A Simplified Gibson Assembly Method for Site Directed Mutagenesis Using Non-Gibson Primers

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Abstract

Background: Site-directed mutagenesis (SDM) is a key method in molecular biology; allowing to modify DNA sequences at single base pair resolution. Although many SDM methods have been developed, methods that increase efficiency and versatility of this process remain highly desired.

Method: We present a versatile and simple method to efficiently introduce a variety of mutation schemes using the Gibson-assembly without the need for unique Gibson primers. The method entails use of standard SDM primers (shorter and completely overlapping in sequences in contrast to Gibson primers) that are separately employed with common primer (~25 bps long) for amplification of fragments flanking the site of mutagenesis, followed by rapid amplification of the Gibson-assembled product for added visualization and sequencing steps for ensuring high success rates.

Results: We find that assembly of the fragments via the Gibson reaction mixture is attainable within as short as 15 minutes, despite the need for extensive digestion of the DNA (by exonuclease) past the entire SDM primer sequence (to expose non-clashing overlap between the fragments). We also find that the amount of the assembled Gibson product is too low to be visualized and assessed on standard agarose gel. We thereby introduce a short amplification step (by use of the same short primers initially employed) to 1) easily resolve whether the product (only the correct size can yield a product) has been obtained, and 2) for isolation of product for DNA-sequencing (to assess whether mutation(s) have been introduced). No other SDM method enables assessment of mutagenesis prior completion of the process.

Conclusion: We employ our approach to delete, replace, insert, and degenerate sequences within target DNA sequences, specifically in DNA sequences that proved very resistant to mutagenesis by multiple other SDM methods (standard and commercial). The entire protocol spans only four days, requires minimal primers sets (as well as can be used with most *in-house* primers) and provides very high yields and success rates (>98%).

Introduction

Site-directed mutagenesis (SDM) is a pivotal molecular biology technique for rationally modifying DNA sequences, for instance substitution deletions or insertions of base pairs (bp) at desired location within a template DNA¹. There are a different approaches to obtain SDM (most are now commercial²⁻⁶), but they all share two common denominators: a set of complementary primers that bear the desired change, mainly, at the center of the primer sequence, and a thermocycler (Polymerase chain reaction; PCR)-based amplification. However, and despite broad-usage, SDM methods provide low efficiency in introducing mutations and, resultantly, multiple trials are often needed to establish optimal settings for the reaction to succeed^{7,8}. The reason for the low efficiency stems from several notable challenges in most steps the process, from the initial design of the primers (requiring lengthy primer of high melting temperature and GC content; yielding high probabilities for primer dimers and strong secondary structures), to the PCR reaction (very prolonged PCR reactions, very low reaction yields that usually cannot be distinguished on

gel, potential occurrence of mutations in large plasmids, high background *dpnI*-evaded template DNA, etc.). Moreover, the resulting SDM-PCR products are typically ligated within the bacteria, which may result in unwarranted additions or deletions in the plasmid⁹. Lastly, the approach is limited in the size of the insertion or deletion that can be introduced by one pair of primers. Other methods, such as seamless introduction of residues by blunt-end ligations, require more expensive primers that require more time for production than standard primers (due 5' phosphate additions), the process is of very low efficiency and may introduce mutations at the ligation site (see ^{10,11}). These acute challenges are the main reasons why most users are deterred from employing SDM, and why new methods are continuously reported^{2,8}. Thus, means to obtain higher efficiency SDM, rapidly and easily with added degrees of versatility, remain warranted.

We sought to develop a rapid, simple and versatile method to improve efficiency of the process and thereby reduce time to completion. These considerations have led us to test whether we could insert, delete, or substitute DNA sequences, as well as degenerate residues, within target sequences by employing the Gibson assembly method but, importantly, without requiring unique Gibson primers. Briefly, the Gibson assembly approach is intended for assembly of multiple DNA-segments in a *one-tube-reaction*¹². Prior assembly, each segment is amplified by use of unique primers (i.e., Gibson primers) to introduce a 15–20 nucleotide sequence at both 5'- and 3'-termini and these added sequences serve for complementation and assembly between the latter. Thereby, Gibson primers are unique to each reaction, often require optimization and are approximately twice longer than standard amplification primers (~40 bp vs ~20 bp)^{13,14}. Importantly, and with respect to our aims, the Gibson Assembly method has not been developed for mutagenesis, insertion or deletion of sequences, though we did find one commercial protocol that employs Gibson primers for single residue mutagenesis¹⁵. However, the latter protocol requires multiple sets of custom and 'lengthy' Gibson primers for a single mutation, the efficiency of the process decreases substantially if additional substitutions are desired and, notably, deletions, insertions or degeneration of sequences remain completely unaddressed by this development¹⁵. Moreover, whether the Gibson assembly method can accommodate other types of primers is less established. Lastly, one major limitation of the process is the user's inability to assess whether the reaction has succeeded until the very last step, namely whether positive bacterial colonies have been obtained the following day.

Here, we present a versatile and simple approach for insertions, deletions, and evolution of DNA sequences by a modified Gibson assembly approach that operates with standard SDM, non-Gibson, primers (Fig. 1). We further introduce two quality control steps to increase the success rate of the process. We show the validity of our approach by deletion and replacement, insertion, and degeneration of residues within target sequences that could not be obtained by standard commercial methods. The entire process spans only four days with very high success rates.

Results

Introducing six bps into dLight— To test the ability of our modified method for insertion of sequences, we opted to insert six bps (TGAATG) at position 50 or 120 at the first and second intracellular loop (ILI or ILII, respectively) of a fluorescent dopamine probe (dLight¹⁶). This insertion should translate into a stop codon followed by a methionine (Stop-Met). We designed SDM primers that containing the insertion (Table 1; **bold**) and tested our procedure to introduce these changes after having repeatedly failed to do so by standard SDM approaches in multiple trials. In fact, these failures could not be remedied by systematic modifications of annealing temperatures, steps’ durations, number of cycles, added reagents (e.g., DMSO), and commercial kits (see **Suppl. Text**).

Table 1
List of the primers employed for this work.

Primer name	Primer sequence (5'-3')
ILI_SDM_F	GGTCTGTGCTGCCGTTATCT TGAATG AGGTTCCGACACCTGCGG
ILI_SDM_R	CCGCAGGTGTCTGGAACCT CATT CAGATAACGGCAGCACAGACC
ILII_SDM_F	CCTCTGTGTGATCAGCGTGT TGAATG GACAGGTATTGGGCTATCTC
ILII_SDM_R	GAGATAGCCCAATACCTGT CATT CACACGCTGATCACACAGAGG
ILIII_R	CGTTAATGAGTGAGCTCAGCATTCACTGTTTCTGAGCAATCCTG
hSyn promoter_F	CGCACCACGCGAGGCGCGAGATAGG
FP_R	CTTGTACAGCTCGTCCATGCC
hChR_203R_F	GCATATATCGAGGGTTATCATACT AGGGT GCCAAAGGGTCGGTGCCGCCAG
hChR_203R_R	CTGGCGGCACCGACCCTTTGGCAC CCT AGTATGATAACCCTCGATATATGC
CAG promoter_F	GCAACGTGCTGGTTATTGTG
hChR_203All_F	GCATATATCGAGGGTTATCATACTTG NNNGT GCCAAAGGGTCGGTGCCG
hChR_203All_R	CGGCACCGACCCTTTGGCAC NNNT CAAGTATGATAACCCTCGATATATGC

We first amplified the sequences flanking the site of insertion (sites A and B) by re-using the complementary SDM primers, even though these have failed by standard approaches (see **Suppl. Text for details**), with general primers from our inventory. Specifically, for amplification of fragment A, we employed the antisense SDM primer (**ILI_SDM_R**) along a sense primer annealing to the promoter of the plasmid (**hSyn promoter_F**) (Fig. 2a **and** Tables 1 and 2). Similarly, amplification of fragment B was obtained by the sense complementary SDM primer (**ILI_SDM_F**) and a standard antisense primer which we have previously used (**ILIII_R**) (Fig. 2a). For an identical insertion at ILII (flanked by sites B and C), we

employed the same strategy; combining ILII_SDM_F/R with another general-use primer that anneals to the 3' terminal of GFP; a sequence that is shared by many other fluorescent proteins (FPs)¹⁷ (Fig. 2a, **FP_R, green**, Tables 1 and 2). A standard amplification protocol yielded two sets of products at very high amounts (and rapidly, <1 hrs, see **methods**; Tables 2 and 3) and these could easily be distinguished and quantified on 1% agarose gel (~300 and ~550 bps, ~500 and ~650 bps for Parts A, B and C of ILI or ILII, respectively) (Fig. 2b). To assemble the fragments (A with B, and B with C), we used the Gibson Assembly mix, even though the extent of complementation between our fragments (obtained by the standard SDM primers) does not meet the requirement of Gibson primers (**Suppl. 1**)¹². Therefore, we assumed that longer DNA-excision times by the T5-exonuclease may be required to remove matching sequences between the fragments, that would otherwise prevent the fragments from annealing to one another. In fact, the fragments need to undergo extensive digestion past the entire sequence of the primer to enable the ligation (**Suppl. 1**). We placed the isolated PCR products from step I within the Gibson reaction mixture for prolonged incubation (two hours; **methods**). Notably, the expected product of this assembly (~800 or ~1150 bps for ILI and ILII, respectively) could not be visualized on 1% agarose gel (see example below in Fig. 4c). We therefore could not assess whether the reaction succeeded and, if it didn't, which step was faulty (for instance, whether the digestion of the overlapping sequence was insufficient). This did not meet our primary goal of providing added quality-control checkpoints throughout the process. We therefore opted to try to detect the potential Gibson-ligated product by amplifying it using primers employed in step I, namely sense hSyn promoter_F with the ILIII_R or FP_R antisense primer, for ILI or ILII, respectively. Importantly, we chose these pairs of primers as they can only amplify the ligated product, if extant in the tube. This rapid PCR reaction (1 hr) yielded easily detectable amplicons of the correct size (Fig. 2c, **step III; ~1 Kbp**). Next, amplicons and plasmid were digested, ligated overnight followed by transformation and plating (Fig. 2d). We isolated DNA from several colonies and visualized them on 1% agarose gel. Though a handful of colonies did not contain the right plasmid ('negative' colonies), all of the 'positive' colonies contained the desired substitutions (Fig. 2e). Together, we found that we could easily introduce the desired six bps at two distinct DNA regions after only four days by the Gibson method without Gibson primers.

Table 2
Reaction mixture for the KAPA HiFi HotStart ReadyMix PCR Kit.

Component	Step I	Step I
2X KAPA HiFi HotStart ReadyMix	12.5 µl	12.5 µl
Template	1 µl (10 ng)	1 µl (Gibson mix)
SDM_Primer_F (10 µM stock)	0.75 µl	0.75 µl
SDM_Primer_R (10 µM stock)	0.75 µl	0.75 µl
ddH2O	Up to 25 µl	Up to 25 µl

Table 3
PCR settings for KAPA HiFi HotStart ReadyMix PCR Kit

Step I			Step III	
Step	Cycles	Temperature/ Duration	Cycles	Temperature/ Duration
Initial denaturation	1	95°C /3 minutes	1	95°C /3 minutes
Denaturation	30	98°C /20 seconds	30	98°C /20 seconds
Annealing		60/56°C /20 seconds		60/56°C /20 seconds
Extension		72°C /1 minutes		72°C /2 minutes
Final extension	1	72°C /5 minutes	1	72°C /5 minutes

Deletion and replacement of residues in Chr2 using SDM primers— We were interested in testing whether the modified procedure could support a slightly more challenging procedure, namely to remove six bps and replace them by three other bps ('TGAATG' to 'AGG') at the ILIII (residue 203) of a humanized Channelrhodopsin2-mCherry variant (hChr2-mCherry). We intentionally designed two standard complementary sets of SDM primers (51 bps each, at 53% GC content) instead of Gibson primers (Table 1). Again, under standard SDM conditions (and by use of different commercial kits), we could not obtain the final product (see **Suppl. Text**). We, therefore, similarly amplified the two segments of hChr2 using each of the SDM primers in two separate PCR reactions, with a standard sense primer annealing to promoter (CAG promoter_F), and a general antisense primer annealing to FP (**FP_R**) (Figs. 3a); yielding correct amplicons sizing at ~700 and ~1040 bps (Part A and B, respectively) (Fig. 3b; **step I**).

Fragments were assembled by the Gibson reaction mixture but, this time around, at various incubation times (15 min to 2 hours), immediately followed by PCR amplification of the potential ligated fragment by use of CAG_promoter and FP_R antisense primers (1.5 hrs, see Tables 2 and 3) (Fig. 3c). Surprisingly, even the shortest Gibson assembly reaction (15 min) yielded the expected ligated product, easily visualized on 1% agarose gel (Fig. 3c, **~1.8 Kbp**). We then added another quality control step by sequencing the amplified ligation-product, a feat that requires a sufficiently large amount of product as obtained here. Indeed, we find the desired changes in the DNA the following day in the sequencing results (**Suppl. 2a**). Then, insert and plasmid were digested, ligated, transformed, and plated (**methods**). We sequenced DNA from only three colonies and find the desired modifications in all three (Fig. 3d).

Gibson assembly using degenerate primers to evolve a single residue in Chr2—

We next examined whether we could evolve a single residue within the third intracellular loop of hChr2-mCherry (residue M203). We designed degenerate primers targeting three bps for evolution (i.e., a mixture of 64 different primers, each 50 bps long; Table 1). Here too, standard SDM conditions repeatedly failed in over 20 different trials (**Suppl. Text**). We then applied our procedure to amplify the DNA flanking the site to be mutated using the degenerate primers separately, combined with sense CAG promoter_F and

antisense FP_R primers (Figs. 4a). Standard amplification (1 hr, **see** Table 3) yielded the expected amplicons (~700 and ~1040 bps for Part A and B; Fig. 4b, **Step I**). Again, the product of the Gibson-assembly of the fragments could not be visualized on gel (Fig. 4c; **Step II, dashed region**) without amplification (Fig. 4c; **Step III, arrowhead, ~1750 bps**).

The next day (following digestion, ligation and bacterial transformation), we isolated DNA from 38 colonies, all of which contained different mutations at the desired site (two colonies contained a mixture of two DNAs); thereby yielding >95% efficiency (Fig. 4e, **dashed regions**). Thus, we have rapidly evolved residue M203; resulting in a small library of 16 different amino acid substitutions. Interestingly, though beyond the scope of this work, we noted that proline was the most abundant substitution and that none of the colonies contained the original M203 (either from residual template DNA or by mutagenesis) (**Suppl. 3**).

Conclusion

Here, we present a step-by-step protocol for employing the Gibson assembly with non-Gibson primers, rather standard SDM primers to insert, delete or substitute sequences, whether rational or random (by degenerate primers), within different genes. Our approach includes two added quality control steps to reduce uncertainties and ensure a high success rate. Our approach is user-friendly, versatile, rapid, and cost-effective (requires standard SDM primers along in-house primers). Importantly, this modified procedure bypasses the need to amplify the plasmid backbone to include overlaps for Gibson assembly. Lastly, we find that 15 minutes are sufficient to digest past the entire sequence of standard ~50 bp-long primers by the Gibson mixture and to assemble the amplicons. Therefore, we demonstrate a simple method for mutagenesis at very high efficiency and at short times to completion (four days).

Methods

Molecular biology and DNA constructs-

dLight, hChR2-mCherry were purchased from Addgene (Cat #125560, Cat #100054, respectively). Thermocycle (ProFlex, Applied Biosystems) settings and primers employed for amplifications are specifically denoted for each reaction (see Tables 1-3). Details on standard SDM settings are provided in **Suppl. Text and Tables**. Restriction enzymes (New England Biolabs; NEB) are denoted for each reaction and were incubated with DNA for 1-2 hrs at 37°C. 2X KAPA HiFi HotStart ReadyMix (Takara) was used for PCR reactions. Ligations were performed at 18°C overnight by T4 ligase (NEB). Omni ultracompetent bacterial cells (Zymo) were used for bacterial transformations. DNA purification was performed by use of DNA isolation/purification kit (Zymo).

Abbreviations

(K)bp- base pairs, K- 1000

hrs- hours

SDM- site directed mutagenesis

FR- forward (i.e., sense primer)

R- reverse (i.e., antisense primer)

hChR2-mCherry- humanized Channelrhodopsin2-mCherry

dLight- Fluorescent (light) Dopamine probe

GFP- Green fluorescent protein

PCR- polymerase chain reaction (i.e., thermocycle-based reaction)

IL(I-III)- intracellular linker I, II or III.

ddH₂O- double distilled water

Declarations

Ethics approval and consent to participate— Not Applicable (no animals or human subjects were used or participated in the study).

Consent for publication—All authors consent the publication of the manuscript.

Availability of data and material— All data are provided in the manuscript and in supplementary material.

Competing interests— The authors declare no competing interests.

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Authors' contributions— S.O. performed all the experiments. S.O. produced all illustrations and figures. S.B. supervised the project. S.O. and S.B wrote the manuscript. All authors have read and approved the manuscript.

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Figures

Figure 1

The modified Gibson assembly method using SDM primers and added quality control steps. Flowchart of the method's main steps. Step I- PCR amplification of the DNA sequence by use of standard SDM primers (50 bp; purple-orange arrows) containing the desired change in sequence, i.e., mutation (green highlight). Each SDM primer (sense, +; antisense, -) is used separately to amplify the fragments (A and B) flanking the site of mutagenesis (green). This is achieved by the additional use of regular amplification primers

(~20 bp, short purple and short orange arrows). Step II- A and B amplicons are assembled using the Gibson reaction mixture. Step III- the resulting assembly is amplified by the two same primers used in step I. Note that these primers can only amplify the assembled fragments. The large amounts of product obtained by this amplification can be used to 1) visualize and examine size of products by electrophoresis (1% agarose gel, cartoon) and 2) products can be isolated and sent to sequencing (cartoon chromatogram). These are the added quality control steps (QC) introduced. Step IV- The amplified assembly product is digested and ligated into a desired plasmid (vector) and transformed into competent cells. The entire process spans four days.

Figure 2

Insertion of six base pairs by the modified Gibson approach into two distinct sites within dLight. a. Depiction of the dLight-GFP (green fluorescent protein) synthetic gene (colored segments, A-C). Fragment A includes sequences spanning from the promoter of the plasmid and intracellular linker I (ILI; purple); fragment B spans from ILI to ILII (orange); fragment C, ILII to the end of the GFP (green). Backbone of plasmid is light grey. The mutations (green or yellow) are situated between the purple and orange fragments, and orange and grey fragments, respectively. Sizes (# of bps) of the fragments are noted within the fragments. Intrinsic and unique digestion sites are also noted (BamHI and BsrGI). Primers used for amplification of each fragment are noted on the right (with corresponding colors). b. Image of PCR products from step I on 1% agarose gel. DNA ladder sizes (in Kbp) are noted on the right of third ladder. c. The assembled Gibson products after amplification by hSyn promoter_F and the ILIII_R primers, and FP_R primers. d. Image of the agar plates with colonies obtained after ligation of assembled Gibson products to the plasmid (at 1:2 ratio, V:V; top—ILI, bottom—ILII), and controls (left images; backbone plasmid without inserts). e. Top- Amino acid sequences of WT, and expected insertion within ILI and ILII, middle and bottom sequences, respectively. Bottom- The resulting sequences and chromatograms from DNA isolated from colonies (dashed boxes shows the correct modifications).

Figure 3

Deletion and replacement of residues within hChR2. a. Depiction of the hChR2-mCherry synthetic gene (colored segments, A and B). Fragment A includes sequences spanning from the standard promoter of the plasmid and intracellular linker III (ILIII; purple); fragment B spans from ILIII to the end of the mCherry (orange). Backbone of plasmid is light grey. The mutations (green highlight) are situated between the purple and orange fragments. Sizes (# of bps) of the fragments are noted within the fragments. Intrinsic and unique digestion sites are also noted (BamHI and BsrGI). Primers used for amplification of each fragment are noted on the right (with corresponding colors). b. Image of PCR products from step I on 1% agarose gel. DNA ladder sizes (in Kbp) are noted on the right of ladder. c. The amplified assembled Gibson products, obtained from varying incubation times (15 to 120 minutes), obtained by CAG promoter_F and the FP_R primers (left) visualized on 1% agarose gel. Restriction analysis of the ligated

products are shown on right lane of the right gel (BamHI and BsrGI). d. Sequences, and matching chromatograms, of DNA sequences obtained from resulting colonies (labeled with dashed box).

Figure 4

Shuffling of residue M203 of hChR2-mCherry by degenerate primers and the modified Gibson assembly method. a. Depiction of the hChR2-mCherry synthetic gene (colored segments, A and B). Fragment A includes sequences spanning from the standard promoter of the plasmid and intracellular linker III (ILIII; purple); fragment B spans from ILIII to the end of the mCherry (orange). Backbone of plasmid is light grey. The degenerate sequence (green) is situated between purple and orange fragments. Sizes (# of bps) of the fragments are noted within the fragments. Intrinsic and unique digestion sites are also noted (BamHI and BsrGI). Primers used for amplification of each fragment are noted on the right (with corresponding colors). b. Image of PCR products from step I on 1% agarose gel. DNA ladder sizes (in Kbp) are noted on the right of ladder. c. Left lane shows the products of the Gibson assembly prior amplification. The bottoms bands shows non-assembled fragments. The expected 'assembled' product is not detectable (dashed box, ~2 Kbp), whereas the amplified assembled Gibson product by CAG promoter_F and the FP_R primers is easily noticeable (right lane; arrowhead). d. Restriction analysis by BamHI and BsrGI of the ligated products. d. Sequences, and matching chromatograms, of DNA sequences obtained from resulting colonies (labeled with dashed box).

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