Improved methods for site-directed mutagenesis using Gibson Assembly™ Master Mix

Introduction

Site-directed mutagenesis (SDM) is a commonly-used technique for introducing mutations into a gene of interest. Existing techniques for SDM, such as whole plasmid SDM, while effective, are time consuming and prone to off-target mutation incorporation. Further, verification of mutation incorporation can be difficult when the insertion site sequence lacks convenient restriction sites for analysis. This can be a serious impediment to the planning and execution of SDM experiments.

Gibson Assembly, developed by Daniel Gibson and his colleagues at JCVI, is a rapid and reliable method for the assembly of DNA fragments. The technique, which involves the design of complimentary flanking primers to align fragments, can be readily adapted for SDM applications. In addition, it is unnecessary to use phosphorylated primers for Gibson Assembly, reducing both cost and time. In one step, two or more PCR products with overlapping ends can be assembled into a pre-cut vector. An exonuclease creates single-stranded 3´ overhangs that promote annealing of complementary fragments at the overlap region. A polymerase then fills in the gaps which are sealed by the DNA ligase. By introducing multiple complementary mutations in the primers at the overlap region, the Gibson Assembly Master Mix forms a single, covalently bonded DNA molecule, containing the desired mutations, that is able to be directly transformed into competent cells and screened or sequenced.

Here we describe the use of the Gibson Assembly Master Mix in two different mutagenesis experiments:

A. Multiple mutations of the the *lacZ* gene (Figure 1)

B. The mutation of 5 of the 6 nucleotides at position 174–179 of eGFP from CTGACC to TTCTAT in order to change the amino acid sequence from LeuThr to PheTyr (Figure 2)

Materials

- Gibson Assembly Master Mix
- Q5™ Hot Start High-Fidelity DNA Polymerase
- Phusion® High-Fidelity PCR Master Mix with GC Buffer
- GFP plasmid
- NcoI and XhoI
- NEBuffer 4 (10X)
- Deoxynucleotide Solution Mix
- Overlapping primers
- *lacZ* in pET21a
- DpnI
- NEB 5-alpha Competent *E. coli*
- LB-Amp plates
- T7 Express *I*^{q} Competent *E. coli*

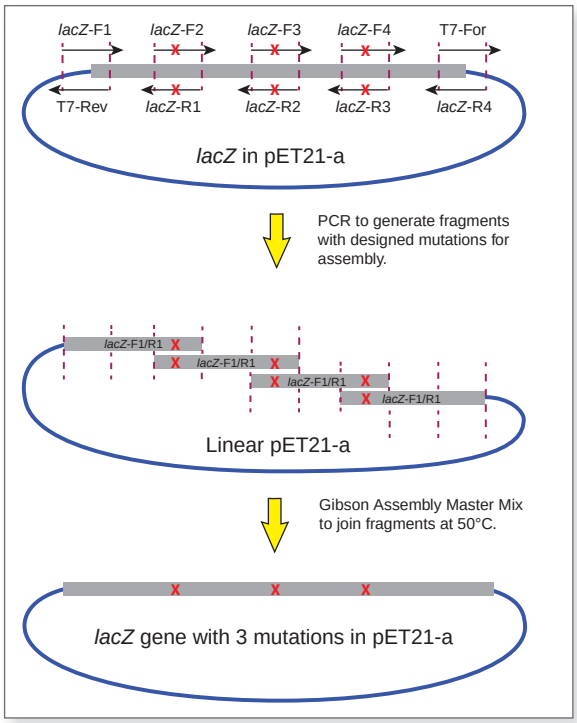
NEBuilder™ for Gibson Assembly

Protocol

IA. Experimental Design – Single bases at multiple sites

In this experiment, multiple primers were designed to incorporate 3 mutations within the gene (Table 1, Note: For help with primer design, we recommend using the NEBuilder primer design tool at NEBGibson.com). The resulting amplicons contained 24 bp overlaps and the desired mutations (Figure 1). The PCR products and linearized vector are treated with the Gibson Assembly Master Mix, and the resulting transformants are screened for mutations by sequencing.

Figure 1. Site-directed mutagenesis of *lacZ* using Gibson Assembly.



In this example, multiple mutations are introduced into the lacZ gene through overlapping primers followed by PCR. Gibson Assembly is then used to join the fragment with linearized vector.

Table 1. Overlapping primers used for SDM of eGFP*

PRIMER	SEQUENCE 5´→ 3´
lacZ-F1	TTTAAGAAGGAGATATACATATGACCATGATTACGGATTC*
lacZ-R1	CACATCTGGAATTCAGCCTCCAGTACAGC**
lacZ-F2	AGGCTGAATTCCAGATGTGCGGCGAGTT
lacZ-R2	GGCCTGATGAATTCCCCAGCGACCAGAT
lacZ-F3	CTGGGGAATTCATCGCCCACGGCGC
lacZ-R3	ACACTGAGGAATTCCGCCAGACGCCA
lacZ-F4	TGGCGGAATTCCTCAGTGTGACGCTCCC
lacZ-R4	TTTGTTAGCAGCCGGATCTCATTTTTGACACCAGACCAACT
T7-For	TGAGATCCGGCTGCTAACAAAG
T7-Rev	ATGTATATCTCCTTCTTAAAGTTAAACAAAAT

*Red indicates changed nucleotides.

IIA. Fragment Preparation:

1. The following reaction conditions were used to amplify fragments with designed mutations using the following primer pairs: *lacZ*-F1/R1, *lacZ*-F2/R2, *lacZ*-F3/R3, *lacZ*-F4/R4 and T7-For/T7-Rev.

COMPONENT	VOLUME (µl)
Forward primer (10 µM)	2.5
Reverse primer (10 µM)	2.5
lacZ in pET21a (5 ng/µl)	1.0
ddH_2O	19.0
Phusion High-Fidelity PCR Master Mix with GC Buffer	25.0
Total Volume	50.0

STEP	TEMP	TIME
Initial denaturation	98°C	1 minute
30 Cycles	98°C	10 seconds
	55°C	15 seconds
	72°C	40 sec. or 3 min
Final extension	72°C	5 minutes
Hold	4°C	∞

2. Following PCR, 1 µl of DpnI was added to each tube and incubated at 37°C for an additional 30 minutes.
3. After DpnI treatment, all products were cleaned up using Qiagen QIAquick™ PCR purification columns.

IIIA. Fragment Assembly:

1. Concentration of the fragments was determined by Nanodrop™ instrument or estimated by agarose gel electrophoresis.
2. The 2X Gibson Assembly Master Mix was thawed at room temperature.
3. Gibson Assembly reaction was set up as follows:

COMPONENT	AMOUNT
Vector	0.05 pmols
PCR products (for each fragment)	0.15 pmols
2X Gibson Assembly Master Mix	10 µl
H_2O	10-x µl
Total volume	20 µl

x = total volume of fragments (including vector)

4. The reaction was incubated at 50°C in a thermocycler for 1 hour.
5. 2 µl of the reaction was transformed into 50 µl of NEB 5-alpha Competent *E. coli* (High Efficiency), plated on LB-Amp plates and incubated overnight at 37°C.

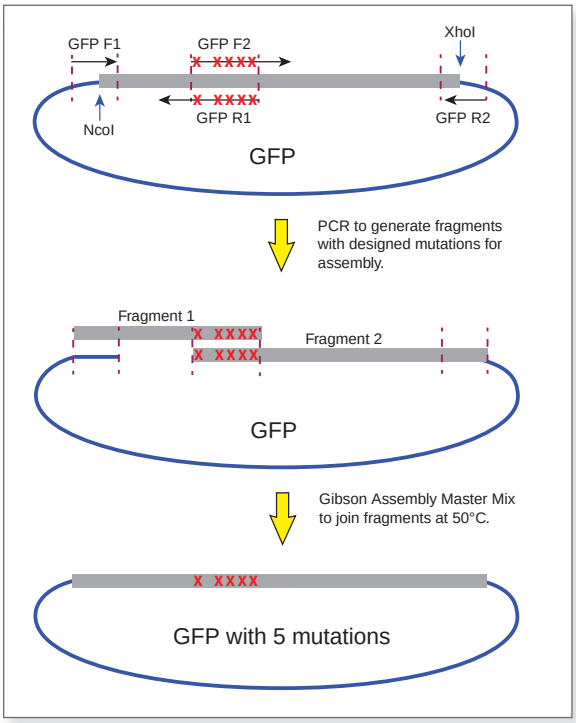
IVA. Results:

Transformation resulted in several thousand colonies. Ten colonies were screened by sequencing, 8 of which contained the desired mutations.

IB. Experimental Design – Multiple bases at a single site:

For this experiment, two overlapping primer sets were designed to incorporate appropriate mutations to alter the two amino acid residues (Table 2, Note: For help with primer design, we recommend using the NEBuilder primer design tool at NEBGibson.com). After amplification, the two resulting amplicons (210 and 528 bp) will overlap by 24 bp and contain the desired mutations (Figure 2). The 5′ end of the first amplicon and the 3′ end of the second amplicon overlap with the vector sequence, which has been digested with NcoI and XhoI. No gel purification is necessary, as the Gibson Assembly Master Mix will not religate the linearized vector. After amplification, the PCR products and linearized vector are treated with the Gibson Assembly Master Mix, and the resulting transformants are screened by sequencing.

Figure 2. Site directed mutagenesis of eGFP using Gibson Assembly



In this example, 5 nucelotides were changed by introducing the mutations into the overlapping primer, followed by PCR. Gibson Assembly is then used to join the fragments with enzyme-digested vector.

Table 2. Overlapping primers used for SDM of eGFP*

PRIMER	SEQUENCE 5′→ 3′
GFP-F1	TTAAGAAGGAGATATACCATGGAGCTTTTCACT
GFP-R1	CACGCCGTAATAGAAGGTGGTCACGAGGGTGGG
GFP-F2	GTGACCACCTTCTATTACGGCGTGCAGTGCTTC
GFP-R2	GATCTCCTAAGGCTCGAGTTAGATCCCGGCGGCGGTCAC

**red indicates changed nucleotides*

IIB. Fragment Preparation:

1. The following reaction conditions were used to amplify the two fragments which contain the desired mutations in the overlapping region:

COMPONENT	VOLUME (µl)
Forward primer (10 µM)	2.5
Reverse primer (10 µM)	2.5
Template (GFP plasmid, 1ng)	1.0
dNTPs (10 mM)	1.0
Q5 5X Buffer	10.0
Q5 Hot Start High-Fidelity DNA Polymerase	0.5
H_2O	32.5
Total	50.0

STEP	TEMP	TIME
Initial denaturation	98°C	30 seconds
30 Cycles	98°C	10 seconds
	70°C	10 seconds
	72°C	20 seconds
Final extension	72°C	2 minutes
Hold	4°C	∞

2. After the PCR was completed, all products were cleaned up using Qiagen QIAquick™ PCR purification columns.

3. Vector was digested as follows:

COMPONENT	VOLUME (µl)
2 µg GFP Plasmid	2.0
10X NEBuffer 4	3.0
100X BSA	0.3
NcoI	1.0
XhoI	1.0
H_2O	22.7
Total Volume	30.0

4. Reactions were incubated at 37°C for 2 hours.

5. Restriction endonucleases were inactivated by incubation at 65°C for 20 minutes.

IIIB. Fragment Assembly:

1. Concentration of fragments was estimated by agarose gel electrophoresis.

2. The 2X Gibson Assembly Master Mix was thawed at room temperature.

3. Gibson Assembly reaction was set up as follows:

COMPONENT	AMOUNT
Vector	0.05 pmols
PCR products (for each fragment)	0.15 pmols
2X Gibson Assembly Master Mix	10 µl
H_2O	10-x µl
Total volume	20 µl

x = total volume of fragments (including vector)

4. The reaction was incubated at 50°C in a thermocycler for 1 hour.

5. 2 µl of the reaction was transformed into 50 µl of T7 Express I^q Competent *E. coli* (High Efficiency, NEB# C3016), plated on LB-Amp plates and incubated overnight at 37°C.

6. 10 colonies were screened by sequencing.

IVB. Results:

Transformation resulted in several thousand colonies. Ten colonies were screened by sequencing, 5 of which contained the desired sequence changes.

Summary

These results demonstrate the versatility of the Gibson Assembly Master Mix in both single and multiple site-directed mutagenesis. In the first example, three mutations were introduced into the *lacZ* gene simultaneously. Four overlapping PCR amplicons were assembled with a linearized vector in one step. Resulting colonies were screened by sequencing, 80% of which contained the desired mutations. This represents a substantial improvement upon earlier methods of multi-site mutagenesis. Whereas previously, one may have had to create mutations sequentially, a significant increase in the length of the experiment, SDM using Gibson Assembly can be done in one step, and in much less time. In the second example, the Gibson Assembly Master Mix was used to successfully incorporate 5 single base changes within a six base sequence of the *GFP* gene. Two PCR amplicons, overlapping at the location of the altered bases, were assembled with a restriction enzyme-cleaved vector in one step. The resulting colonies were screened by sequence analysis, which demonstrated that 50% of the colonies contained the desired sequence change. In both cases, the Gibson Assembly Master Mix represents a substantial improvement over traditional methods, specifically in time savings, ease-of-use and cost.

Ordering Information

PRODUCT	NEB #	SIZE	PRICE
Gibson Assembly Master Mix	E2611S/L	10/50 reactions	$150/$600
COMPANION PRODUCTS			
Gibson Assembly Cloning Kit	E5510S	10 reactions	$180
Q5 Hot Start High-Fidelity DNA Polymerase	M0493S/L	100/500 units	$125/$500
NEB 5-alpha Competent *E. coli* (High Efficiency)	C2987I/H	6 x 0.2 ml/ 20 x 0.05 ml	$138/$176
Deoxynucleotide Solution Mix	N0447S/L	8/40 µmol of each	$54/$216
Phusion® High-Fidelity PCR Master Mix w/GC Buffer	M0532S/L	100/500 rxns	$167/$668
DpnI	R0176S/L	1,000/5,000 units	$59/$236
XhoI	R0146S/L/M	5,000/25,000/ 25,000 units	$64/$256/$256
NcoI	R0193S/L/T/M	1,000/5,000/ 1,000/5,000 units	$57/$228/ $57/$228