Molecular biology

Site-directed mutagenesis using the GeneArt Gibson Assembly HiFi Cloning Kit

Introduction

Site-directed mutagenesis (SDM) is a powerful technique used in molecular biology to introduce specific and precise mutations into a DNA sequence of interest. SDM is a versatile technique that allows scientists to investigate gene and protein functions, and is a tool used in synthetic biology to alter protein properties. By introducing site-specific mutations, scientists can gain insights into the role of specific amino acids or nucleotides in biological processes and disease mechanisms.

Cloning with Gibson Assembly™ kits simplifies the SDM process by enabling the direct assembly of mutated DNA fragments into a vector without the need for restriction enzymes or ligase-mediated cloning steps. Paired with high-fidelity Invitrogen™ Platinum™ SuperFi™ II DNA Polymerase, the Invitrogen™ GeneArt™ Gibson Assembly™ HiFi Cloning Kit offers a reliable, efficient, and convenient approach for introducing precise mutations such as insertions, substitutions, or deletions into DNA sequences. Using the GeneArt Gibson Assembly cloning kit, even multiple alterations can be easily introduced simultaneously into a sequence (Figure 1).

Here we describe how to use the GeneArt Gibson Assembly HiFi Cloning Kit in several SDM experiments with a plasmid containing the Green Fluorescent Protein (GFP) gene:

- Creating single mutations of the eGFP gene
- Creating a double mutant of the eGFP gene
- Introducing multiple mutations into the eGFP gene

Enhanced GFP (eGFP) is a well-described protein, and various mutants of GFP with different fluorescence properties have been discovered using random or site-directed mutagenesis [1,2]. A selected number of mutants have been reproduced by modifying the GeneArt Gibson Assembly cloning protocol for SDM. Our protocol consists of the following steps:

1. A PCR reaction using primers containing multiple mutations and overlaps for the Gibson assembly
2. A DpnI treatment to digest template DNA
3. Assembly of the fragments
4. Bacterial transformation

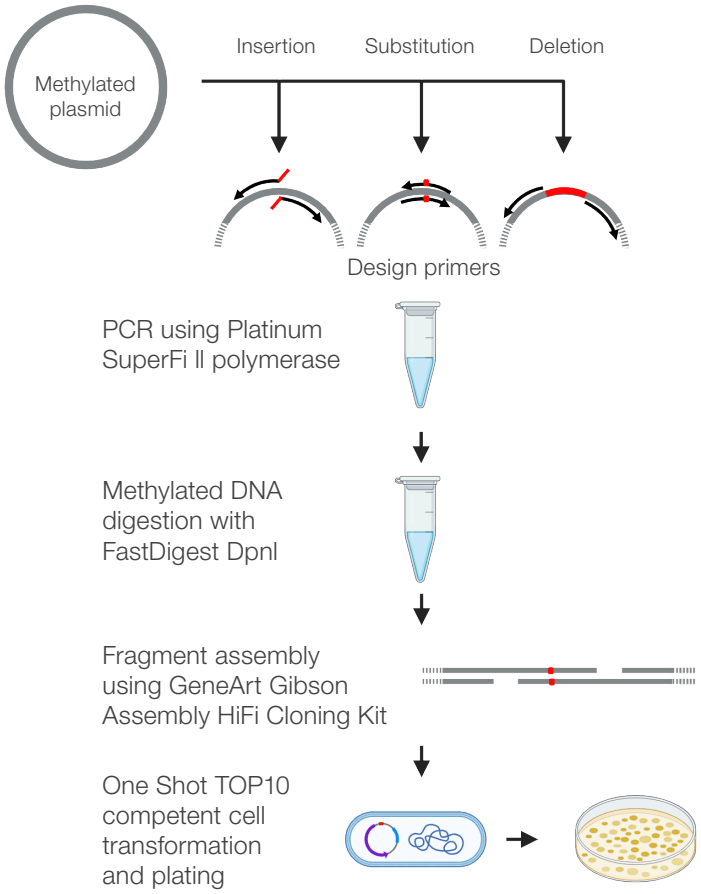

Figure 1. Overview of SDM using the GeneArt Gibson Assembly cloning kit. Following mutagenesis, DNA fragments of various lengths are uniformly assembled using complementary overlaps between fragments. Image created with BioRender.com.

Methods

Strategy of mutagenesis

Overall, the strategy of SDM using the Gibson Assembly method depends on the availability of the target DNA sequence. Single or multiple mutations can be introduced using an existing plasmid construct encoding the target sequence, or mutagenesis can be performed during subcloning if there is a need to transfer the gene sequence into a new DNA vector. We have used both strategies to mutate the eGFP gene in a Thermo Scientific™ pUC19 plasmid.

Primer design

To introduce mutations into an existing construct using the Gibson Assembly method, primers containing an overlapping homologous region for DNA assembly should be designed. Special attention should be given to the primer design if a reassembly (or recircularization) reaction using Gibson Assembly is planned. To prevent formation of primer-dimers during PCR and ensure efficient DNA fragment amplification, the overlapping region should be no longer than 8–10 bases at the primer 5′ end. Desired mutations should be introduced into an overlap sequence for the Gibson Assembly process. However, for introduction of multi-site mutations into an available DNA construct or for subcloning of a target DNA sequence, primer design can follow the common recommendations for the Gibson Assembly process: primers should have a longer homologous region of 20–40 bases added to the 5′ ends. This homologous region should contain introduced mutations when necessary. To design the most optimal primer, note that:

- For higher-order assembly, longer overlap regions tend to result in improved efficiency.
- The primer should not contain tandem repeats, homopolymers, or sequences with high secondary structure. Avoid GC content below 40% or above 60%.
- Overlapping homology regions with T_m higher than 50°C will improve efficiency.
- Complementary mutations in overlapping homologous regions should be introduced into both adjacent fragments.

Fragment preparation

For the single-mutation introduction experiments, primers were designed to incorporate Y66W and T203Y mutations into the eGFP gene cloned into the pUC19 plasmid (Table 1). The assembly scheme for creating the eGFP/T203Y variant is shown in Figure 2A. The eGFP/Y66W variant was similarly created using the primers listed in Table 1. To generate the double mutant (Y66W/T203Y) and the pentuple mutant (Y66W/N146I/M153T/V163A/N164), overlapping primer sets were designed (Table 1). The assembly schemes for these mutants are illustrated in Figures 2B and 2C.

Table 1. Primers used for fragment preparation.

Protein	Primer name	Sequence* 5′ → 3′	Fragment length
eGFP/T203Y	F2_T203Y R2_T203Y	gagctaccagtccgccctgagcaag ggtagctcaggtagtggttgtcgg	3,082 bp
eGFP/Y66W	F2_Y66W R2_Y66W	cccaggtcagggtggtcacg gacctggggcgtgcagtgcttc	3,082 bp
eGFP/Y66W/T203Y	F_Y66W GFP_RpUC	gcactgcacgcccccaggtcagggtggt atttcacacaggaaacagctatggtgagcaagggcgag	233 bp
	F_T203Y R_Y66W	gggcggactggtagctcaggtagtg ctgacctggggcgtgcagtgcttc	430 bp
	pUC_F R_T203Y	agctgtttcctgtgtgaaattg ctacctgagctaccagtccgccctgagcaa	2,483 bp
eCFP (eGFP/Y66W/N146I/M153T/V163A/N164H)	F_V163A_N164H R_N146I_M153T	ggatcttgaagtgcgccttgatgccg atcagccacaacgtctatatcacggccgac	67 bp
	GFP_F R_V163A_N164H	gggtgtcggggctggcttaattacttgtacagctcgtccatg catcaaggcgcacttcaagatccgcc	258 bp
	F_N146I_M153T R_Y66W	ccgtgatatagacgttgtggctgatgtagttg ctgacctggggcgtgcagtgcttc	271 bp
	F_Y66W pUC_R	gcactgcacgcccccaggtcagggtggt ttaagccagccccgacac	2,575 bp

* For help with primer design, we recommend SnapGene™ software. Mutations are indicated in red.

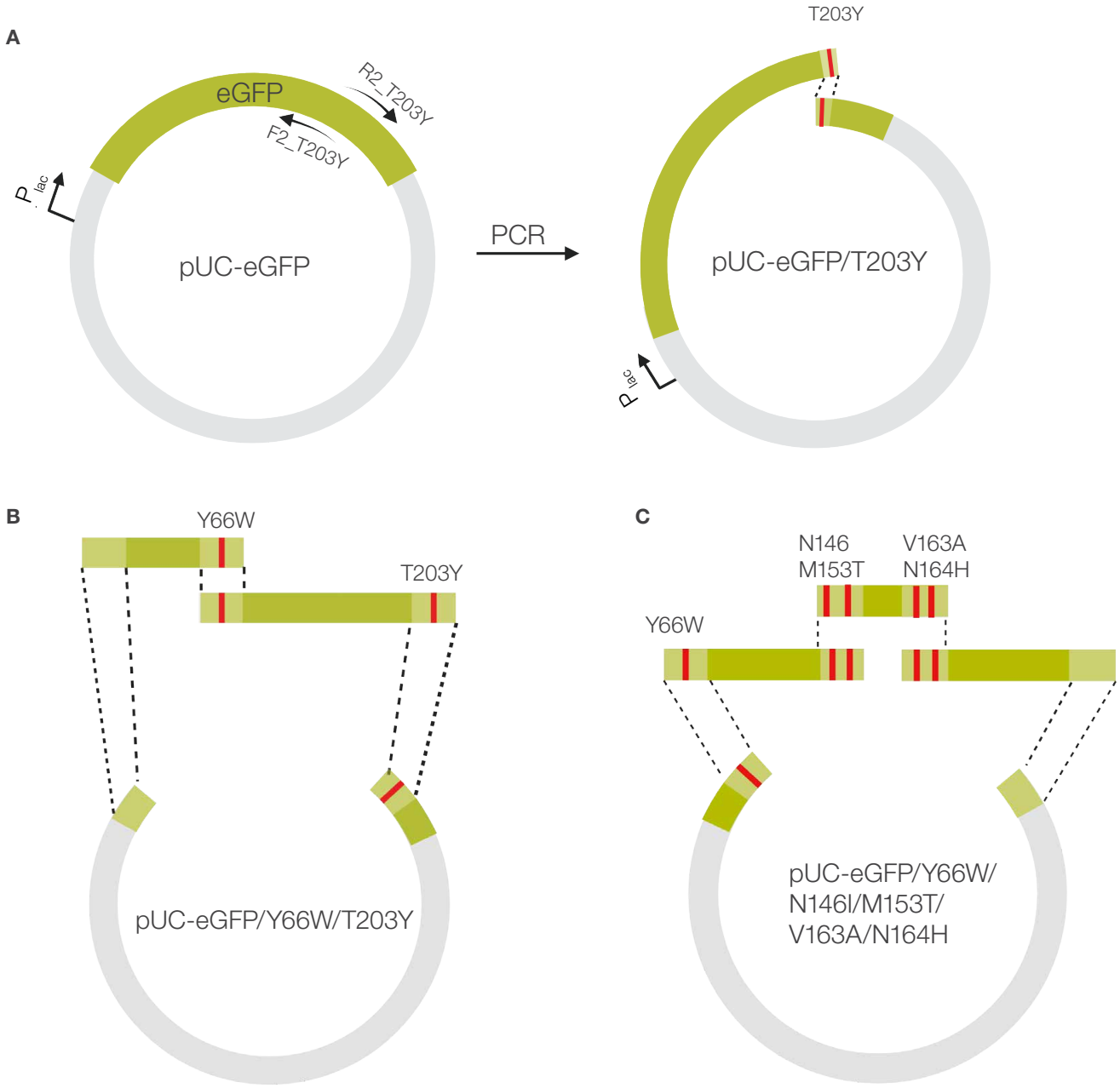

Figure 2. Assembly scheme for plasmids encoding eGFP mutants. (A) Single mutant; **(B)** double mutant; **(C)** pentuple mutant.

DNA fragments containing overlaps with introduced mutations were amplified using high-fidelity Platinum SuperFi II DNA Polymerase in a PCR reaction. The unique composition of SuperFi II Buffer allowed for a universal PCR protocol, eliminating the need to adjust the annealing temperature based on the T_m of the primers. The reaction setup and the universal cycling protocol are provided in Tables 2 and 3, respectively.

Table 2. PCR setup.

Component	Amount
Invitrogen™ Platinum™ SuperFi II Green PCR Master Mix (2X)	25 µL
Forward primer, 10 µM	2.5 µL
Reverse primer, 10 µM	2.5 µL
pUC-eGFP plasmid, 1 ng/µL	1 µL
Thermo Scientific™ Water, nuclease-free	To 50 µL

Table 3. Universal cycling protocol.

Step	Temperature	Time	Cycles
Initial denaturation	98°C	10 sec	1
Denaturation	98°C	10 sec	35
Annealing	60°C	10 sec	
Extension	72°C	15–30 sec/kb	
Final extension	72°C	5 min	1
Hold	4°C	Hold	–

After PCR, the template pUC-eGFP plasmid was digested using 1 µL of Thermo Scientific™ FastDigest™ DpnI. The digestion reaction was incubated at 37°C for 15 minutes and the enzyme then inactivated at 85°C for 5 minutes.

PCR products were analyzed on 1% agarose gels in Invitrogen™ TAE buffer and purified using the Thermo Scientific™ GeneJET™ PCR Purification Kit following user guide recommendations. Concentrations of the purified DNA were determined using a Thermo Scientific™ NanoDrop™ spectrophotometer.

Fragment preparation by fusion PCR

The pentuple eGFP mutant (Y66W/N146I/M153T/V163A/N164) contains mutations in close proximity to each other at positions 146, 153, 163, and 164 within a 55 nt DNA region. To introduce these mutations, a short amplicon (67 bp) was generated that results in less efficient DNA assembly when a standard approach is used. However, the number of transformant colonies and the cloning efficiency can be improved if the short fragment is attached to the adjacent fragment by fusion PCR before proceeding to the final Gibson Assembly reaction (Figure 3). The fusion of two short DNA fragments (67 bp and 258 bp) containing mutations within the overlap was performed as follows using the same universal cycling protocol as described in Table 3 and the setup parameters shown in Table 4.

Table 4. Fusion PCR setup.

Component	Amount
Platinum SuperFi II Green PCR Master Mix (2X)	25 µL
Primer GFP_F, 10 µM	2.5 µL
Primer R_N146I_M153T, 10 µM	2.5 µL
Insert 1 (67 bp)	0.05 pmol
Insert 2 (258 bp)	0.05 pmol
Water, nuclease-free	To 50 µL

The PCR product was analyzed on a 1% agarose gel in TAE buffer and purified using the GeneJET PCR Purification Kit following user guide recommendations. The concentration of the purified DNA was determined on a NanoDrop spectrophotometer.

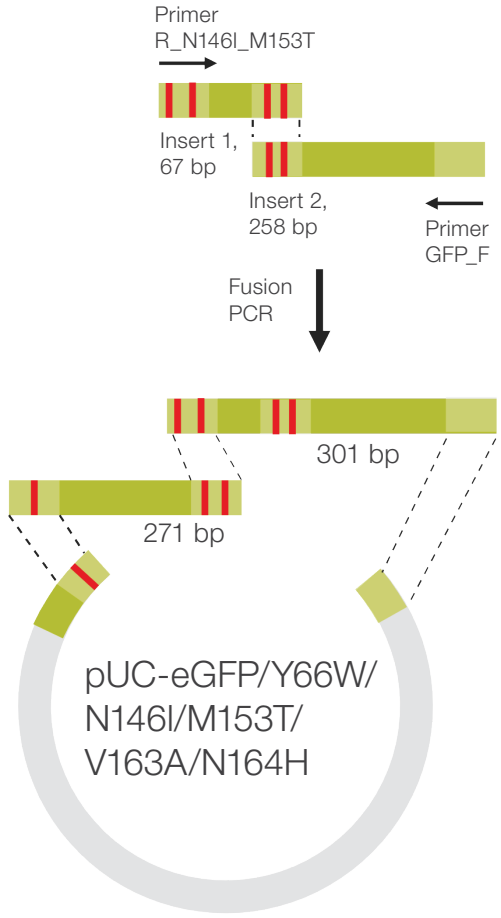

Figure 3. Pentuple eGFP mutant assembly scheme using three DNA fragments of 271, 301 (fusion PCR product), and 2,575 bp.

Fragment assembly

The DNA fragments generated above can be used directly for assembly with the GeneArt Gibson Assembly HiFi Cloning Kit as shown in Table 5.

Table 5. GeneArt Gibson Assembly reaction setup.

Component	Amount
Vector*	0.08 pmol
Each insert*	0.08 pmol
GeneArt™ Gibson Assembly™ HiFi Master Mix	10 µL
Water, nuclease-free	To 20 µL
Incubate at 37°C for 15 min**	

* In the recircularization reaction (see Figure 2A), 0.08 pmol of the fragment should be used.

** Longer incubation times may result in fragment degradation because of exonuclease activity.

After incubation, place the reaction mixes on ice and proceed to the transformation step.

Transformation with HiFi Master Mix

The GeneArt Gibson Assembly reactions were diluted 5-fold in nuclease-free water, and 1 µL of each dilution was added to a vial of Invitrogen™ One Shot™ TOP10 Chemically Competent *E. coli* and mixed gently. The transformation mixes were incubated on ice for 20–30 minutes and heat-shocked for 30 seconds at 42°C.

After the heat shock treatment, the tubes were immediately transferred to ice and incubated there for 2 minutes; then 450 µL of room temperature Invitrogen™ S.O.C. medium was added to the transformation mixes, which were then shaken at 300 rpm and incubated at 37°C for 1 hour to allow the cells to recover.

From each tube of recovered cells, 100 µL was plated on an LB agar plate containing 100 µg/mL ampicillin, and incubated at 37°C overnight. One Shot TOP10 Chemically Competent *E. coli* allows expression from the *lac* promoter without the use of IPTG. In these experiments, the pUC19 vector was used. Expression of eGFP and its mutants was successful if the circular plasmid was generated during assembly and subsequently transformed the cells.

To detect eGFP mutants with known differences in fluorescence shift, transformants were analyzed using an Amersham™ Typhoon™ 5 biomolecular imaging system. Plates were scanned using 488 nm and 532 nm lasers, images were overlaid, and results were visually analyzed.

Note: To increase transformation efficiency, electroporation may be used instead. Invitrogen™ ElectroMAX™ DH10B electrocompetent *E. coli* cells are compatible with the given Gibson Assembly reaction protocol.

DNA sequencing

Plasmid DNA from selected transformant colonies was amplified using rolling circle amplification (RCA), and sequenced.

Using a pipette tip, a transformant colony was picked from a plate and resuspended in 10 µL of nuclease-free water in a thin-walled, nuclease-free PCR tube. To lyse cells, the tubes were placed in a thermal cycler with the lid set at 105°C, for 3 minutes at 95°C. The tubes were then transferred to ice. RCA reactions were prepared as described in Table 6. The reactions were mixed thoroughly but gently by pipetting or vortexing, then centrifuged briefly to collect the contents at the bottoms of the tubes.

Table 6. RCA reaction setup.

Component	Amount
Lysed bacteria	5 µL
Thermo Scientific™ EquiPhi29™ DNA Polymerase Reaction Buffer	2 µL
Thermo Scientific™ dNTPs (10 mM each)	2 µL
Exonuclease-resistant random primers (500 µM)	2 µL
Thermo Scientific™ EquiPhi29™ DNA Polymerase (10 U/µL)	1 µL
Thermo Scientific™ DTT (100 mM)	0.2 µL
Water, nuclease-free	To 20 µL

Reactions were incubated in the thermal cycler with the lid set at ≥75°C, for 1 hour at 42°C, followed by 10 minutes at 65°C to inactivate the DNA polymerase. The RCA products can be kept at 4°C overnight or at –20°C for long-term storage.

Note: The RCA products may be viscous due to the high yield of high molecular weight DNA; 50-fold dilution with nuclease-free water is recommended before treatment with phosphatase and exonuclease I.

Primers and unincorporated nucleotides should be removed from the RCA mixture. For this reason, digestion with Thermo Scientific™ FastAP™ Thermosensitive Alkaline Phosphatase and Exonuclease I was performed as described in Table 7.

Table 7. Cleanup reaction.

Component	Amount
RCA reaction product (1:50 diluted)	5 µL
FastAP Thermosensitive Alkaline Phosphatase, 1 U/µL	1 µL
Exonuclease I, 20 U/µL	0.5 µL

Samples were incubated in the thermal cycler with the lid set at ≥75°C, for 15 min at 37°C, followed by 15 min at 85°C to inactivate the enzymes. The whole sample was directly used for DNA sequencing without further purification.

Results and discussion

DNA fragments for assembly were successfully generated by PCR using Platinum SuperFi II DNA Polymerase. All amplicons were obtained with high yields and specificity, using a universal 60°C annealing protocol with all primers. The PCR fragments were digested with FastDigest DpnI to remove templates, followed by column purification and assembly using the GeneArt Gibson Assembly HiFi Cloning Kit.

Hundreds of transformant colonies were obtained for single eGFP mutants—eGFP/Y66W and eGFP/T203Y (Table 8). Preliminary screening of correct transformants was performed by analyzing fluorescence. Reduced fluorescence or an emission shift compared to eGFP suggested a recombinant clone. Colonies containing incorrect fragment assemblies usually did not generate fluorescence due to deletions in the GFP gene. To evaluate the accuracy of the DNA assembly reaction, 20 colonies of each reaction were amplified by RCA and subjected to Sanger sequencing. Data analysis confirmed that 90–95% of colonies contained the desired sequence changes.

Table 8. Assembly of eGFP and mutants, and fluorescence characteristics.

Protein/mutation	Assembly fragment length (bp)	CFU/plate	Cloning efficiency (%)	Excitation* (nm)	Emission* (nm)
eGFP	–	–	–	488	509
eGFP/Y66W	3,082	882	95	450	494
eGFP/T203Y	3,082	553	85	514	524
eGFP/Y66W,T203Y	233, 430, 2,483	63	37	458	504
eCFP (eGFP/Y66W/N146I/M153T/V163A/N164H)	67, 258, 271, 2,575	41	55	434	476
	301, 271, 2,575	375	67	434	476

* Reported by Sawano and Miyawaki [2].

The assembly of the pentuple eGFP mutant was the most challenging, as the N146I, M153T, V163A, and N164H mutations were in close proximity and required careful consideration in designing assembly junction and overlapping sequences. Different approaches could be used to generate the pentuple mutant: (i) generation of a short dsDNA fragment (67 bp as indicated above), (ii) bridging of two double-stranded DNA fragments with an oligonucleotide, or (iii) fusion of the short fragment (insert 1) to the adjacent fragment (insert 2 in Figure 3) by fusion PCR before proceeding to the assembly reaction [3]. All of these approaches were tested during this study, and transformant colonies were successfully obtained. However, the results demonstrated that fusion of the 67 bp with the 258 bp DNA fragment to generate a longer 301 bp fragment allowed more efficient assembly: 10 times as many transformant colonies were obtained, and sequencing revealed that a higher proportion of them (67% vs. 55%) contained the correct gene structure. We have noticed that a higher error rate of transformants generated by bridging of two double-stranded DNA fragments with an oligonucleotide in the assembly experiment was associated with indel mutations in the long oligonucleotide (55 nt) (data not shown).

Conclusions

These results demonstrate how the GeneArt Gibson Assembly HiFi Cloning Kit can be used to perform SDM to introduce single or multiple mutations simultaneously. For challenging mutagenesis experiments—e.g., when mutations are in close proximity—a significant improvement in cloning performance and accuracy was achieved using the fusion PCR approach. Single mutations (Y66W and T203Y), double mutations (Y66W/T203Y), and multiple mutations (Y66W/N146I/M153T/V163A/N164H) were introduced into the eGFP protein to shift the fluorescence intensity or emission wavelength (Figure 4).

For introducing both single and multiple mutations through SDM, the GeneArt Gibson Assembly HiFi Cloning Kit offers significant advantages over conventional mutagenesis methods in terms of time efficiency, user-friendliness, and cost-effectiveness.

A **B**

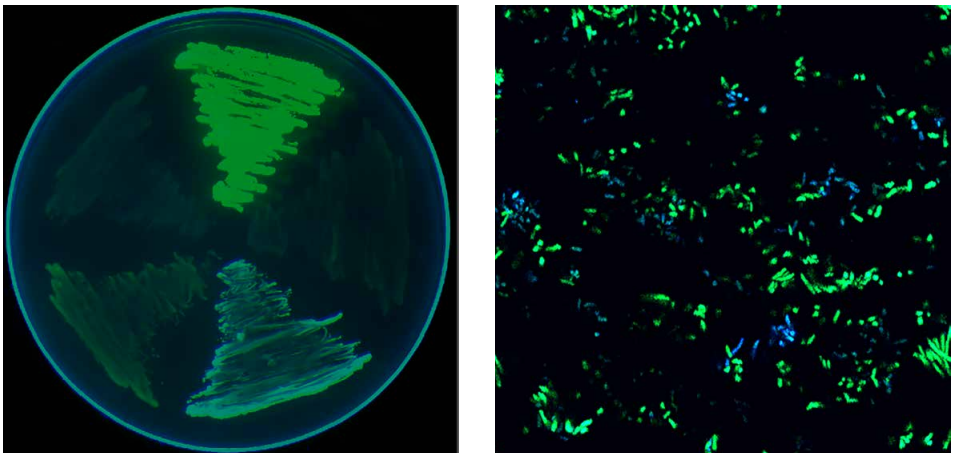

Figure 4. *E. coli* strains containing eGFP and various mutants created by Gibson Assembly cloning. (A) Fluorescence image of streaked *E. coli* strains producing eGFP, eGFP/Y66W, eGFP/T203Y, eGFP/Y66W/T203Y, and eCFP. **(B)** Confocal fluorescence microscopy image of *E. coli* population expressing eGFP (green) and eGFP/T203Y (blue).

References

1. Heim R, Tsien RY (1996) Engineering green fluorescent protein for improved brightness, longer wavelengths and fluorescence resonance energy transfer. *Curr Biol* 6(2):178–182. doi: 10.1016/S0960-9822(02)00450-5
2. Sawano A, Miyawaki A (2000) Directed evolution of green fluorescent protein by a new versatile PCR strategy for site-directed and semi-random mutagenesis. *Nucleic Acids Res* 28(16):E78. doi: 10.1093/nar/28.16.e78
3. Hilgarth RS, Lanigan TM (2020) Optimization of overlap extension PCR for efficient transgene construction. *MethodsX* 7:100759. doi: 10.1016/j.mex.2019.12.001

Ordering information

Product	Cat. No.
GeneArt Gibson Assembly HiFi Cloning Kit	A46624
Platinum SuperFi II PCR Master Mix	12368010
Platinum SuperFi II Green PCR Master Mix	12369010
FastDigest DpnI	FD1703
GeneJET PCR Purification Kit	K0701
One Shot TOP10 Chemically Competent *E. coli*	C404010
ElectroMAX DH10B Cells	18290015
One Shot LB Agar Plates with 100 µg/mL Ampicillin	A55802
EquiPhi29 DNA Amplification Kit	A65393
FastAP Thermosensitive Alkaline Phosphatase (1 U/µL)	EF0651
Exonuclease I (20 U/µL)	EN0581

Learn more at **thermofisher.com/gibsonassembly**