Chapter 5

High-Throughput Allelic Replacement Screening in *Bacillus subtilis*

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Abstract

Site-directed mutagenesis is a key tool in the analysis of biological mechanisms. We have established an efficient and systematic gene targeting strategy for *Bacillus subtilis* based on the Golden Gate cloning methodology. Our approach permits the introduction of single or multiple point mutations or of heavily engineered alleles into the endogenous gene locus in a single step using a 96-well microtiter plate format. We have successfully applied this system for high-throughput functional screening of resized variants of the Structural Maintenance of Chromosome (Smc) protein and for exhaustive cysteine cross-linking mutagenesis. Here we describe, in detail, the experimental setup for high-throughput introduction of modifications into the *B. subtilis* chromosome. With minor modifications, the approach should be applicable to other bacteria and yeast.

Key words Golden Gate assembly, *Bacillus subtilis*, Gene targeting, High-throughput screening, Cysteine scanning

1 Introduction

The study of protein function and activity in the natural context of a living cell, sometimes referred to as 'in vivo biochemistry', has become feasible in many model organisms. Experimental readouts range from simple growth assays, protein cross-linking to high-end microscopy using fluorescent tags and reporters. Several techniques offer fast turn-around and thus become limited by the generation of genetic modifications. Here, we combine Golden Gate DNA assembly with natural genetic competence of *B. subtilis* to quickly generate large collections of mutations in any gene of interest.

Many bacteria exhibit *natural competence*, that is, the ability to incorporate DNA of exogenous origin into the genome by replacing homologous segments of the chromosome [1–5]. In *B. subtilis*, competence is naturally induced under starvation conditions by the expression of the DNA uptake machinery [6, 7]. Natural competence enables efficient replacement of an endogenous

Anjana Badrinarayanan (ed.), *SMC Complexes: Methods and Protocols*, Methods in Molecular Biology, vol. 2004,
https://doi.org/10.1007/978-1-4939-9520-2_5,

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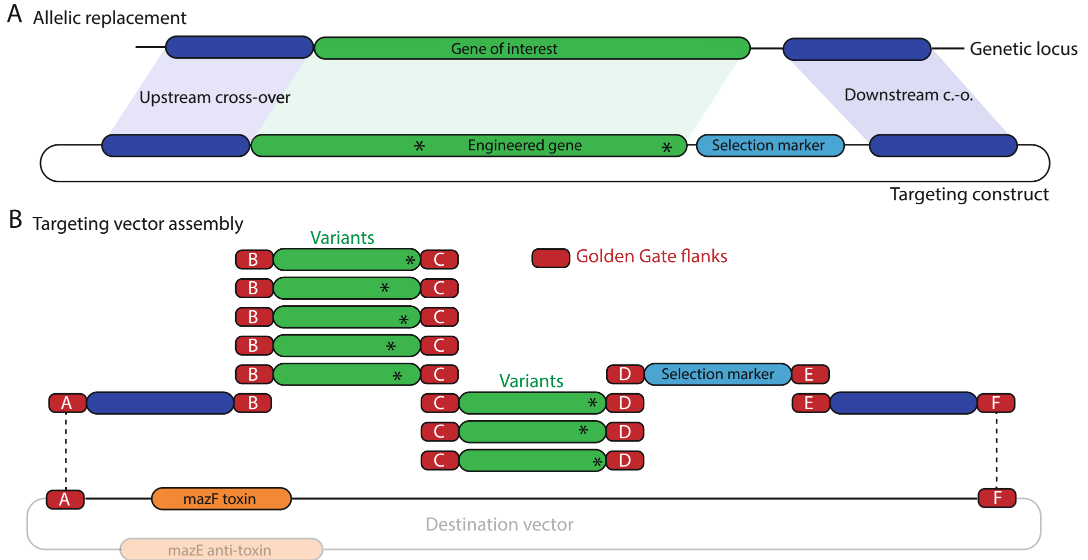


Fig. 1 (**a**) Principle of allelic replacement of the genetic locus by the targeting construct in *B. subtilis*. Selection marker allows for the selection of clones having incorporated the targeting construct. (**b**) Assembly of the targeting vector. DNA fragments are flanked by unique Golden Gate overhangs that allow for the directional assembly of multiple DNA fragments into a targeting construct by the Golden Gate reaction

gene locus by recombinant DNA via double crossover recombination (Fig. 1a). This allelic replacement strategy ensures that gene dosage (on replicating bacterial chromosomes) of recombinant alleles are as close to wild-type levels as possible. Inserting a selection marker upstream or downstream of the operon limits any effects on the expression of neighboring genes. In case of large operons, a toxin-antitoxin system can be used to genetically link the desired mutations with a more distant marker to facilitate the isolation of mutant clones (*see* below). Performing direct transformation of recombinant DNA targeting constructs into *B. subtilis* bypasses the need for cloning in *E. coli*, an endeavor which is often hampered by gene toxicity. However, if needed, the use of suitable destination vectors (e.g., pSG436, Table 1) enables the recovery of gene targeting constructs from *E. coli*. Altogether, allelic replacement by natural competence provides an efficient and precise means for genetic alterations.

We have based the generation of recombinant DNA molecules on *Golden Gate cloning* [8, 9]. It achieves high-efficiency, directional assembly of multiple DNA fragments in a single reaction—leaving no cloning marks (Fig. 1b). Assembly does not involve DNA synthesis and is virtually free of point mutations. At first, the desired gene-of-interest is split into smaller DNA fragments (typically ~1 kb long). All fragments are flanked at both ends by recognition sites for a Type IIS restriction enzyme (such as BsaI), which cleaves outside the recognition site and produces

Table 1
Vectors

Gene	Plasmid name	5′ BsaI overhang	3′ BsaI overhang	Addgene deposition
Backbone vectors				
Destination vector pET-Gate2 ccdB	pSG436	CGAG (RU)	CCAT (RD)	#117116
Destination vector with mazF toxin pET-Gate2 mazEF	pSG1525	CGAG (RU)	CCAT (RD)	#117117
Vectors encoding antibiotic resistance gene				
Chloramphenicol resistance cassette pJet1.2 cat	pSG408	AATA (Rup)	TCTA (Rdo)	#117119
Spectinomycin resistance cassette pJET1.2 spcR	pSG487	AATA (Rup)	TCTA (Rdo)	#117120
Erythromycin resistance cassette pJET1.2 ermB	pSG682	AATA (Rup)	TCTA (Rdo)	#117121
Kanamycin resistance cassette pJET1.2 kanR	pSG723	AATA (Rup)	TCTA (Rdo)	#117122
Vectors encoding tag cassettes				
C-terminal GFP tag pJET1.2 sfGFP	pSG710	AGCG (RtagN)	TAAG (RtagC)	#117123
C-terminal Tev-HaloTag pJET1.2 TEV-HaloTag	pSG711	AGCG (RtagN)	TAAG (RtagC)	#117124
C-terminal HA6 tag pJET1.2 HA6-tag	pSG1106	AGCG (RtagN)	TAAG (RtagC)	#117126
C-terminal Pk6 tag pJET1.2 Pk6-tag	pSG1107	AGCG (RtagN)	TAAG (RtagC)	#117127
C-terminal Tev-AviTag tag pJET1.2 TEV-AviTag	pSG1249	AGCG (RtagN)	TAAG (RtagC)	#117128
Constant vectors for reconstitution of the Smc locus				
smc upstream homology region pJET1.2 ’rncS	pSG1745	CGAG (RU)	TTCC	#117129
ftsY gene cassette pJET1.2 ftsY	pSG849	CCTG (R1)	AATA (Rup)	#117130
smc downstream homology region pJET1.2 sivC	pSG841	TCTA (Rdo)	CCAT (RD)	#117131

DNA overhangs of freely designable composition. Importantly, mutations, truncations and insertions are generated at the junction of two Golden Gate DNA fragments. Ideally, all fragments are devoid of internal restriction sites for the chosen Type IIS enzyme (*see* **Note 1**). Fragments are PCR-amplified, separately cloned into donor vectors, and sequenced. DNA fragments for selection markers and tags are reused for several genes of interest (Fig. 1b), whereas upstream and downstream homology regions are specific for a certain gene of interest. Single-use fragments carrying a specific mutation, the "variable fragments" (Fig. 1b), are typically used for assembly directly as PCR product without prior cloning and sequencing. All fragments are directionally assembled in a one-pot DNA cleavage/ligation reaction to reconstitute the targeting construct that is directly mixed with competent *B. subtilis* cells.

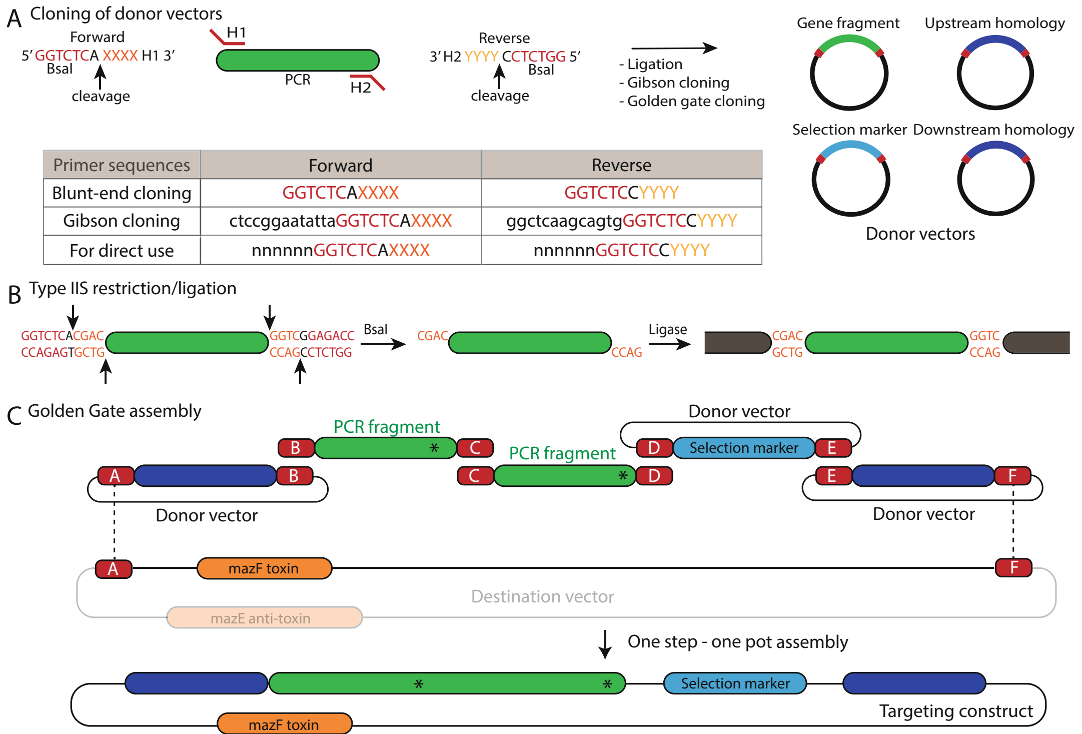
Here, we provide an example for the allelic replacement strategy based on the *B. subtilis smc* gene. Using this strategy, we have performed systematic screens expressing single cysteine Smc mutants and internally truncated versions of Smc in vivo, respectively [10, 11].

1.1 Generation of Donor Vectors and DNA Fragments

The *B. subtilis smc* targeting constructs contain flanking upstream and downstream homology regions of roughly half a kilobase, covering the *rncS* and *sivC* genes, respectively, to direct recombination to the *smc* locus (Figs. 2 and 3). A marker cassette for selection of recombinant clones is placed in-between the convergent *smc-ftsY* and *sivC* transcription units, thus avoiding detrimental impact on gene regulation. Fragments for upstream and downstream homology sequences, the *ftsY* gene and the resistance marker—all flanked by BsaI recognition sites (*see* **Note 1**)—are first introduced into donor vectors using a convenient method of choice, such as conventional cloning, Gibson cloning or Golden Gate cloning (Fig. 2a). The variable fragments typically divide the full *smc* coding sequence into two or more parts, whereby the fragment junctions introduce the desired modifications. Systematic mutation of *smc* is achieved by gradually shifting the position of the junction. Matching variable fragments that reconstitute *smc* alleles are typically generated by PCR in a 96-well format.

1.2 Golden Gate Assembly Reaction and Strain Generation

The Golden Gate reaction enables the directional assembly of the different fragments in a single reaction (Fig. 2c). Equimolar amounts of the different donor vectors and PCR fragments are mixed with the type IIS restriction enzyme BsaI, T4 DNA ligase and a suitable destination vector. During the assembly reaction, BsaI generates matching 4-bp overhangs which are joined together by the ligase to generate recombinant DNA devoid of the particular IIS recognition sites (Fig. 2b). The targeting construct is assembled into a large (>5 kb) circular nonreplicating DNA molecule, which



Primer sequences	Forward	Reverse
Blunt-end cloning	GGTCTCAXXXX	GGTCTCCYYYY
Gibson cloning	ctccggaatattaGGTCTCAXXXX	ggctcaagcagtgGGTCTCCYYYY
For direct use	nnnnnnGGTCTCAXXXX	nnnnnnGGTCTCCYYYY

Fig. 2 (**a**) DNA fragments are generated by PCR amplification using primers containing the appropriate Golden Gate DNA flanks. Blunt end cloning (e.g., using pJET2.1) does not require extra bases, while Gibson cloning relies on specific 5′ overhang sequences. For direct use of PCR products in Golden Gate assembly, six additional 5′ flanking bases are added to allow for efficient restriction digest. (**b**) Principle of Golden Gate restriction/ligation. (**c**) Golden Gate assembly of donor vectors and linear DNA fragments (PCR products) in a one step—one pot assembly reaction to generate the targeting construct. The *mazF* toxin gene encodes for an RNAse that will kill *B. subtilis* cells having incorporated the vector backbone during transformation. Asterisk indicates the position of the mutations introduced in the variants

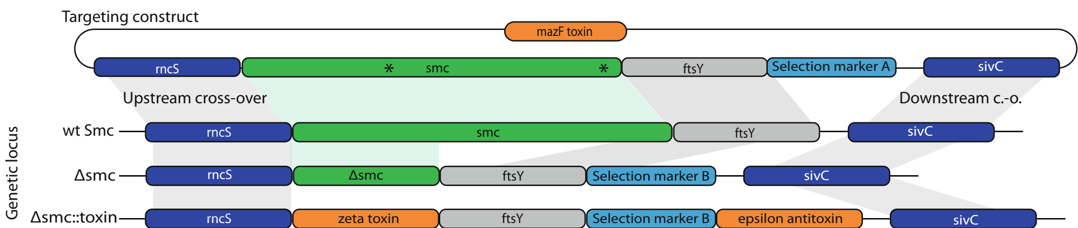


Fig. 3 Allelic replacement of the targeting construct in different *B. subtilis* background. Transformation of a wild-type strain ("wt Smc") results in a mixture of transformants having wild-type or mutant *smc* alleles. Transformation in *smc* null strains ("Δsmc") allows for the isolation of *smc* mutants by selecting for Smc function by growth on nutrient rich medium. If nonfunctional *smc* alleles are generated, the zeta/epsilon toxin/antitoxin system at the *smc* locus ("Δsmc::toxin") ensures that only clones that have incorporated the modified *smc* allele are able to grow. Incorporation of the full targeting construct will remove both toxin and antitoxin from the endogenous locus. If only the selection marker is replaced, the zeta toxin will kill the recipient cell

facilitates efficient uptake by *B. subtilis*. Finally, constructs are transformed into a suitable *B. subtilis* strain in 96-well format. To counter-select single crossover events the backbone of the destination vector harbors the *E. coli mazF* gene, coding for an RNase toxin which kills cells that have incorporated the vector backbone into the genome (Fig. 3 and Table 1, pSG1525) [12, 13]. Recombinant clones can then be verified by PCR and DNA sequencing or can be used directly for downstream high-throughput experiments.

Targeting constructs are typically transformed into strains where the endogenous gene has been deleted (*smc* null) and replaced by a selection marker to select for marker exchange in the recombinant clone (Fig. 3). However, due to stochastic recombination site selection in the homology regions, a certain fraction of transformants will incorporate the selection marker but not the desired mutation(s). To improve linkage between the marker and the *smc* mutations, we have designed a counter-selection strategy based on the zeta/epsilon toxin/antitoxin system [14, 15]. We have replaced the *smc* open reading frame by the coding sequence of the zeta toxin and integrated an epsilon antitoxin cassette downstream of *ftsY* (Fig. 3). Upon recombination, the antitoxin cassette gets replaced by the resistance marker from the targeting construct. Clones that are not recombinant for *smc* will thus be killed by unsuppressed zeta toxin activity.

Overall, our genetic engineering approach offers an efficient way of generating strains for testing functional properties of the *B. subtilis* Smc protein. The approach is particularly suited for high-throughput experiments and has allowed us to generate hundreds of cysteine mutants for site-specific in vivo cross-linking as well as nearly thousand mutants with internal truncations and/or replacements. We have also applied this approach to other *B. subtilis* genes. Extension of the methodology to other organisms and most particularly the ones exhibiting efficient DNA transformation seems feasible and may enable similar screening experiments in other contexts.

2 Materials

2.1 Growth Medium

1. LB medium: 10 g/L tryptone, 5 g/L yeast extract, 10 g/L sodium chloride (*see* **Note 2**).
2. Nutrient agar: 5 g/L peptone, 2 g/L yeast extract, 1 g/L meat extract, 5 g/L sodium chloride, 15 g/L Difco Bacto Agar.
3. SMM solution: 17.9 g/L dipotassium hydrogen phosphate trihydrate or 14 g/L dipotassium hydrogen phosphate, 6 g/L potassium dihydrogen phosphate, 2 g/L ammonium sulfate, 1 g/L trisodium citrate dihydrate, 0.2 g/L magnesium sulfate heptahydrate.
4. 50 % (w/v) glucose solution.

5. 10 % (w/v) glutamate solution.
6. 0.2 % (w/v) L-tryptophan solution.
7. 30 % (w/v) Difco Bacto Agar (called 2× Agar).
8. 246.47 g/L magnesium sulfate heptahydrate (1 M).
9. 20 % (w/v) casamino acids solution.
10. 0.22 % (w/v) ferric ammonium citrate solution.

2.2 PCR and Cloning

1. High-fidelity DNA polymerase, for example Phusion Hot Start II polymerase.
2. 10 mM dNTP mix.
3. 96-PCR well plate and 200 μL PCR strips.
4. Adhesive PCR plate seals.
5. Suitable restriction enzymes for checking constructs.
6. 0.8 % agarose gels in TBE buffer containing a suitable DNA stain (e.g., SYBR Safe).
7. PCR product and plasmid purification kits.
8. NucleoFast 96 PCR Plate.
9. Nanodrop spectrophotometer.
10. Quant-iT kit.
11. Components specific for the chosen PCR fragment cloning method (e.g., pJET blunt-end ligation kit and Gibson Master mix) (*see* Subheading 2.4).
12. Competent DH5α *E. coli* cells or alternative cloning host.

2.3 Golden Gate Assembly Reaction

1. 10 U/μL BsaI (or 20 U/μL BsaI-HFv2. Do not use BsaI-HF enzyme!).
2. 1 U/μL T4 DNA ligase.
3. 10× T4 DNA ligase buffer.
4. Thermocycler.

2.4 Gibson Master Mix (2×)

1. Buffer (2×): 200 mM Tris–HCl pH 7.5, 20 mM $MgCl_2$, 20 mM DTT, 2 mM NAD, 0.4 mM each dNTPs; 10 % (w/v) PEG 8000.
2. 10 U/μL T5 Exonuclease: final concentration in Gibson Master mix: 8 U/mL.
3. 2 U/μL Phusion High-Fidelity DNA Polymerase: final concentration in Gibson Master mix: 0.5 U/mL (*see* **Note 3**).

2.5 *Bacillus subtilis* Transformation

1. 100 mL Erlenmeyer flasks.
2. 96-well flat bottom microplates.
3. Air-permeable membrane seals.

3 Methods

3.1 Medium Preparation

1. SMG medium: mix 100 mL of SMM solution with 1 mL glucose solution, 1 mL glutamate solution, 1 mL L-tryptophan solution. Add antibiotics as required.
2. SMG agar: 2× Agar is mixed with an equal amount of 2× SMM solution (*see* **Note 4**) immediately after autoclaving and stored at 55 °C until use. Before pouring plates, 100 mL of the mixture is supplemented with 1 mL glucose solution, 1 mL glutamate solution, 1 mL L-tryptophan solution. Add antibiotics as required.
3. Competence medium: 100 mL of SMM solution is mixed with 1 mL glucose solution, 1 mL L-tryptophan solution, 600 μL of magnesium sulfate solution, 100 μL of casamino acids solution and 50 μL ferric ammonium citrate solution.
4. Starvation medium: 100 mL of SMM medium is mixed with 1 mL glucose solution, 600 μL of 1 M magnesium sulfate solution. All solutions are autoclaved before mixing.

3.2 Construction of the Donor Vectors

The initial step requires the generation of vectors encoding the DNA fragments for gene targeting, that is, upstream and downstream homology regions and the selection marker, plus any other parts used in multiple constructs such as tagging cassettes (Fig. 2a). We strongly recommend reading instructions on Golden Gate cloning (such as Engler et al. [8]) as well as assembling the DNA sequence of the complete gene targeting construct in silico before designing DNA primers.

1. Design oligonucleotide primers containing a BsaI recognition site, the 4 nt overhang sequence (*see* **Note 5**), and about 15–20 nt (H1 and H2) homologous to the target sequence (Fig. 2a). Depending on the cloning strategy used for generating the donor vectors, the 5′ end of the primer may vary (Fig. 2a).
2. Perform PCR reactions using a high-fidelity DNA polymerase, following the manufacturer's instructions. Check the size of the amplified DNA fragment by running an agarose gel.
3. Purify the PCR products and clone into the donor vectors. Set up reactions depending on the cloning strategy chosen (e.g., conventional cloning, Golden Gate cloning or Gibson cloning).
4. For Gibson cloning: 12.5 nmol of PCR amplified pIDC destination vector [16] (*see* **Note 6**) is mixed in a 2:1 molar ratio of insert PCR DNA and diluted with water to 5 μL. DNAs are mixed with 5 μL of 2× Gibson master mix and incubated for 1 h at 50 °C. 2 μL of the reaction is transformed into *E. coli*

cells expressing the pir replication initiator protein (e.g., *pirHC*).

5. Plate the transformation reaction on nutrient agar plates containing the appropriate antibiotic. Incubate at 37 °C overnight.
6. Inoculate colonies in 5 mL LB medium with appropriate antibiotics and incubate overnight at 37 °C in a shaker.
7. Purify plasmids according to the manufacturer's instruction for the purification kit. Check plasmids by restriction digest/agarose gel electrophoresis and sequencing.

3.3 Generation of a PCR Product Library

The target gene is divided into two or more fragments which are amplified by PCR, whereby the primers at the fragment junction ("variable primers") introduce the desired mutations.

1. PCRs are typically performed using a constant and a variable primer. The constant primer is reused, whereas the variable primer is unique for each fragment/mutation and is located at the seam of two fragments.
2. To ensure efficient PCR and assembly, design PCR primers with BsaI recognition sites according to the following rules (*see* **Note 7**):
   - If possible, use a 3′ homology region with a melting temperature of more than 50 °C.
   - If possible, avoid self-complementarity of the 4–17 bases at the 3′ end (to prevent primer dimerization).
   - If possible, avoid GC or CG dinucleotides at the 3′ end (to prevent primer dimerization).
   - If possible, avoid a T at the 3′ position (to prevent promiscuous annealing).
   - If possible, have at least one G/C within the last four bases (to prevent promiscuous annealing).
   - To reduce variations in ligation efficiency, try to make BsaI overhangs for the different mutants of a screen as similar as possible. For example, if a cysteine is to be introduced, this can be done by constraining the overhangs at the mutation site to GTGC and TTGC, whereby the last three nucleotides (TGC) encode cysteine and the first nucleotide is part of the upstream codon. Recode the upstream codon if required to limit the number of different BsaI overhangs.
   - Make sure that BsaI overhangs at the variable fragment seam are unique, that is, not used in any of the other fragments.
   - Make sure that BsaI overhangs are nonpalindromic (e.g., GATC would not be a valid overhang).

- Add at least 6 nt of random sequence to the 5′ end of the BsaI recognition site to ensure efficient cleavage.
- When incorporating point mutations or insertions ensure that about 15–20 nt template homology are present at the 3′ end (H1 and H2).
- For larger scale experiments organize variable primers in 96-well plates for convenience.

3. Dilute variable primers to 2.5 μM in 96-well format. Store at −20 °C for reuse.
4. 25 μL PCR reactions are set up in 96-well format using a high-fidelity enzyme according to the manufacturer's instruction. For high-throughput experiments, the use of a Hot-Start polymerase is highly recommended. Prepare a sufficient volume of 1.25× concentrated master mix containing all PCR components except for the variable primers. Distribute 20 μL aliquots into 96-well PCR plates.
5. Add 5 μL of the variable primer dilutions to the wells.
6. Use PCR conditions as specified by the polymerase manufacturer.
7. Check success of the reactions by running 2 μL of PCR product on an agarose gel.
8. Purify PCR products on NucleoFast 96 PCR plates and quantify using Nanodrop or Quant-iT fluorescence spectroscopy (*see* **Note 8**).
9. Dilute PCR products in a 96-PCR well plate to 20 nM in water.

3.4 Golden Gate Assembly Reactions

Once all vectors and PCR products are obtained, the Golden Gate assembly reactions are set up and transformed into *B. subtilis.*

1. Measure plasmid concentration with a NanoDrop spectrophotometer.
2. Program the thermocycler. The cycle is composed by the following steps: a loop composed of 2 min at 37 °C followed by 5 min at 16 °C repeated 50 times, one step at 50 °C for 5 min and one step at 80 °C for 5 min. Reaction is then stored in the thermocycler at 4 °C until it is picked up.
3. Mix the donor vectors at 2 nM each. Distribute 10 μL aliquots into 96-well plates.
4. Add 1 μL of each of two diluted PCR product (1 nM final in 20 μL) to each well.
5. Prepare 2.5× enzyme master mix: 1.25 U/μL BsaI (or 0.5 U/μL BsaI-HFv2) and 0.125 U/μL T4 Ligase in 2.5× T4 DNA ligase buffer.

6. Add 8 μL enzyme mix to each well. Mix by pipetting up and down.
7. Place the plates in the thermocycler and start the run.

3.5 Transformation of Bacillus subtilis

Any method for generating naturally competent cells of *B. subtilis* should be suitable for transformation. The success of high-throughput applications relies on efficient transformation and may thus require prior optimization. The following protocol yields good results for *smc* mutant strains.

1. Patch the *B. subtilis* strain to transform on an SMG agar plate and incubate overnight at 37 °C (*see* **Note 9**).
2. Inoculate multiple flasks containing 10 mL of competence medium with cells from the patch. Incubate at 37 °C overnight in a shaker.
3. In the morning, dilute 600 μL of the overnight cultures into separate flasks with 10 mL of fresh competence medium. Grow at 37 °C for 3 h with shaking.
4. Add 10 mL of starvation medium, incubate for 2 h at 37 °C with shaking.
5. Pool cultures (*see* **Note 10**).
6. Transfer the Golden Gate assembly reactions to 96-well flat bottom plates.
7. Add 200 μL of competent *B. subtilis* to each well and seal with an air-permeable membrane.
8. Incubate for 3 h at 37 °C with shaking.
9. Prepare nutrient agar plates with the appropriate antibiotic (*see* **Note 11**).
10. Plate the transformation mixtures and incubate at 37 °C overnight.
11. If required, purify colonies by streaking to singles and confirm recombinants by PCR and sequencing. Alternatively, process colonies in custom downstream high-throughput experiments.

4 Notes

1. If the template sequence contains endogenous BsaI sites, split the sequence into multiple fragments. Flanking primers are positioned at the endogenous BsaI sites such that silent mutations removing the recognition site are introduced. Alternatively, BsaI restriction sites can be left in the construct and ligated after Golden Gate assembly by an additional T4 ligation step.
2. LB medium, SMM, 2× SMM, glucose, and magnesium sulfate heptahydrate solutions are autoclaved and stored at room

temperature. Nutrient agar and SMG Agar are autoclaved and stored at 55 °C. L-tryptophan, glutamate, and ferric ammonium citrate solutions are sterile filtered and stored at 4 °C. L-tryptophan should be kept in the dark. Casamino acids solution is kept at −20 °C for long-term storage and can be stored at 4 °C for a few weeks.

3. Efficient Gibson cloning of donor vectors is achieved in the absence of Taq ligase, the most expensive ingredient in standard Gibson reactions [17]. 1 mL of 2× Gibson Master mix is prepared and stored as 50 μL aliquots at −80 °C.
4. 2× SMM solution is prepared by weighing twice the amount of each salt comprising the SMM solution.
5. BsaI overhangs should be designed so that each overhang is unique. Thus, the 16 palindromic sequences should be avoided as they interfere with directional assembly [8].
6. Linear destination vector for Gibson reaction is generated by PCR amplification of the circular vector pIDC (available from Geneva Biotech) with primers generating the appropriate Gibson overhangs: Forward primer TGAGACCTAATATTCCG-GAGTAGGTCGCG and reverse primer GGAGACCCACTG CTTGAGCCTAGAAGATCCG. The amplified vector DNA is purified on PCR purification kit and stored.
7. A Wolfram Language package and accompanying tutorial for the automated design of mutagenesis primers is available on GitHub (https://github.com/fbuermann/CysteineScreen). The package automatically generates forward and reverse primers for the systematic mutation of target residues to any residue of choice (e.g., cysteine).
8. PCR efficiency is usually similar between similar reactions (running an agarose gel should give an indication). For convenience, group the reactions by approximate product length, determine the concentration for a subset and use the average to determine an appropriate dilution.
9. *B. subtilis* strain is patched the day before the Golden Gate reaction is set so that the transformation can be performed on the day following the Golden Gate assembly reaction.
10. Pooling multiple independent batches of competent cultures increases the reproducibility of experiments.
11. If *smc* alleles are transformed that do not support growth on nutrient-rich medium, cells are plated on SMG agar plates.

Author Contributions F.B. and S.G. designed, established, and optimized the Golden Gate cloning and allelic replacement strategy. F.B. wrote code for automated primer design. M.-L.D.-D. wrote the draft manuscript, while F.B. and S.G. commented on the manuscript.

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