Molecular cloning using the Gibson Assembly cloning kit (NEB E5510S)

Adapted from the NEB Gibson Assembly Instruction Manual, www.neb.com

Overview

Molecular cloning refers to the process by which recombinant DNA molecules are produced and transformed into a host organism, where they are replicated. A molecular cloning reaction is usually comprised of two components:

- The DNA fragment of interest to be replicated
- A vector/plasmid backbone that contains all the components for replication in the host

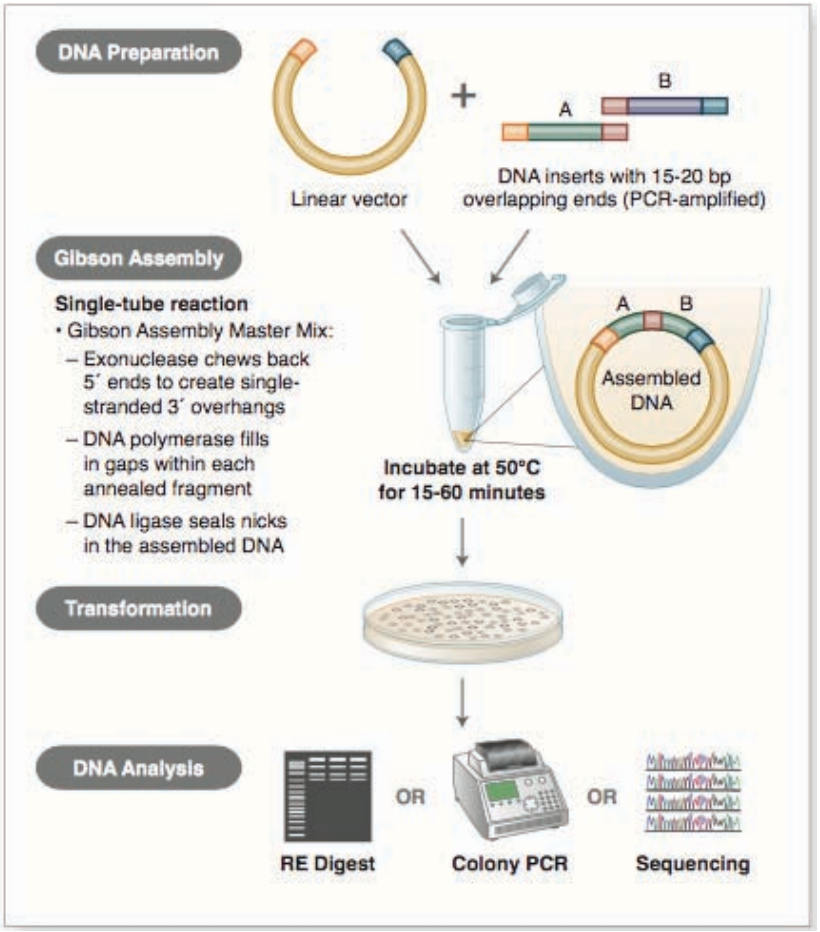
DNA of interest, such as a gene, regulatory element(s), operon, etc., is prepared for cloning by either excising it out of the source DNA using restriction enzymes, copying it using PCR, or assembling it from individual oligonucleotides. At the same time, a plasmid vector is prepared in a linear form using restriction enzymes or PCR. The plasmid is a small, circular piece of DNA that is replicated within the host and exists separately from the host's chromosomal or genomic DNA. By physically joining the DNA of interest to the plasmid vector through phosphodiester bonds, the DNA of interest becomes part of the new recombinant plasmid and is replicated by the host. Plasmid vectors allow the DNA of interest to be copied easily in large amounts, and often provide the necessary control elements to be used to direct transcription and translation of the cloned DNA. As such, they have become the workhorse for many molecular methods such as protein expression, gene expression studies, and functional analysis of biomolecules.

During the cloning process, the ends of the DNA of interest and the vector have to be modified to make them compatible for joining through the action of a DNA ligase, recombinase, or an *in vivo* DNA repair mechanism. These steps typically utilize enzymes such as nucleases, phosphatases, kinases and/or ligases. Many cloning methodologies and, more recently kits have been developed to simplify and standardize these processes.

The Gibson Assembly method, originally described by Daniel G. Gibson, of the J. Craig Venter Institute, is an exonuclease-based method performed under isothermal conditions after linear insert and vector have been prepared by PCR and/ or restriction digestion. Three enzymatic activities are employed: a 5´ exonuclease generates terminal cohesive ends (overhangs), a polymerase fills in the gaps of the annealed single-stranded regions, and a DNA ligase seals the nicks. The assembled, fully-sealed construct is then transformed into NEB 5-alpha competent *E. coli*. The entire protocol, from assembly to transformation, takes just under two hours.

The Gibson Assembly method has several advantages compared to traditional cloning methods. It 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.

Figure 1. Overview of Gibson Assembly cloning method



Protocol

A. Primer design for PCR amplification of inserts

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

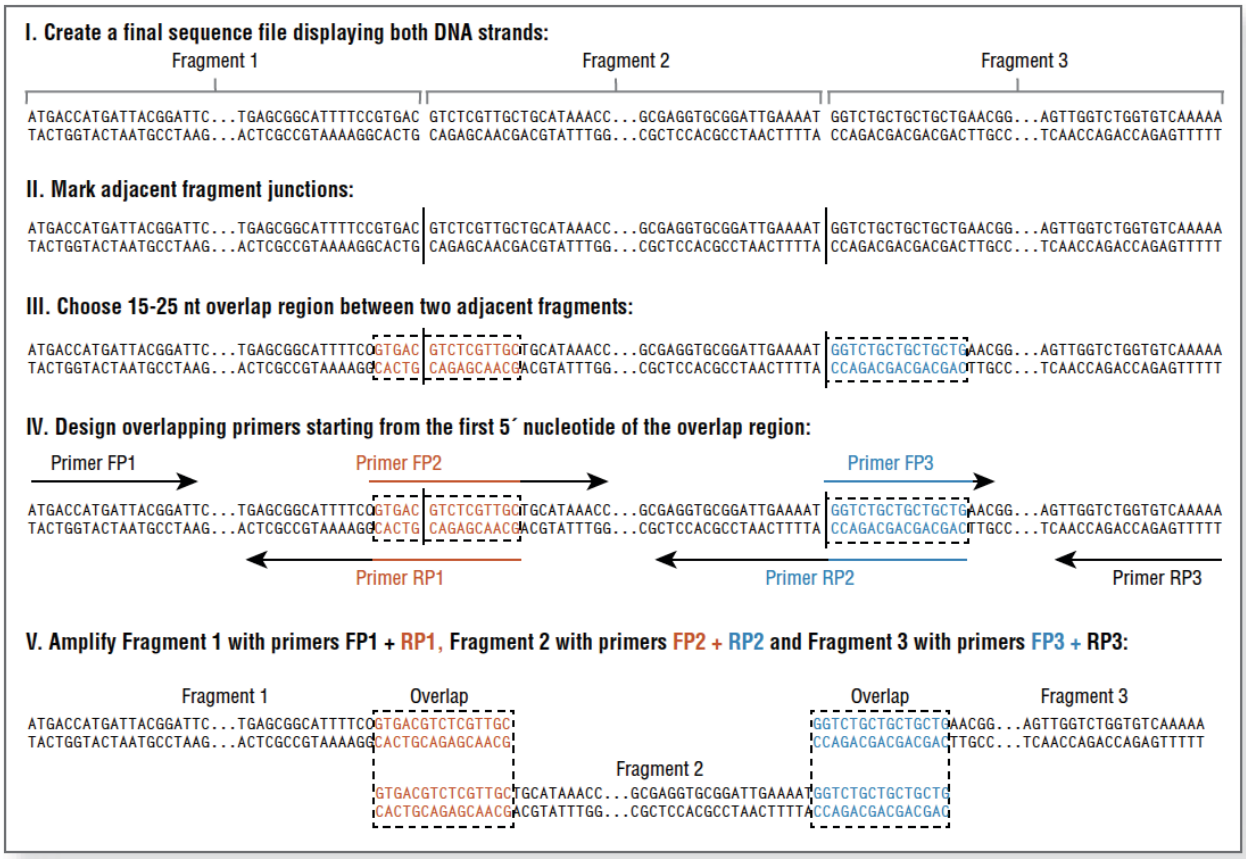
• an overlap sequence, required for the assembly of adjacent fragments;
• a gene-specific sequence, required for template priming during PCR amplification;

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:

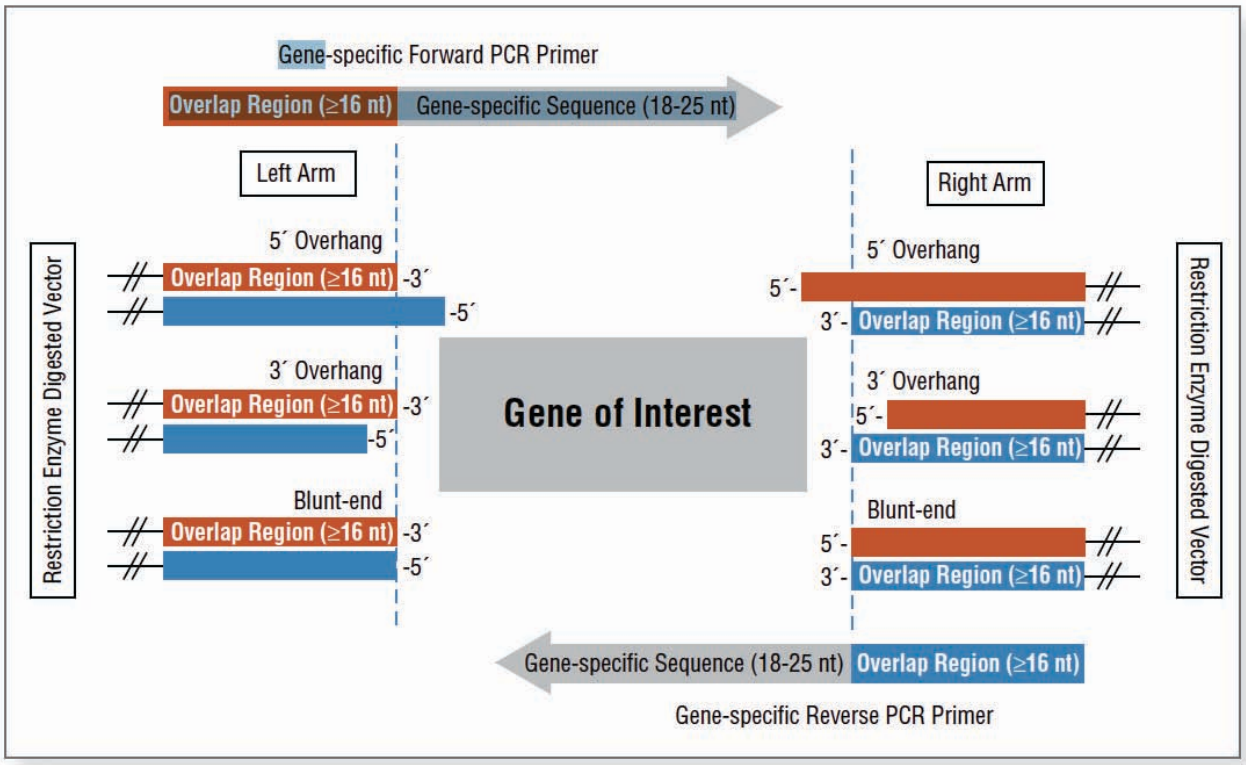
To achieve efficient assembly of PCR fragments into a vector, we suggest using a 15–25 nt overlap with a Tm equal to or greater than 48°C (assuming A-T pair = 2°C and G-C pair = 4°C). To prevent errors in primer design it is highly recommended to first perform DNA fragment assembly *in silico* and create 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. 2, 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. 2, 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. 2, 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. 2, step V).

Figure 2A. *In silico* primer design using DNA sequence file.



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.3, 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. 3, Right side).

Figure 3. Assembly of restriction enzyme-digested vector and PCR-derived insert



B. PCR amplification of fragments for Gibson Assembly

High Fidelity DNA Polymerases are important for applications in which the DNA sequence needs to be correct after amplification. Phusion High-Fidelity DNA Polymerase offers both high fidelity and robust performance, and thus can be used for all PCR applications. Its unique structure, a novel *Pyrococcus*-like enzyme fused with a processivity-enhancing domain, increases fidelity and speed. Phusion DNA Polymerase is an ideal choice for cloning and can be used for long or difficult amplicons. In addition, the use of this high-fidelity DNA polymerase yields PCR products with blunt ends, thereby reducing the error rates at the fragment junctions.

The following guidelines are provided to ensure successful PCR using Phusion Master Mixes. These guidelines cover routine PCR reactions. Amplification of templates with high GC content, high secondary structure, low template concentrations or long amplicons may require further optimization.

Reaction Setup:

Component	20 µl Reaction	50 µl Reaction	Final Concentration
Nuclease-free water	to 20 µl	to 50 µl	-
5X Phusion HF Buffer	4 µl	10 µl	1 X
10 mM dNTPs	0.4 µl	1.0 µl	200 µl
10 µM Forward Primer	1 µl	2.5 µl	0.5 µM
10 µM Reverse Primer	1 µl	2.5 µl	0.5 µM
Template DNA	Variable*	Variable*	0.1-0.5 ng plasmid /50 µl PCR
DMSO (optional)	(0.6 µl)	(1.5 µl)	3%
Phusion DNA polymerase	0.2 µl	0.5 µl	1 U/ 50 µl PCR

Procedure:

1. Calculate how much master mix is needed based on the number of PCR reactions.

2. Thaw, mix and centrifuge all components prior to use.

3. Assemble all the reaction components on ice.

4. Add Phusion DNA polymerase last in order to prevent any primer degradation caused by the 3´→ 5´ exonuclease activity.

5. Gently mix the reaction. Collect all liquid to the bottom of the tube by a quick spin if necessary.

6. Quickly transfer the final mix to a thermocycler preheated to the denaturation temperature (98°C) and begin thermocycling.

7. 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.

*Note: 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).

Thermocycling conditions for a routine PCR using Phusion DNA polymerase:

1. initial denaturation at 98°C for 30 seconds

2. followed by 34 cycles of:

    a) denaturation at 98°C for 10 seconds

    b) annealing at 45-65°C for 20 seconds (depends on primers Tm)

    c) extension at 72°C for 30 seconds per kb

3. final extension at 72°C for 10 minutes

4. Hold at 12°C

C. (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 restriction endonuclease in order to destroy plasmid template before setting up the Gibson Assembly reaction. DpnI cleaves only *E. coli* Dam methylasemethylated 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.

D. 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 pmol

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:

Component	**Amount per reaction**
PCR Fragment	Variable
Linearized vector	Variable (50 ng)
Gibson assembly Master Mix (2X)	10 µl
H2O	To 20 µl

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 at –20°C or proceed with transformation.

E. Transformation of chemically competent cells

Note: Be extremely gentle when working with competent cells. Competent cells are highly sensitive to changes in temperature or mechanical lysis caused by pipetting. Transformation should be started immediately following the thawing of the cells on ice. **Mix by swirling or tapping the tube gently, not by pipetting or vortexing.**

Protocol:

1. Thaw chemically competent cells on ice.

2. Add 2 µl of assembled product to NEB competent cells. Mix gently by pipetting up and down or flicking the tube 4–5 times. Do not vortex.

3. To determine the transformation efficiency, add 5 µl (50 pg) pUC19 control DNA to one tube containing 50 µl competent cells. Move the pipette through the cells while dispensing. Gently tap tube to mix.

4. 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.

12. The next day, select colonies and analyze by plasmid isolation, PCR, or sequencing.

Calculating transformation efficiency

Count the number of colonies in the LB plate of cells transformed with pUC19. Calculate transformation efficiency using the formula below. This control enables you to be confident of the quality of the cells, and whether the technique was performed correctly (ie., was the heat shock too prolonged).

Calculating Transformation Efficiency

Transformation efficiency (# transformants/µg DNA) =

$$\frac{\text{\# of colonies}}{\text{pg pUC19 DNA}} \times \frac{10^6 \text{ pg}}{\mu\text{g}} \times \frac{\text{volume of transformants}}{\text{X µl plated}} \times \frac{\text{dilution}}{\text{factor}}$$

For example, if transformation of 250 pg of pUC19 DNA yields 100 colonies when 100 µl of the transformation is plated, then the transformation efficiency is:

$$\frac{100 \text{ colonies}}{250 \text{ pg DNA}} \times \frac{10^6 \text{ pg}}{\mu\text{g}} \times \frac{1000 \text{ µl}}{100 \text{ µl plated}} \times 1 = 4.0 \times 10^6$$

Expected transformation efficiency: >1 x 106 cfu/µg supercoiled pUC19 plasmid

Note: Transformation efficiencies for cDNA and ligation of inserts to vectors will be lower than for a supercoiled control plasmid such as pUC19.

• For cDNA, transformation efficiencies may be 10- to 100-fold lower.

• For ligation of inserts to vectors, transformation efficiencies may be 10-fold lower.

Additional controls:

In addition to controlling for the health of the competent cells and the proper performance of the technique, there are additional controls that should be done in order to solve any problems that might occur.

1. If you are transforming with a ligation reaction, you should include a second ligation reaction

of the cut vector alone (without insert). This "negative" control is especially important if the vector is cut with only one enzyme (probability of ligation between ends of vector), as well as to control for contamination of preparations of isolated linearized vector with circular vector. The number of colonies resulting from a transformation with this control is the background – transformation with the ligated vector-insert combination should produce more colonies than the background.

2. Another negative control to include is simply to "transform" the competent cells without plasmid DNA or ligation reaction. All the steps of the procedure are carried out and the cells are plated on the agar containing selective antibiotic. In this case, there should not be any colonies unless the cells are "contaminated" with a plasmid that confers resistance.

Recipes

Preparation of agar plates

LB agar (2%) agar can be obtained from the kitchen. Pour plates at least 1 day before required (they need time to dry).

1. Use the microwave to melt agar.

2. Allow it to cool until hand warm/hot, about 55°C (agar should still be molten!).

3. Add the appropriate antibiotic (100 µg/ml ampicillin or 50 µg/ml kanamycin).

4. Pour into petri dishes (~25 ml / 100 mm plate). Leave to dry 1 day.

5. Store upside down in the bags the plates came in at 4°C. Always label the bags with the antibiotic included and the date of preparation. Use within a month.

SOC Recipe (per one liter)

2% tryptone
0.5% yeast extract
10 mM sodium chloride
2.5 mM potassium chloride
10 mM magnesium chloride
10 mM magnesium sulfate
20 mM glucose

SOB medium is SOC medium without the addition of glucose. SOB medium and sterile glucose solutions are available from the kitchen.

MIT OpenCourseWare
http://ocw.mit.edu

7.15 Experimental Molecular Genetics

Spring 2015

For information about citing these materials or our Terms of Use, visit: http://ocw.mit.edu/terms.