Can longer or shorter incubation times be used?

Yes. For assembling 2–3 fragments, 15 minute incubation times are sufficient. For assembling 4–6 fragments, 60 minute incubation times are recommended. Reaction times less than 15 minutes are generally not recommended. Extended incubation times (up to 4 hours) have been shown to improve assembly efficiencies in some cases. Do not incubate the Gibson Assembly reaction overnight.

Will the reaction work at other temperatures?

The reaction has been optimized at 50°C, but it has been shown to work between 40°C and 50°C.

Is it necessary to purify PCR products?

Purification of PCR products is generally not necessary. You can use unpurified PCR products directly, as long as the total volume of PCR products in the Gibson Assembly reaction is 20% or less. If greater amounts of PCR products are used, a column cleanup kit is sufficient. It is advantageous to gel-purify the target DNA fragment if the PCR product is contaminated by either non-specific amplification products, primer-dimers or large quantities of unused PCR primers.

Is it necessary to inactivate restriction enzymes after vector digestion?

No, unless the insert also carries the restriction site that was used to linearize the vector. You may still linearize the vector with such restriction enzymes, but it is necessary to heat inactivate the restriction enzyme before mixing the linearized vector with the insert in Gibson Assembly. If a heat-resistant restriction enzyme was used to linearize the vector, then vector should be purified by DNA columns, phenol-chloroform extraction or extracted from agarose gel after electrophoresis, before coming into contact with the insert.

I would like to produce overlapping dsDNA fragments by PCR. Do I need to use PCR primers that have been purified by PAGE or HPLC?

No. Standard, desalted primers may be used.

I would like to assemble ssDNA oligonucleotides into dsDNA fragments. Do I need to use oligonucleotides that have been purified by PAGE or HPLC?

No. Standard, desalted primers may be used.

Can I use a 15-nt overlap that is entirely composed of His-tag repeats (i.e. CACCACCACCACCAC)?

No, after the His-tag, you must include at least 3 nucleotides, that are not part of the His-tag repeating sequence. Avoid repeating sequences at the end of an overlap.

Can you PCR-amplify the assembled product?

Yes. The assembled DNA molecule is covalently joined and may be PCR-amplified. Additionally, if the final product is a closed circular DNA molecule, it may be used as a template in rolling-circle amplification (RCA).

The Gibson Assembly Master Mix control reaction is not giving me any colonies. Why?

Our testing indicates that the choice of competent cells is critical. We recommend the use of high efficiency chemically competent cells such as NEB 5-alpha Competent *E. coli* (High Efficiency) (NEB #C2987). The reaction can be added directly to the cells without any dilution, although further dilution of the reaction mix may improve transformation efficiency. However when using high efficiency chemically competent cells from some other vendors, if you did not get any colonies, we recommend a 1:4 dilution of the reaction prior to transformation. For transformation into all high efficiency electrocompetent cells, including NEB's, we recommend a 1:3 dilution of the reaction.

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?

Assemble and transform the positive control provided with the Gibson Assembly Master Mix (see page 11,12). Successful assembly of a 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 disappearance of fragments.

Check the primer design of the overlapping DNA fragments to ensure that there is sufficient overlap to facilitate assembly.

Consider whether the cloned insert may be toxic to *E. coli* and a low-copy vector, such as a BAC, should be used. Because the assembled product is a covalently closed molecule, it may be alternatively amplified by PCR or RCA.

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

To significantly reduce the background of unwanted vector-only colonies, the vector should be a PCR product rather than a restriction fragment. If background continues to be a problem, the PCR-amplified vector can be treated with DpnI to remove the template carry-over, if applicable, extracted from an agarose gel following electrophoresis.

What type of competent cells are suitable for transformation of DNA constructs created using Gibson Assembly?

The resulting DNA constructs are compatible with most *E. coli* competent cells. NEB recommends using NEB 5-alpha Competent *E. coli* (High Efficiency, NEB #C2987). If the assembled products are larger than 10 kb, NEB recommends using NEB 10-beta Competent *E. coli* (High Efficiency, NEB #C3019) or NEB 10-beta Electrocompetent *E. coli* (NEB #C3020).

Can I use electroporation instead of chemical transformation?

Yes, but it is necessary to dilute the Gibson Assembly reaction product 3-fold, and use only 1 µl for electroporation.

Are there any differences between the Gibson Assembly Master Mix (NEB #E2611) and Gibson Assembly Master Mix included in the Gibson Assembly Cloning Kit (NEB #E5510)?

No, the master mix is the same in both kits. The Gibson Assembly Cloning Kit (NEB #E5510) includes NEB 5-alpha chemically competent *E. coli*.

Are there any differences between the requirements for 2–3 fragment assemblies versus 4–6?

The major differences between the two are the length of overlapping sequences between the adjacent fragments and the incubation time of the assembly reaction. The 15 minute assembly reaction protocol is recommended for assembly of 2–3 fragments that are flanked by 15–25 nt overlaps. The 1 hour assembly protocol is recommended for the assembly of up to 6 fragments, flanked by 20–80 nt overlaps. The total amount of DNA in a 4–6 fragment assembly is also higher than a 2–3 fragment assembly (see the chart on page 11).

Troubleshooting

Positive Control Yields No Colonies Following Transformation into *E. coli*

- Be sure to perform the positive control assembly reaction with 2X Gibson Master Mix, followed by transformation using competent cells from (NEB #C2987) as described on page 11,12. If competent cells are purchased from other manufacture, dilute assembled products 4 times with H_2O prior transformation. This can be achieved by mixing 5 µl of assembled products with 15 µl of H_2O. Add 2 µl of the diluted assembled product to competent cells.
- Competent cells may be thawed only once and cannot be repeatedly frozen and thawed without extreme loss in competency. Cells are best thawed on ice and DNA added as soon as the last bit of ice in the tube disappears.
- Do not vortex competent cells. Mix cells and DNA by gently pipetting up and down. Check cell competency by transforming 100 pg of pUC19 plasmid provided with the kit. Expect 1–3 x 10^9 colonies formed/µg DNA after overnight incubation on LB-ampicilin plates at 37°C.

Gibson Assembly Reaction Yields No Colonies Following Transformation into *E. coli*.

- Assemble and transform the positive control provided with the Gibson Assembly Cloning Kit. Successful assembly of a positive control will demonstrate that the Gibson Assembly Master Mix is functional and the transformation conditions are suitable.
- Check the primer design of the overlapping DNA fragments to ensure that there is sufficient and correct overlap to facilitate assembly.
- Avoid overlaps with highly palindromic sequences as they may cause up to a 10-fold reduction in recombinant colonies. When assembling fragments into a multiple cloning site (MCS) of a cloning vector, it is strongly recommended to use restriction sites that are located on the edges of the MCS to avoid overlap regions with highly palindromic sequences. Plate higher amounts of transformation reaction when using restriction sites that are located in the middle of the MCS of the cloning vector.
- Repeat the Gibson Assembly reaction using higher concentrations of fragments and/or vector. Make sure that the total volume of PCR-amplified products does not exceed 20% of Gibson Assembly reaction. If necessary, purify PCR fragments and/or PCR-amplified vector before the assembly reaction.
- Some DNA structures, including inverted and tandem repeats, are selected against by *E. coli*. Some recombinant proteins are not well tolerated by *E. coli* and can result in poor transformation.

- Test the success of the assembly by performing PCR with primers that flank the assembled product.
- Consider whether the cloned insert may be toxic to *E. coli*, and a low-copy vector, such as a BAC, should be used. Because the assembled product is a covalently-closed molecule, it may be alternatively amplified by PCR or rolling-circle amplification (RCA).

Gibson Assembly Reaction Yields High Number of Clones with Incorrect Inserts

- Make sure that your PCR product is a single band of the correct size. If the PCR product is contaminated with non-specific bands, it is necessary to gel-purify the PCR product to ensure cloning of the correct insert.
- Consider whether the cloned insert may be toxic to *E. coli* and a low-copy vector, such as a BAC, should be used. Because the assembled product is a covalently closed molecule, it may be alternatively amplified by PCR or RCA.

Gibson Assembly Reaction Yields High Number of Small Colonies

- Some recombinant proteins are not well-tolerated by *E. coli* and can result in poor transformation efficiency or small colonies. Use a low copy number vector (i.e. pACYC184) or a vector with tight control of protein expression. When assembling into the pUC19 vector, make sure that your gene is not in frame with *lacZ* alpha fragment.

Gibson Assembly Reaction Yields a High Number of Clones without the Insert

- PCR products may carry over large quantities of uncut plasmid template. To remove plasmid template, treat PCR products with DpnI restriction endonuclease before performing Gibson Assembly. Protocol for DpnI digestion can be found on page 10.
- Restriction enzyme digested vector may carry over large quantities of uncut plasmid. Some restriction enzymes do not cut supercoiled plasmids to completion. The best way to reduce uncut vector background is to digest the vector with two different restriction endonucleases. If a single enzyme must be used, avoid restriction enzymes that leave four-base single-stranded overhangs rich in C/G (i.e. CCGG overhang). These overhangs may self-anneal to form the transformable form of the vector molecule. Also, increase units and/or incubation time and/or purify the linear vector from agarose gel.

References:

1. Gibson, D.G. et.al. (2009) *Nature Methods,* 343–345.
2. Gibson, D.G. et al. (2010) *Nature Methods*, 901–903.
3. Barnes, W.M. (1994) *Proc. Natl. Acad. Sci.* 91, 2216–2220.

For additional references and information on Gibson Assembly Master Mix, visit www.sgidna.com. and www.jcvi.org.

Ordering Information

PRODUCT	NEB #	SIZE
Gibson Assembly Master Mix	E2611S/L	10/50 reactions
COMPANION PRODUCTS		
Gibson Assembly Cloning Kit	E5510S	10 reactions
Q5 High-Fidelity DNA Polymerase	M0491S/L	100/500 units
Q5 High-Fidelity 2X Master Mix	M0492S/L	100/500 reactions
Q5 Hot Start High-Fidelity DNA Polymerase	M0493S/L	100/500 units
Q5 Hot Start High-Fidelity 2X Master Mix	M0494S/L	100/500 reactions
NEB 5-alpha Competent *E. coli* (High Efficiency)	C2987I/H	6 x 0.2 ml/ 20 x 0.05 ml
NEB 10-beta Competent *E. coli* (High Efficiency)	C3019I/H	6 x 0.2 ml/ 20 x 0.05 ml
NEB 10-beta Electrocompetent *E. coli*	C3020K	6 x 0.1 ml
SOC Outgrowth Medium	B9020S	4 x 25 ml

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