SYNTHETIC GENOMICS®

Gibson Assembly® Site-Directed Mutagenesis Kit

Instructions

Catalog Numbers GA2100-S and GA2100-10

REV. 1.0 08.10.15
Part number 40016

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Gibson Assembly®: US Patent Nos. 7,776,532 and 8,435,736.

Table of Contents

Kit Information

Gibson Assembly® Site-Directed Mutagenesis Kit Contents

Component	Quantity	Cat. GA2100-S (5 Reactions)	Cat. GA2100-10 (10 Reactions)	Storage Temperature
		Volume		
GA SDM PCR Mix (2X)	1 each	315 µL	625 µL	−20°C
GA SDM Assembly Mix A (2X)		25 µL	50 µL	
GA SDM Assembly Mix B (2X)		50 µL	100 µL	
Control Plasmid* (5 ng/µL)		5 µL	5 µL	
Control Primer Mix A (25 µM)		5 µL	5 µL	
Control Primer Mix B (25 µM)		5 µL	5 µL	
GA SDM Quick Reference Manual				

*Sufficient Positive Control is included for 2 control reactions per kit.

Gibson Assembly® Site-Directed Mutagenesis Kit Reaction Components

The Gibson Assembly® Site-Directed Mutagenesis (GA SDM) Kit contains the following components to facilitate site-directed mutagenesis:

Step	Component	Reaction
1	GA SDM PCR Mix (2X)	Generates amplicon fragments, incorporating mutation(s) through PCR amplification
2	GA SDM Assembly Mix A (2X)	Enzymatic assembly of SDM fragments
3	GA SDM Assembly Mix B (2X)	

Positive Control

The positive control DNA supplied with this kit is sufficient for 2 reactions.

The 2.8 kb positive Control Plasmid (at 5 ng/µL) contains an ampicillin resistance gene (*amp*^{R}) and a mutated *lacZ* gene, which allows for blue/white screening of colonies on LB agar plates containing 100 µg/mL ampicillin, 0.1 mM IPTG, and 40 µg/mL X-Gal. Performing site-directed mutagenesis of the Control Plasmid with Control Primer Mixes A and B restores the functional *lacZ* sequence. Following assembly and transformation, colonies containing the revertant positive control are blue.

Additional Required Materials

- Thermocycler
- Luria-Bertani (LB) plates with appropriate antibiotic
- SOC Outgrowth Medium
- High efficiency electrocompetent cells

  Recommended: TransforMax™ EPI300™ Electrocompetent *E. coli*
- Gene Pulser®/MicroPulser™ Cuvettes with a 0.1 cm Gap Width
- Gene Pulser® Xcell Microbial System

Additional Optional Materials

- QIAquick PCR purification Kit
- Spectrophotometer

Overview

Introduction

The Gibson Assembly® Method is a well-established assembly reaction that can be leveraged to join multiple, mutagenized DNA fragments with overlapping ends. DNA fragments of various lengths are uniformly assembled using complementary overlaps between fragments. The inherent flexibility of this approach lends itself to small and large constructs and encompasses both single and multiple insert assemblies. The resulting products may be used for a variety of downstream applications including protein engineering, error-free cloning, and gene editing (SNP corrections, insertion/deletion or tag insertion).

Key Features

Key Features of the Gibson Assembly® Site Directed Mutagenesis Kit
• Accurate • Seamless and scarfree cloning • Efficient • Incorporate multiple mutation sites in a single round • Accommodates large insertions (up to 40 nucleotides) per single round of mutagenesis • Accommodates large deletions (no size limitation) • Optimal for 1–5 inserts • Suitable for fragments ranging from 500 bp – 5.5 kb

Synopsis

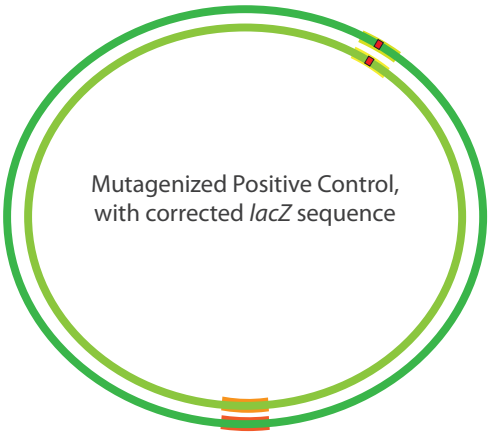
Site-Directed Mutagenesis of the Positive Control

The Control Plasmid contains a single base pair mutation in the *lacZ* gene. Site-directed mutagenesis of the Control Plasmid with the Control Primer Mixes restores the correct sequence in the *lacZ* gene and yields two linear fragments with homologous overlapping ends.

Gel Purify the PCR product(s) or treat the PCR product with DpnI

Perform assembly of the mutagenized positive control. Homologous overlapping ends, denoted in yellow and orange, are required for proper assembly. Red denotes the single nucleotide correction in the *lacZ* gene.

Gibson Assembly® Site-Directed Mutagenesis yields a mutagenized GA SDM Positive Control.



Mutagenesis Primer Characteristics

Considerations

- Proper design of primers for mutagenesis is critically important for the success of the reaction.
- Each primer should be at least 40–60 nucleotides (nt) long, with overlap regions that are at least 30 bp long.
- The length of the homologous overlap sequence is dependent on the GC content at the junction and the length of fragment. See the following sections for detailed instructions.

Homologous Overlap Regions

- The optimal length of the overlap region depends on the number and length of the fragments in the assembly reaction.

Recommended Length of the Overlap Region

Number of fragments	Length of overlap region
1–2	30–40 bp
3–5	40 bp

- For higher order assembly, longer overlap regions result in higher efficiency reactions.
- You may need to optimize PCR amplification reactions when using PCR primers with long homologous overlap regions.

Mutagenesis Primer Design

1. Optimally, design primers for site-directed mutagenesis such that the sequence change resides in the middle of the 40 nt homologous region.
2. Create a file containing the putative final product by cutting and pasting the source DNA sequences into your new file. Make the intended sequence change(s) within the file and annotate the sequence to identify junctions and sequence changes.
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 next sections.

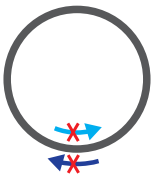
Note: Confirm that the termini of your SDM PCR products do not contain stable single stranded DNA secondary structure, such as a hairpin, stem loops, or repeated sequences, which would directly compete and interfere with the single-stranded annealing and priming of neighboring assembly fragments. Most primers will contain some hairpin secondary structure, but in general, make certain that any hairpin with a calculated T_m° > 30°C is > 5 bp from the 3′ terminus of the primer.

Mutagenesis Primer Design

Single Site Mutations (less than 5 bp)

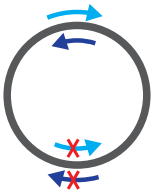
Single Site Mutation Primer Design Key Points
Design complementary 40-mer primers
Center the intended mutation(s) within the complementary primers

Single Site Mutation of a Circular Plasmid (<5.5 kb)



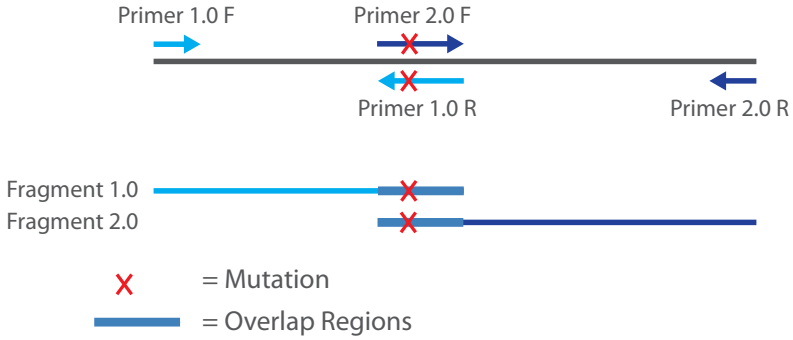
For single-site substitutions (SNPs), deletions, and insertions ≤4 nucleotides, design a single set of complementary 40-mer primers that include the intended mutation centered within the primers as depicted in the illustration above.

Single Site Mutation of a Circular Plasmid (>5.5 kb)



For templates >5.5 kb, design additional primers to generate subfragments, such that each subfragment will be less than 5.5 kb.

Single Site Mutation of Linear Template

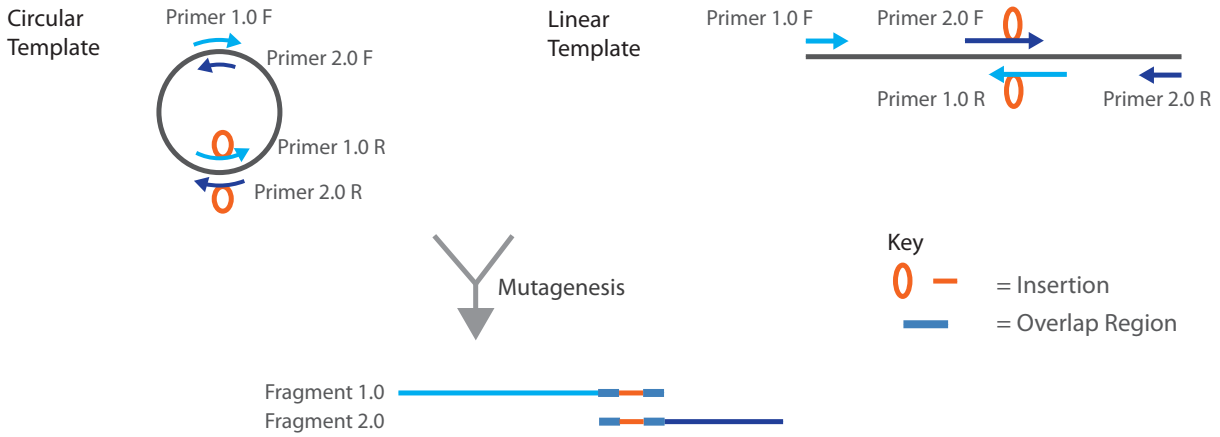


Linear templates require at least two primer pairs. In this scenario, you will set up the following 2 PCR mutagenesis reactions:

Reaction	Primer Pair
1	Primer 1.0 F + Primer 1.0 R
2	Primer 2.0 F + Primer 2.0 R

Single Site Mutations (greater than 4 bp)

Single Site Mutation (greater than 4 bp) Primer Design Key Points
Design overlapping 60-mer primers
Design each 60-mer primer with 20 nt of gene specific sequence for primer binding and 40 nt of homologous overlap sequence for assembly
Include substitutions or insertions up to 40 nt in the homologous overlap sequence



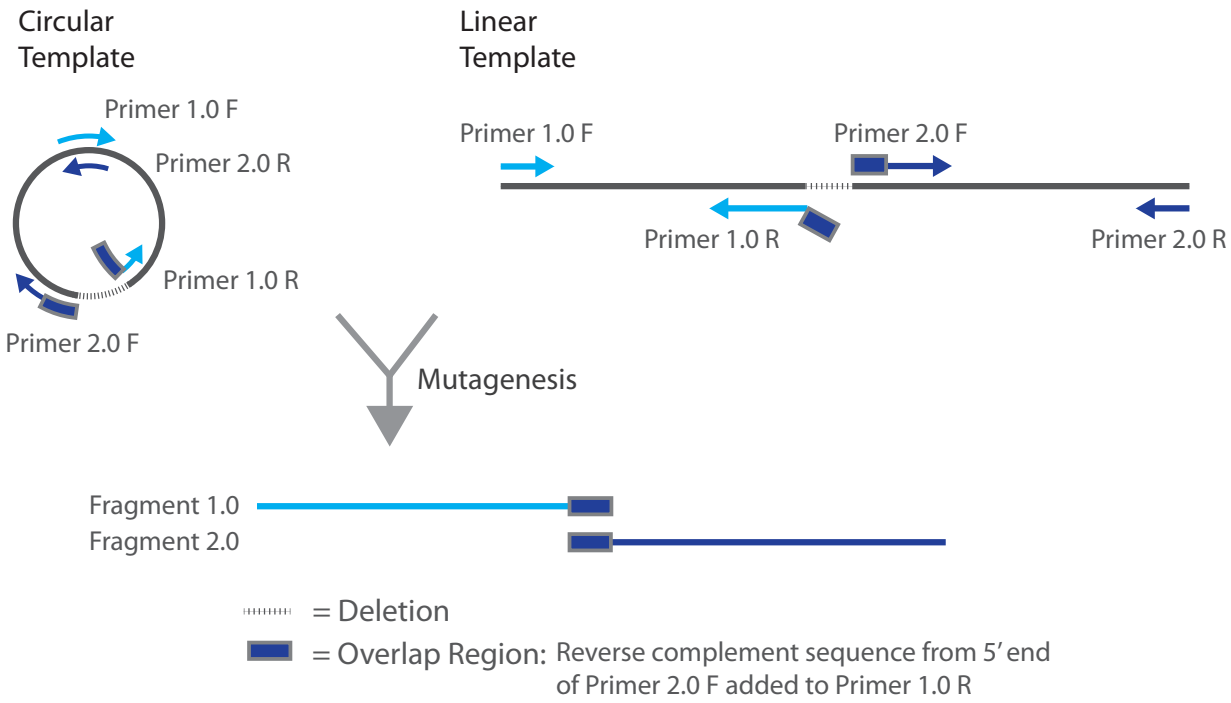
For single-site SNPs and insertions from 5–40 bp long, use the following guidelines:

- Circular templates less than 5.5 kb may be mutagenized with a single set of primers.
- For circular templates >5.5 kb, design additional primers to generate subfragments such that each subfragment will be less than 5.5 kb.
- Linear templates require at least two primer pairs.
- Design overlapping 60-mer primers which you will use to amplify template in separate PCR mutagenesis reactions (see the illustration above)
- The complementary primers may be staggered, but be certain to include a 20 nt primer binding sequence on the 3' end of each primer

Note: To introduce mutations > 40 bp long, design multi-stage mutagenesis with multiple rounds of mutagenesis.

Large Deletions (greater than 4 bp)

Large Deletions: Primer Design Key Points
Design 60-mer primers flanking the intended deletion
Design each 60-mer with 20 nt of gene specific sequence for primer binding and 40 nt of homologous overlap sequence for assembly



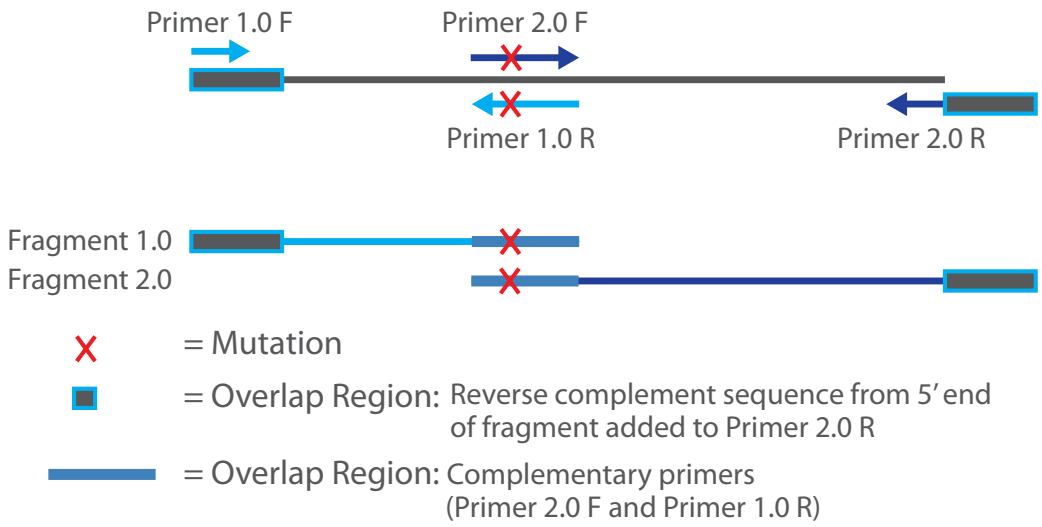
For deletions >4 nucleotides, create a homologous overlap region by adding 40 nt of the 5' terminal Primer 2.0 F reverse complement sequence to the 5' end of Primer 1.0 R as depicted in the illustration above.

Circularizing SDM Fragments

Circularizing Fragments: Primer Design Key Points
Design SDM primers according to the guidelines for your particular mutation of interest as outlined in the preceding sections
Add homologous overlap sequence to the reverse terminal primer.

To circularize SDM fragment(s) generated from linear templates, design the following terminal primers:

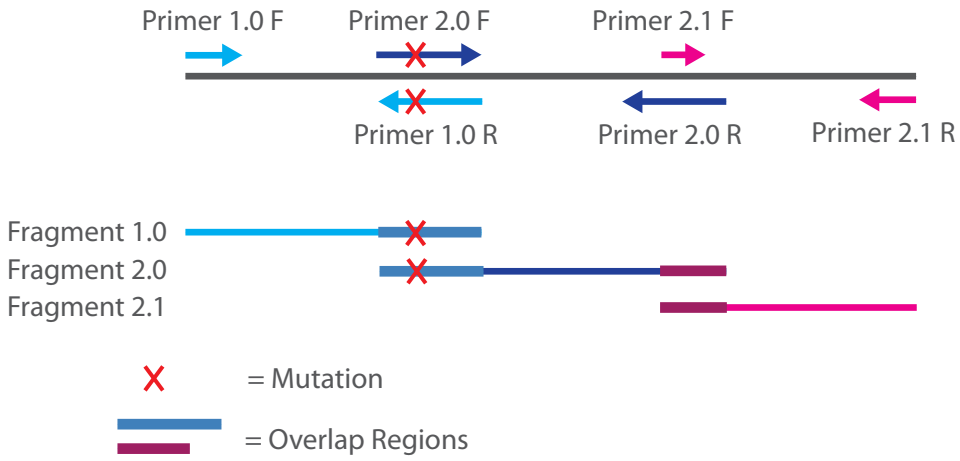
- Primer 1.0 F (5' terminal primer): comprised of 20 nt of 5' fragment terminal sequence
- Primer 2.0 R (3' terminal primer): comprised of 20 nt of 3' terminal reverse complement sequence with 40 nucleotides of the reverse complement of the fragment 5' terminal sequence added at the 5' end (shown in the following illustration).



Mutations of Large Fragments (greater than 5 kb)

Large Fragments: Primer Design Key Points
Forward primer(s) are 20 nt long
Reverse primer(s) are 60 nt long
Add homologous overlap sequence to the reverse primer

For site-directed mutagenesis of fragments greater than 5 kb, we recommend designing additional internal primers to avoid incorporating additional, unwanted mutations during mutagenesis PCR.



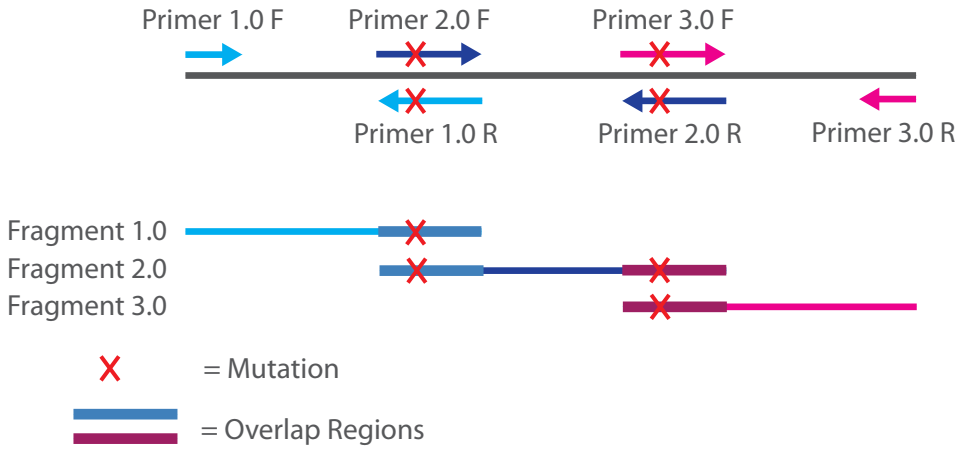
In this example, you will set up the following 3 PCR mutagenesis reactions:

Reaction	Primer Pair
1	Primer 1.0 F + Primer 1.0 R
2	Primer 2.0 F + Primer 2.0 R
3	Primer 2.1 F + Primer 2.1 R

Multi-site Mutations

Multi-Site Mutations: Primer Design Key Points
Design 40- or 60-mer primers according to the intended mutation
For highest mutation efficiencies, design primer(s) to generate similar sized fragments

For the introduction of multiple mutations at multiple locations, design multiple sets of primers as shown in the following illustration. Multiple rounds of mutagenesis are recommended for mutation sites that are 40–100 bp apart.



In this example, you will set up the following 3 PCR mutagenesis reactions:

Reaction	Primer Pair
1	Primer 1.0 F + Primer 1.0 R
2	Primer 2.0 F + Primer 2.0 R
3	Primer 3.0 F + Primer 3.0 R

DNA Preparation

Gibson Assembly® SDM Reaction Substrate DNA: Considerations

Generate double-stranded DNA fragments (including vectors) for the Gibson Assembly® Reaction using either PCR or restriction enzyme digestion. For optimal results:

- Only use DNA samples with $A_{260/280}$ > 1.8.
- Use DNA at a concentration ≥ 40 ng/µL.

Note: 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.

PCR Amplification of the Substrate DNA

To minimize the introduction of errors, we recommend:

- Reducing the number of PCR cycles to 25−30 cycles
- Using amplicons with a maximum length of 5.5 kb.

Purifying Vector and Insert DNA

- Following amplification, column-purify or ethanol-precipitate PCR reactions to remove enzymes that could interfere with the Gibson Assembly® method.
- If plasmid templates are used for amplification, treating the PCR reactions with DpnI will reduce template carryover.
- If your PCR reaction or synthetic assembly yields non-specific fragments, purify the fragment of interest using gel extraction or another size fractionation method.

  Note: Gel extraction minimizes template background and reduces the number of observed non-mutagenized colonies.

Protocols

Guidelines for the Gibson Assembly® Site-Directed Mutagenesis Procedure

- For multiple reactions, create a master mix to minimize pipetting error.
- Use approximately 100 pg – 30 ng of template DNA.

  Note: To achieve the most robust PCR performance, consider linearizing DNA by restriction enzyme digestion or PCR amplification prior to mutagenesis.
- To minimize the production of non-specific PCR products and primer-dimers, prepare reactions on ice.

Gibson Assembly® Site-Directed Mutagenesis Procedure

Site-Directed Amplification: Reaction Setup

1. Thaw the GA SDM PCR Mix (2X) on ice.
2. For each fragment, prepare the following reaction mixture in a thin-walled PCR tube:

Component	Final Concentration	Volume
GA SDM PCR Mix (2X)	1X	12.5 µL
dsDNA template*	100 pg –30 ng	χ µL
SDM Forward Primer* (5 µM)	0.5 µM	2.5 µL
SDM Reverse Primer* (5 µM)	0.5 µM	2.5 µL
PCR water*	—	7.5–χ µL
Total		**to 25 µL**

* User supplied

3. (Optional) For the positive control, prepare the following reaction mixtures in thin-walled PCR tubes. Prepare both amplicons (Control Amplicon A and Control Amplicon B) for the positive control reaction.

   To generate Control Amplicon A:

Component	Final Concentration	Volume
GA SDM PCR Mix (2X)	1X	12.5 µL
Control Plasmid (5 ng/ µL)	5 ng	1.0 µL
Control Primer Mix A (25 µM)	1 µM (0.5 µM of each primer)	1.0 µL
PCR water	—	10.5 µL
Total		**25 µL**

   To generate Control Amplicon B:

Component	Final Concentration	Volume
GA SDM PCR Mix (2X)	1X	12.5 µL
Control Plasmid (5 ng/ µL)	5 ng	1.0 µL
Control Primer Mix B (25 µM)	1 µM (0.5 µM of each primer)	1.0 µL
PCR water	—	10.5 µL
Total		**25 µL**

4. Mix the reactions with pipetting.

Site-Directed Amplification Reaction

1. Transfer tubes to a thermocycler. Program and start the thermocycler using the following conditions:

Initial Denaturation	98°C	1 minute	1 Cycle
Amplification	98°C	10−30 seconds	30 Cycles
	Primer T_m*	30 seconds	
	72°C	30 seconds per kb	
Final Extension	72°C	5 minutes	1 Cycle
Hold	4°C	as necessary	1 Cycle

* The T_m of the Control Primers is 60°C.

2. After the reaction is complete, analyze 5 µL of the product on a 0.8 % agarose gel.

   Note: See "Site-Directed Mutagenesis PCR Product Gel Electrophoresis" on page 24 for an example gel image.

3. Minimize original template carryover by choosing from one of the following options:

   - Gel-purify the PCR product

   OR

   - Treat the PCR product with DpnI and column-purify the DpnI-treated reaction.

4. Proceed directly to "Gibson Assembly® Reaction of Mutagenized Fragments Setup" on page 18.

Gibson Assembly® Reaction of Mutagenized Fragments Setup

1. Thaw GA SDM Assembly Mix A (2X) on ice.
2. Adjust the concentration of the purified, mutagenized fragments according to the guidelines listed in the following table. In a PCR tube, combine the insert(s) and vector for assembly, bringing the total volume of the fragments for assembly to 5 µL with nuclease-free water.

Fragment	Size	Amount	Insert:Vector Molar Ratio*
Insert	≤ 1 kb	10–40 ng	1:1 to 5:1
	1–5.5 kb	10–25 ng	1:1 to 2:1
Vector	2–10 kb	25 ng	—

* Use the following formula for calculations:

$$\frac{\text{pmol}}{\mu\text{L}} \cong 1.55 \times \frac{\text{ng}/\mu\text{L}}{\text{bp}}$$

Note: To assemble multiple, dilute fragments, it may not be feasible to combine samples and maintain a maximum 5 µL volume. For dilute fragments, you may increase the total combined sample volume to 10 µL. For 10 µL samples, increase all subsequent assembly volumes 2-fold [i.e., use 10 µL total sample volume, 10 µL GA SDM Assembly Mix A (2X) and 20 µL GA SDM Assembly Mix B (2X)] in the "Gibson Assembly® Reaction of Mutagenized Fragments Setup" and "Performing the Gibson Assembly® Reaction of Mutagenized Fragments".

3. (Optional) For the positive control, combine 25 ng of gel- or column-purified Control Amplicon A with 25 ng of gel- or column-purified Control Amplicon B. Bring the total volume to 5 µL with nuclease-free water.
4. Vortex the GA SDM Assembly Mix A (2X) immediately before use (after it is thawed).
5. In a tube on ice, combine 5 µL of volume-adjusted, purified mutagenized fragments from step 2 of this procedure and 5 µL of thawed GA SDM Assembly Mix A (2X). Mix the reaction by pipetting.
6. (Optional) For the positive control, while keeping tubes on ice, combine the 5 µL mixture from step 3 with 5 µL of thawed GA SDM Assembly Mix A (2X). Mix the reaction by pipetting.
7. Vortex and spin down all reactions.

Performing the Gibson Assembly® Reaction of Mutagenized Fragments

1. Transfer tubes to a thermocycler. Program and start the thermocycler using the following conditions (1 cycle):

3’ end Chew Back	37°C for 5 minutes
Inactivation	75°C for 20 minutes
Slowly Cool	0.1°C/second to 60°C
Anneal	60°C for 30 minutes
Slowly Cool	0.1°C/second to 4°C

2. Thaw GA SDM Assembly Mix B (2X) on ice. **Vortex and briefly centrifuge thawed GA SDM Assembly Mix B (2X) immediately before use**.

3. While keeping the tubes on ice, add 10 µL of GA SDM Assembly Mix B (2X) to the completed reaction from step 1. Mix samples by pipetting.

4. Incubate samples using the following conditions:

Repair	1 cycle	45°C for 15 minutes

5. After the incubation is complete, store the reactions at –20°C or proceed to downstream applications such as *E. coli* transformation (see the protocols on pages 20–23).

6. (Optional) Analyze the assembly reaction with electrophoresis of 50% of the assembly reaction (e.g. 10 µL of a 20 µL reaction or 20 µL of a 40 µL reaction) on a 0.8%–2% agarose gel.

Guidelines and Recommendations for Transformation

- We recommend using Epicentre TransforMax™ EPI300™ electrocompetent cells with the Bio Rad Gene Pulser Xcell electroporator.
- For transformation with electrocompetent cells other than EPI300™ cells, we recommend using the following guidelines:
    - Electrocompetent cells with a transformation efficiency ≥ 1×10^9 CFU/µg pUC19 are preferred.
    - Use 2% (v/v) of the assembly reaction per transformation as a starting point (e.g. use 1 µL of the assembly reaction per 50 µL of high efficiency competent cells). If you need to optimize transformation conditions, use < 2% of the assembly reaction.
    - Follow the protocol and electroporation parameters supplied with your electrocompetent cells
- For transformation using chemically competent cells, follow the procedure "Transformation using Chemically Competent Cells" on page 22.
- Observe the value of the TC (Time Constant) for each transformation, which conveys efficiency (4.5–5 is ideal, ≤4 is not acceptable).
- Before starting, prepare ice buckets, tubes, and pipettors so that the transformation steps can be completed quickly and efficiently.

Transformation Procedure for Electrocompetent EPI300™ Cells (Recommended Procedure)

1. Prepare 15 mL snap cap tubes with 1 mL SOC per tube for each transformation reaction.
2. Chill electroporation cuvettes on ice.
3. While keeping the tubes on ice, pipet 1–2.5 µL of the SDM assembly reaction into a clean 1.5-mL microfuge tube.
4. Thaw TransforMax™ EPI300™ Electrocompetent *E. coli* (Epicentre® EC300110) on ice. Mix gently.
5. Add 30 µL of thawed, electrocompetent cells to each cold tube containing the SDM assembly reaction (from step 3, above). Mix gently with the end of a pipette tip and return the tube to ice.
6. Incubate the cells and DNA on ice for one minute.
7. After the incubation, pipet the cell/DNA mixture into a chilled cuvette. Tap the cuvette gently onto the benchtop 2–3 times, insert the cuvette into the electroporator, close the lid, and press the pulse button.

   Note: The pulse settings for electrocompetent TransforMax™ EPI300™ cells are 1200 V, 25 µF, 200 Ω, 0.1 cm cuvette.
8. During the pulse (≈ 2 seconds), remove about 800 µL SOC from the snap cap tube (step 1). Add the SOC to the cuvette immediately after the end of the pulse.
9. Thoroughly pipet the mixture up and down. Add the mixture back to the snap cap tube containing about 200 µL SOC, and repeat steps 5–8 for the remaining tubes. Work as quickly as possible until the cells are transferred into the snap cap tube.
10. Incubate the tubes with shaking at about 200 RPM for 1 hour at 37°C to allow the cells to recover.
11. Pre-warm LB plates in an incubator upside down for 10–15 minutes.
12. After the 1 hour incubation, plate 1/2–1/50 of the transformation reaction (20–500 µL of 1 mL) onto LB agar plates with appropriate antibiotics.

    Note: See "Recommended Plating Volume" on page 23 for more information.
13. (Optional) For the positive control, plate 1/100 volume of the transformed reaction onto LB plates containing 100 µg/mL ampicillin or carbenicillin with 40 µg/mL X-Gal and 0.1 mM IPTG.
14. Incubate plates at 37°C upside down, overnight.
15. Pick colonies for screening. We typically pick 6–10 colonies and screen the clones by colony PCR or by plasmid DNA purification followed by restriction enzyme digestion.

Transformation using Chemically Competent Cells

Note: Use the following procedure as a starting point. For optimal efficiency, you may need to adjust the amount of the assembly reaction used for transformation, as well as transformation conditions and parameters according to the competent cell manufacturer's recommendations.

1. While keeping tubes on ice, pipet 1 µL of the assembly reaction (from step 5 of "Performing the Gibson Assembly® Reaction of Mutagenized Fragments" on page 19) into a clean 1.5 mL microfuge tube.
2. Thaw chemically competent cells on ice. Mix gently.
3. Add 50 µL of thawed, chemically competent cells to each cold tube containing the assembly reaction (from step 1, above). Mix gently with the end of a pipette tip.
4. Incubate the cells and DNA on ice for 30 minutes without mixing.
5. Heat shock the cell/DNA mixture according to the instructions provided with your competent cells.

   Note: Typical heat shock parameters are 42°C for 30 seconds.
6. Return tubes to ice for 2 minutes
7. Add room temperature SOC media (250–950 µL) according to the instructions provided with your competent cells.
8. Incubate the tubes with shaking at about 200 RPM for 1 hour at 37°C to allow the cells to recover.
9. Pre-warm LB plates in an incubator upside down for 10–15 minutes.
10. After the 1 hour incubation, plate 1/2–1/50 of the transformation reaction (20–500 µL out of 1 mL) onto LB agar plates with appropriate antibiotics.

    Note: See "Recommended Plating Volume" below for more information.
11. (Optional) For the positive control, plate 1/100 volume of the transformed reaction onto LB plates containing 100 µg/mL ampicillin or carbenicillin with 40 µg/mL X-Gal and 0.1 mM IPTG.
12. Incubate plates at 37°C upside down, overnight.
13. Pick colonies for screening. We typically pick 6–10 colonies and screen the clones by colony PCR or by plasmid DNA purification followed by restriction enzyme digestion.

Recommended Plating Volume

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

Plating Volume for EPI300™ *E. coli* and Competent Cells with Transformation Efficiencies > 1×10^9 CFU/µg pUC19			
Number of fragments	Plating volume*	For example, we normally plate... †	Expected number of colonies ‡
1–2	1/50	20 µL and 100 µL	> 100
3–5	1/10	100 µL and 500 µL Note: Spin down the reaction before plating	

* 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.

Plating Volume for Competent Cells with Transformation Efficiencies ≥ 1×10^8 CFU/µg pUC19*		
Number of fragments	Plating volume †	For example, we normally plate... ‡
1–2	1/50	20 µL and 100 µL
3–5	1/2	100 µL and 500 µL Note: Spin down the reaction before plating

* Lower colony output is expected with lower transformation efficiency competent cells.
† The plating volume is the fraction of transformation reaction plated per the total transformation mixture.
‡ Volumes are based on a 1000 µL transformation mixture.

Transformation Results and Analysis

For the positive control, blue colonies indicate successful mutagenesis and assembly; white colonies indicate the presence of assembly of unmutagenized fragments or template carryover. Calculate cloning efficiency using the following formula:

Mutagenesis efficiency (ME, %) = Number of blue colonies/ Total colonies x 100

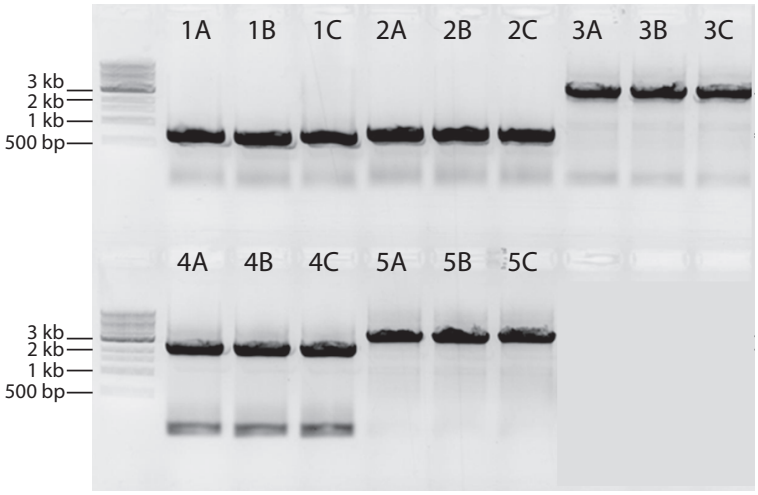
We typically observe positive control mutation efficiencies > 90%. Colony output is dependent on several factors, including transformation efficiency. Note that low colony output is not necessarily indicative of low cloning efficiency.

Expected Results

Site-Directed Mutagenesis PCR Product Gel Electrophoresis

Multi-site Mutagenesis Results: Introducing 9 mutations within 5 sites of an 11.2 kb plasmid.

The image below shows the gel electrophoresis of 2 µL of each SDM PCR product (reactions performed in triplicate, prior to assembly) on a 1% TAE gel. The ladder shown is the Quick-Load® 1 kb DNA Ladder (NEB). Lane designations correspond to the table in Experimental Details, below.



Experimental Details

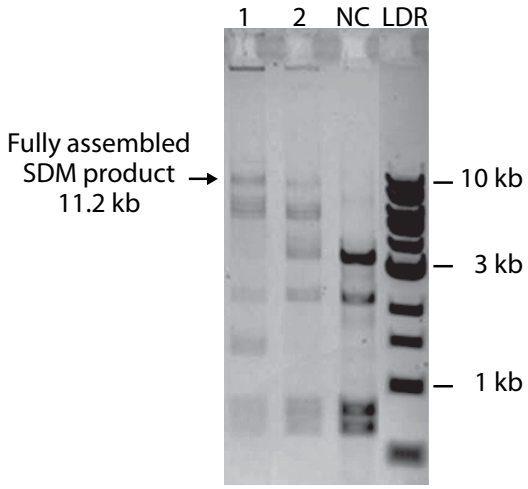
Lane (from gel image, above)	Mutations	Expected Mutagenized Amplicon Size
1A, 1B, 1C	G1013T, T1015C	727 bp
2A, 2B, 2C	G1664T, A1665T, A1666C	871 bp
3A, 3B, 3C	A2456T, A2457T	4.1 kb
4A, 4B, 4C	T6326G	2.5 kb
5A, 5B, 5C	C8696A	3.9 kb

To perform mutagenesis, 5 ng of a circular 11.2 kb plasmid was used as the template with the following reaction conditions in a 25 µL PCR mutagenesis reaction:

Initial Denaturation	98°C	1 minute	1 Cycle
Amplification	98°C	30 seconds	30 Cycles
	60°C	30 seconds	
	72°C	30 seconds – 2:15 minutes	
Final Extension	72°C	5 minutes	1 Cycle
Hold	4°C	as necessary	1 Cycle

Multi-Site Mutagenesis and Assembly Gel Electrophoresis

A representative image for multi-site mutagenesis and assembly using the Gibson Assembly® Site-Directed Mutagenesis Kit.



Five amplicons generated from the protocol described in this manual were used to assemble an 11.2 kb construct. Duplicate assembly reactions are shown in the image (Lanes 1 and 2). Without GA SDM Assembly (Negative control, Lane NC), five amplicons (4.1, 3.9, 2.5, 0.8 and 0.7 kb, respectively) appear as distinct bands. With GA SDM Assembly (Lanes 1 and 2), multiple bands indicate the progression of assembly. The final 11.2 product indicates the success of the Gibson Assembly® reaction.

Lanes 1 and 2 are the samples, NC = Negative Control (no GA SDM Assembly Mix A or B added to the reaction), LDR = Quick-Load® 1 kb DNA Ladder (NEB).

Mutagenesis Efficiencies Greater Than 90% with Either Purification Method

Following 5-site mutagenesis of the 11.2 kb plasmid, PCR samples were either treated with DpnI or gel-purified. Fragments were pooled in equal molar ratios and assembled. Eight DpnI-treated samples (8 duplicates; 64 colonies in total) or nine gel-purified samples (9 duplicates; 72 colonies in total) were picked and sequence-verified. As indicated in the following graph, both purification methods can be used for 5 site mutagenesis with >90% success rate.