# Chapter 12

# Combinatorial DNA Assembly Using Golden Gate Cloning

Carola Engler and Sylvestre Marillonnet

## Abstract

A basic requirement for synthetic biology is the availability of efficient DNA assembly methods. We have previously reported the development of Golden Gate cloning, a method that allows parallel assembly of multiple DNA fragments in a one-tube reaction. Golden Gate cloning can be used for different levels of construct assembly: from gene fragments to complete gene coding sequences, from basic genetic elements to full transcription units, and finally from transcription units to multigene constructs. We provide here a protocol for DNA assembly using Golden Gate cloning, taking as an example the level of assembly of gene fragments to complete coding sequences, a level of cloning that can be used to perform DNA shuffling. Such protocol requires the following steps: (1) selecting fusion sites within parental sequences (sites at which parental sequences will be recombined), (2) amplifying all DNA fragments by PCR to add flanking restriction sites, (3) cloning the amplified fragments in intermediate constructs, and (4) assembling all or selected sets of intermediate constructs in a compatible recipient vector using a one-pot restriction-ligation.

**Key words** Synthetic biology, DNA assembly, DNA shuffling, Combinatorial, Hierarchical, Type IIS restriction enzymes, Seamless cloning, Modular cloning

## 1 Introduction

The emerging field of synthetic biology promises the production of organisms with novel phenotypes useful for medicine, agriculture, and industry. Unlike traditional biotechnology, which has so far produced organisms containing relatively low numbers of modified genes, synthetic biology aims to engineer organisms with larger numbers of modified genetic elements, potentially at up to genome scale. Such endeavor requires methods that can allow parallel assembly of multiple DNA fragments very efficiently. Fortunately, several methods that provide this capability have been developed recently [1]. Most of these methods are based on homologous recombination between sequences present at the ends of the fragments to assemble [2–4]. Methods that would allow assembly of multiple DNA fragments without a requirement for sequence homology would be particularly useful for combinatorial assembly

Karen M. Polizzi and Cleo Kontoravdi (eds.), *Synthetic Biology,* Methods in Molecular Biology, vol. 1073, DOI 10.1007/978-1-62703-625-2_12, 

of libraries of standard basic genetic elements, especially at the level of transcription units, where fixed junctions between various genetic elements need to be as small as possible.

We have recently developed a cloning method, called Golden Gate cloning, that is suitable for this purpose [5, 6]. The principle of this method is based on the ability of type IIS enzymes to cleave outside of their recognition site, allowing two DNA fragments flanked by compatible restriction sites to be digested and ligated seamlessly [7–10] (Fig. 1a). Since the ligated product of interest does not contain the original type IIS recognition site, it will not be subject to redigestion in a restriction-ligation reaction. However, all other products that reconstitute the original site will be redigested, allowing their components to be made available for further ligation, leading to formation of an increasing amount of the desired product with increasing time of incubation. Since the sequence of the overhangs at the ends of the digested fragments can be chosen to be any 4-nucleotide sequence of choice, multiple compatible DNA fragments can be assembled in a defined linear order in a single restriction-ligation step.

Golden Gate assembly can be used for assembly of multigene constructs from libraries of standardized modules containing basic genetic elements such as promoters, gene coding sequences, and terminators using a succession of one-pot assembly reactions [11, 12]. A first Golden Gate cloning reaction is used for assembly of transcription units from standard basic genetic elements, while a second reaction is used for generation of multigene constructs from individual transcription units (levels 1 and 2, respectively, Fig. 2). At a lower level of assembly, Golden Gate cloning can also be used to generate new biological parts (such as new promoters or gene coding sequences) by DNA shuffling of several parental sequences (assembly from level −1 to level 0, Fig. 2). An example of novel coding sequences created by combinatorial assembly of variant gene fragments is the construction of DNA binding proteins with user-defined binding specificities [13–17]. We provide here a protocol for gene shuffling, but the conditions described for the assembly step can also be applied for other levels of cloning (i.e., for assembly of transcription units or of multigene constructs). Performing gene shuffling using Golden Gate cloning basically requires four steps: (1) defining a set of fusion sites that will be used to link the various DNA fragments to be assembled, (2) amplifying all DNA fragments by PCR to add flanking restriction sites, (3) cloning the amplified fragments in intermediate constructs, and (4) assembling all or selected sets of intermediate constructs in a compatible recipient vector using a one-pot restriction-ligation.

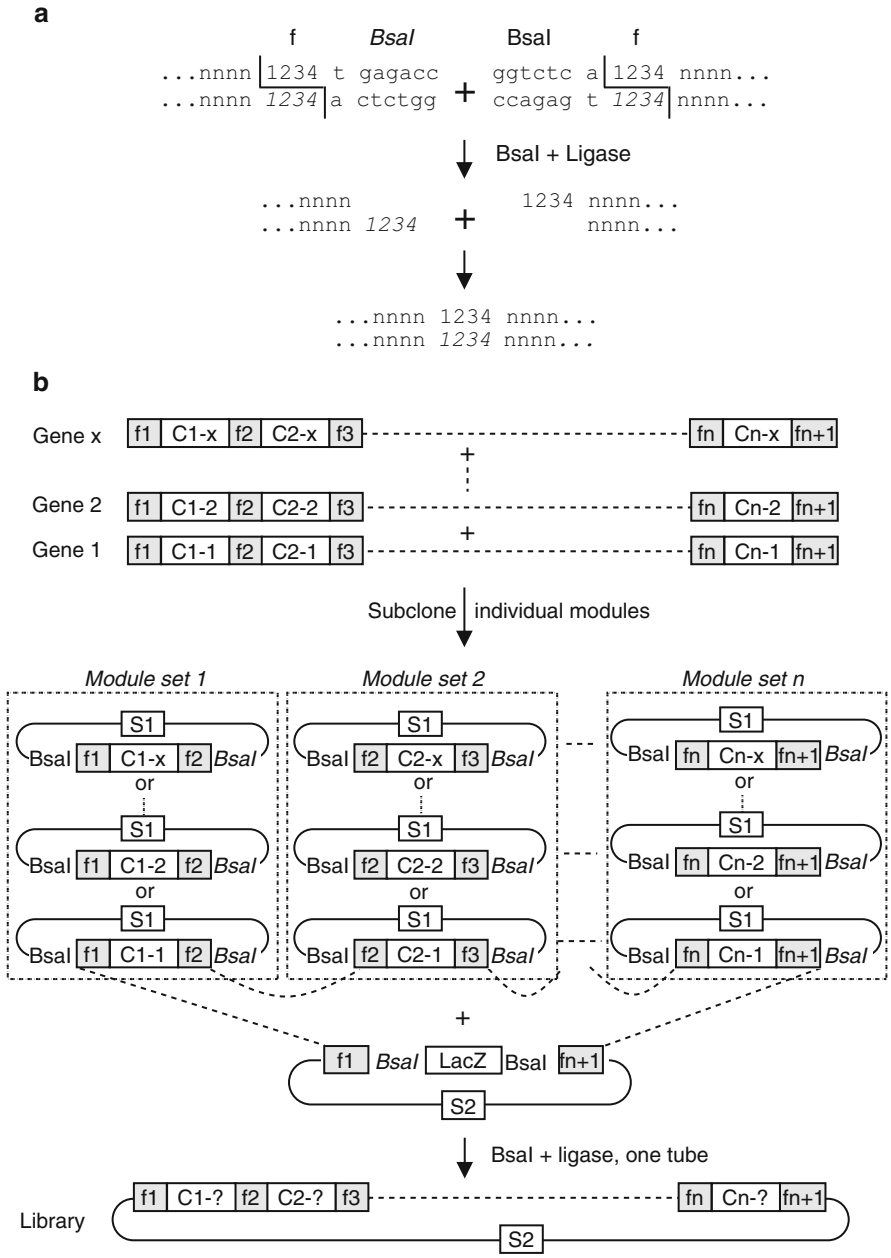


**Fig. 1** DNA shuffling strategy. (**a**) Two DNA ends terminated by the same 4 nucleotides (sequence f, composed of nucleotides 1234, complementary nucleotides noted in *italics*) flanked by a BsaI recognition sequence, ggtctc, form two complementary DNA overhangs after digestion with BsaI. (**b**) For DNA shuffling, genes of interest are aligned, and fusion sites consisting of 4-nucleotide sequences (f1 to fn + 1) are defined on conserved sequences. Module fragments (core sequence, C1 to Cn, plus flanking 4-nucleotide sequences) are amplified by PCR and cloned in an intermediate cloning vector. Module fragment plasmids and the acceptor vector are assembled with a restriction-ligation using BsaI and ligase. S1 and S2, two different selectable markers

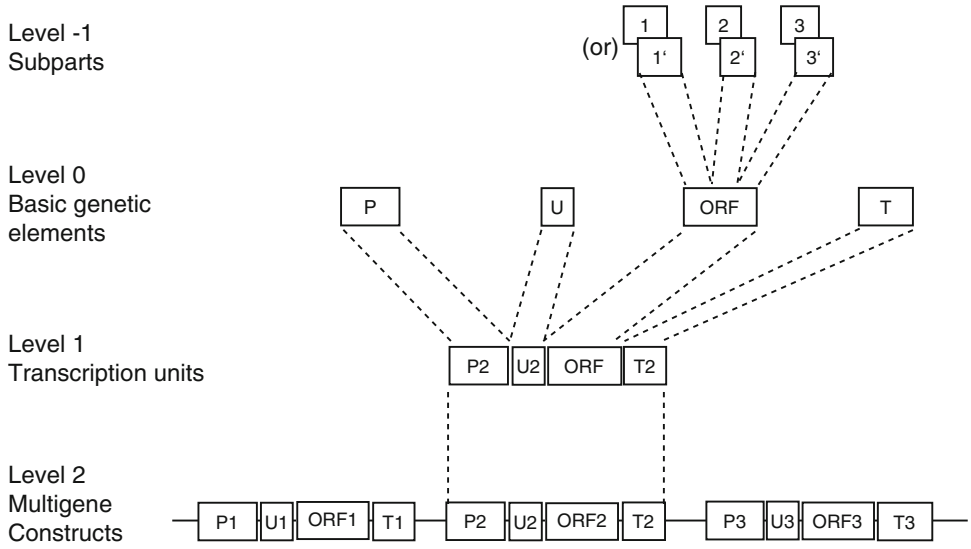


**Fig. 2** Golden Gate cloning can be used for different levels of construct assembly. Cloning at each step is performed using a similar assembly reaction, except that different type IIS enzymes must be used for successive levels of assembly. This is because each cloning step results in constructs that lack restriction sites for the type IIS used. Cloning from level −1 to level 0 can be used for gene or promoter shuffling, or to make various gene fusions

## 2 Materials

### 2.1 PCR

1. Novagen KOD Hot Start DNA polymerase (Merck KGaA, Darmstadt), supplied with 10× buffer, 25 mM $MgSO_4$, and 2 mM dNTPs.
2. Custom-made primers can be ordered from many commercial vendors (e.g., Invitrogen, Karlsruhe).
3. NucleoSpin® Extract II kit (Macherey Nagel, Düren), for purification of PCR products.

### 2.2 Cloning

1. Restriction endonuclease SmaI (10 U/μL) (NEB, New England Biolabs Inc., Ipswich, MA, USA), supplied with 10× NEBuffer 4 (200 mM Tris–acetate pH 7.5, 100 mM magnesium acetate, 500 mM potassium acetate, 10 mM dithiothreitol).
2. Restriction endonuclease BsaI (10 U/μL) (NEB), supplied with 10× NEBuffer 4.
3. T4 DNA Ligase 3 U/μL or T4 DNA Ligase (HC) 20 U/μL (Promega, Mannheim), both supplied with 10× ligation buffer (300 mM Tris–HCl pH 7.8, 100 mM $MgCl_2$, 100 mM DTT, 10 mM ATP).

4. For measuring DNA concentration, we use the NanoDrop ND2000 (Peqlab, Erlangen).
5. Luria-Bertani (LB) Medium: 1 % bacto-tryptone, 0.5 % yeast extract, 1 % NaCl in deionized water, adjusted to pH 7.0 with 5 N NaOH. For plates, 1.5 % agar is added.
6. Antibiotics carbenicillin (used instead of ampicillin) and kanamycin: filter-sterilized stocks of 50 mg/mL in $H_2O$ (stored in aliquots at −20 °C) are diluted 1:1,000 (final concentration: 50 μg/mL) in an appropriate amount of medium after the medium has been autoclaved and cooled down. For spectinomycin, a stock of 40 mg/mL is made and is used at a final concentration of 100 μg/mL (dilution 1:400).
7. 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal): stock solution of 20 mg/mL in dimethylformamide (DMF). For preparation of plates, the stock is diluted 1:500 (final concentration: 40 μg/mL) in an appropriate amount of LB agar after autoclaving/melting and cooling down.

### 2.3 Preparation of Chemically Competent Cells

1. Solution TFB1: 30 mM potassium acetate, 10 mM $CaCl_2$, 50 mM $MnCl_2$, 100 mM RbCl, 15 % glycerol; adjust to pH 5.8 (with 1 M acetic acid), filter-sterilize, and store at 4 °C (ready to use) or at room temperature (cool down before use).
2. Solution TFB2: 100 mM MOPS (or PIPES), 75 mM $CaCl_2$, 10 mM RbCl, 15 % glycerol; adjust to pH 6.5 (with 1 M KOH), filter-sterilize, and store at 4 °C (ready to use) or at room temperature (cool down before use).
3. The $OD_{600}$ of bacterial cultures is measured in a SmartSpec™ 3000 spectrophotometer (Biorad, Muenchen).

### 2.4 Screening of Colonies

1. DNA minipreps: NucleoSpin® Plasmid Quick Pure (Macherey Nagel, Dueren).
2. Restriction endonucleases (NEB or Fermentas, St. Leon-Rot), all supplied with 10× buffer and if necessary also with 100× BSA (dilute 1:10 and store in aliquots at −20 °C).
3. DNA ladder: GeneRuler™ 1 kb DNA Ladder Plus (Fermentas) is used as marker for gel electrophoresis.
4. 50× TAE buffer: 242.0 g Tris–HCl, 57.1 mL acetic acid, and 100 mL 0.5 M EDTA, pH 8.0, in 1 L of deionized water.
5. Gels: agarose (0.7–1.5 %) in 1× TAE is melted in a microwave oven and one drop of a 0.025 % ethidium bromide solution (Carl Roth GmbH, Karlsruhe) is added per 100 mL of melted agarose solution.
6. Running buffer of agarose gels is 1× TAE.

7. Gels are checked visually using a Syngene GelVue transilluminator (VWR, Darmstadt), and pictures are taken by using a Quantity one® gel analysis software (Biorad).
8. DNA maps of plasmids are made by using the Vector NTI software (Invitrogen).

### 2.5 Sequencing

1. DNA/constructs to sequence are sent to an external contractor (GATC Biotech, Konstanz). Sequence data are analyzed using the DNASTAR's Lasergene software.
2. Primers M13RP (CAGGAAACAGCTATGACC) and/or M13FP (TGTAAAACGACGGCCAGT) are used for sequencing of inserts cloned in pUC19-derived vectors.

## 3 Methods

### 3.1 Selection of Fusion Sites

Fusion sites consist of 3- or 4-nucleotide sequences that will be used for restriction enzyme digestion and ligation for the DNA assembly step. Fusion sites of 4 nucleotides are selected if a type IIS restriction enzyme such as BsaI is used for DNA assembly, or of 3 nucleotides if an enzyme of the type of SapI is used (*see* **Note 1**). For DNA shuffling, fusion sites are selected from sequences conserved among all parental sequences (sequence f1 to fn + 1, Fig. 1). For assembly reactions of a higher order (i.e., for assembly of transcription units or of multigene constructs, levels 1 and 2, Fig. 2), fusion sites do not necessarily consist of native sequences, since these will serve to link various genetic elements of different sequences and origins. In contrast to fusion sites selected for gene shuffling, which need to be defined for each gene individually, these fusion sites can be universally defined and are reused for all assembly reactions of the same level (*see* **Note 2**).

Fusion sites used in the same assembly reaction must follow several requirements:

1. A first requirement is to avoid selecting the same sequence twice, as this could lead to annealing of the ends of inappropriate DNA fragments during the restriction-ligation step (**step 3.5**), resulting in deletion of the sequences between these two sites. It is also important to make sure that the sequence of any site does not match the sequence of any of the other chosen sites on the complement strand. For example, choice of the sequence ATTC will preclude the choice of the sequence GAAT for any of the other fusion sites used for this shuffling experiment. Use of two such sites would sometimes lead to ligation of two inappropriate fragments, one in the opposite orientation. This would lead to formation of molecules that will not be able to form circular plasmids, but that would continue to ligate to further fragments and form linear concatemers.

2. A second requirement is to avoid the 16 palindromic sequences (for 4-nucleotide fusion sites), since any palindromic DNA end can be ligated to another copy of the same DNA fragment in the opposite orientation, and lead to the same problem as described above. For enzymes that produce a 4-nucleotide extension, 240 different sequences are, therefore, available.
3. Finally, a third but optional requirement can be defined to maximize the efficiency of DNA shuffling. We have observed that inappropriate ligation of fragments can sometimes occur between ends in which 3 out of 4 consecutive nucleotides are identical, for example, as in sequences GGTG and AGTG, or GGTG and CACT. Therefore, combination of two such sites should be avoided if possible.

The number of fusion sites, as well as their position within the gene to be shuffled, is chosen depending on the needs of the user for each specific protein. Therefore, the size (*see* **Note 3**) and number of fragments to shuffle/assemble will vary for each gene and each experiment. We have tested up to eight recombination points within a gene (for assembly of nine fragments), but a higher number is possible as well, although with reduced efficiency (*see* **Note 4**).

The following steps consist of amplifying the defined fragments by PCR, cloning them in intermediate vectors, and sequencing them. Alternatively, the fragments amplified by PCR can be used directly for gene assembly or DNA shuffling without an intermediate cloning step, but this approach may lead to reduced cloning efficiency (*see* **Note 5**). Alternatively, these steps can also be replaced by simply ordering the fragments of interest from a gene synthesis company (*see* **Note 6**).

### 3.2 PCR Amplification of the Modules

Modules defined by the position of the fusion sites are amplified by PCR using primers designed to add two BsaI sites flanking each module. Primers are designed such that the overhangs created by digestion of the amplified products with BsaI (or any other type IIS enzyme chosen) correspond to the sequence of the chosen fusion sites. Therefore, the sequence ttggtctca is added to each primer sequence, for example, ttggtctca CAGG nnnnn (CAGG being the fusion site, followed by 16–20 nucleotides of target sequence). For nine modules prepared from three homologous sequences, 54 primers need to be made.

A requirement for Golden Gate assembly is to not have any internal BsaI sites present within any of the DNA fragments to assemble. Indeed, the presence of a BsaI site within one of the modules would lead to redigestion of the shuffled DNA sequences containing such fragment, at the end of the assembly step. These linear molecules will of course not transform *E. coli*. Therefore, any internal site needs to be removed upon generation of the entry

clones. Removal of internal BsaI sites from PCR fragments can be done easily using gene SOEing [18] (*see* **Note 7**). An alternative strategy has also been described elsewhere [11].

PCR can be performed using any source of DNA, but for small modules, template DNA is not necessary (*see* **Note 8**). For amplification, we use the enzyme KOD Hot Start DNA polymerase since it has a very low error rate and, unlike Taq polymerase or several enzyme mixes, produces DNA products with blunt ends. Blunt ends are advantageous, as the products can be easily cloned in any standard vector such as pUC19 by blunt-end cloning (see below).

1. The PCR mix is set up following the manufacturer's instructions, for example, using KOD polymerase, with the following conditions: 1 μL plasmid DNA (5–20 ng/μL), 5 μL of 10× buffer, 3 μL of 25 mM $MgSO_4$, 5 μL of 2 mM dNTPs, 1.5 μL each of 10 μM sense and antisense primers, and 1 μL of KOD Hot Start DNA polymerase (10 U/μL, final concentration 0.02 U/μL) in a total reaction volume of 50 μL.
2. PCR is performed using the following cycling conditions: (1) incubation at 95 °C for 2 min for polymerase activation, (2) denaturation at 95 °C for 20 s, (3) annealing at 58 °C for 10 s—the temperature for the annealing step can be adjusted for specific primers, but the temperature of 58 °C usually works well for primers designed as described above, (4) extension at 70 °C, the duration depends on the length of the expected fragment (from 10 s/kb for fragments smaller than 500 bp up to 25 s/kb for fragments larger than 3 kb, see manufacturer's instructions); **steps 2–4** are repeated 35 times and are followed by a final extension step at 70 °C for 20 s–2 min (depending on fragment length). The reaction is then kept at 12 °C until taken out of the thermocycler.
3. Of the PCR product obtained, 2 μL is then analyzed by gel electrophoresis to make sure that a product of the correct size has been amplified.
4. The amplified fragment is purified from remaining primers, potential primer dimers, and remaining polymerase enzyme by using the NucleoSpin® Extract II kit following the kit protocol. DNA is eluted from the column with 30–50 μL of elution buffer (5 mM Tris–HCl, pH 8.5). In case several bands were amplified rather than only the expected fragment, the same kit can also be used to cut and extract the appropriate DNA fragment from an agarose gel.

### 3.3 Blunt-End Cloning of the Modules

Many commercial kits are available for cloning PCR products, including the pGEM-T kit (Promega), pJET (Fermentas), and TOPO® TA kit (Invitrogen). Alternatively, PCR products can also be cloned efficiently using blunt-end cloning with a protocol that

uses restriction-ligation [19, 20]. This method has the advantage that the DNA fragment of interest to be cloned does not need to be flanked by any specific sequence, and therefore primers used for amplification do not need to have special extensions (*see* **Note 9**). Cloning vectors for generating entry clones for shuffling need to fulfill preferentially two requirements: (1) they should preferably not contain any restriction site for the type IIS enzyme chosen for shuffling (*see* **Note 10**), and (2) the antibiotic resistance gene of the entry vector should preferably be different from the one in the destination vector. Since several commercial cloning vectors have a BsaI restriction site in the ampicillin resistance gene (e.g., pGEM-T or pJET), we have made our own entry cloning vector that simply consists of pUC19 lacking a BsaI restriction site (*see* **Note 11**).

1. Add 0.5 μL of vector (50 ng), 1 μL of PCR product (50–100 ng), 2 μL of 10× ligation buffer (Promega), 1 μL of SmaI enzyme (10 U; NEB), 1 μL of ligase (3 U; Promega), and 14.5 μL of water (total volume of 20 μL) into a tube. The reaction mix is incubated for 1–2 h at room temperature or in a 25 °C incubator, if one is available.
2. The entire ligation mix is transformed to DH10B chemically competent cells and plated on LB plates with X-gal and the appropriate antibiotic (the transformation protocol is described below in Subheading 3.6).
3. White colonies (or sometimes pale blue when small inserts are cloned) are picked and inoculated in 5 mL of LB medium containing the appropriate antibiotic.
4. Plasmid DNA is extracted using the NucleoSpin® Plasmid Quick Pure kit from Macherey Nagel following the manufacturer's instructions.
5. Plasmid DNA can be checked by restriction enzyme digestion using BsaI, and analysis of the digested DNA by agarose gel electrophoresis. A fragment of the size of the expected module should be visible.
6. Two minipreps are sent for sequencing using primers M13RP and/or M13FP.
7. When the correct sequence has been verified, DNA concentration of the plasmid prep is measured using the NanoDrop ND2000 (Peqlab).

### 3.4 Construction of the Destination Vector

A destination vector compatible with the entry modules needs to be made. The vector should respect the following criteria. It should contain two BsaI sites (or any other type IIS enzyme chosen) with cleavage sites compatible with the beginning of the first module set and the end of the last module set. The vector backbone should not

contain any BsaI restriction site and should have an antibiotic resistance gene different from the one used for the entry clones. Additionally, the vector may contain a lacZα fragment (Fig. 1b) to allow blue-white selection of the resulting clones. Optionally, restriction sites for a second type IIS enzyme may be added to the vector to allow further subcloning of the sequence that will be formed by assembly of the fragments in the first assembly reaction. An example of destination vector sequence is as follows: gaagac aa abcd 1234 t gagacc, followed by LacZ gene sequences, followed by sequence ggtctc a 5678 efgh tt gtcttc followed by vector backbone sequences. In this example, fusion sites 1234 and 5678 are used for assembly of several fragments using BsaI, while fusion sites abcd and efgh are used for further subcloning of the assembled sequence using BpiI.

### 3.5 Golden Gate DNA Assembly

Once entry constructs and the recipient vector are made and sequenced, performing DNA shuffling/assembly only requires pipetting all components into a reaction mix, incubating the mix in a thermocycler, and transforming the ligation mix into competent cells. An important factor is to use an equimolar amount of DNA for each of the module sets and the destination vector. If a module set contains several modules, the amount of DNA for each module of a set containing $x$ different modules ($x$ alternative homologous sequences) should contain only $1/x$ the amount of DNA compared to the vector; for example, each module from a set containing three modules should have a third the amount of DNA compared with the recipient vector.

1. A restriction-ligation is set up by pipetting 40 fmol (or 100 ng, *see* **Note 12**) of each module set and of the vector, 2 μL 10× ligation buffer, 10 U (1 μL) of BsaI, and either 3 U (1 μL) of ligase for assembly of 2–4 module sets or 20 U (1 μL) HC ligase for assembly of more than four module sets, in a total volume of 20 μL into a tube.
2. The restriction-ligation mix is incubated in a thermocycler. For assembly of 2–4 module sets, incubation for 60–120 min at 37 °C is sufficient. If more module sets are ligated together, the incubation time is increased to 6 h, or cycling is used as following: 2 min at 37 °C followed by 3 min at 16 °C, both repeated 50 times (*see* **Note 13**).
3. Restriction-ligation is followed by a digestion step (5 min at 50 °C) and then by heat inactivation for 5 min at 80 °C. The final incubation step at 80 °C is very important and should not be omitted. Its purpose is to inactivate the ligase at the end of the restriction-ligation. Omitting this step would lead to religation of some of the insert and plasmid backbone fragments still present in the mix, when it is taken out of the thermocycler

before transformation. Such unwanted products might be ligated more efficiently than they are redigested by the type IIS enzyme at room temperature or on ice in the time interval between restriction-ligation and transformation. Therefore, a larger percentage of colonies would contain such type of undesired ligation products.

### 3.6 Transformation of the Library in Competent Cells

The entire ligation is transformed into chemically competent DH10B cells (*see* **Note 14**).

1. Frozen chemically competent cells (100 μL per tube) are thawed on ice.
2. The entire ligation is added to the cells and the mix is incubated on ice for 30 min.
3. The cells and DNA mix is heat shocked for 90 s at 42 °C in a water bath.
4. The cells are allowed to recover on ice for 5 min.
5. To the cells, add 1 μL of LB medium and incubate the tube at 37 °C in a shaker-incubator (150 rpm) for 45 min to 1 h.
6. After incubation, 25–100 μL of the transformation is plated on LB agar plates containing antibiotic and X-gal. Plating of an aliquot of the transformation is necessary to estimate the number of independent constructs that will be obtained. The remainder of the transformation can be inoculated into 5 mL of liquid LB with the appropriate antibiotic.
7. The plates and liquid culture are incubated overnight at 37 °C.
8. Many white and very few blue colonies should be obtained on the plate. A few white colonies from the plate can be picked for preparation of miniprep DNA. Plasmid DNA can be analyzed by restriction digestion and sequencing to estimate the number of correct clones.
9. Miniprep DNA is also prepared from the liquid culture. This DNA prep should represent a library of constructs containing shuffled DNA.

Depending on the specific goal of the shuffling experiment, clones can be functionally screened either individually or as a library. The shuffled plasmid library may be transformed in any target organism of choice for functional screening.

### 3.7 Preparation of Chemically Competent DH10B Cells

Chemically competent or electrocompetent *E. coli* cells can be either purchased from a commercial vendor or made in the laboratory. The protocol that we use is as follows:

1. *E. coli* strain DH10B is inoculated from a glycerol stock onto an LB plate; the inoculum is streaked on the plate using a loop

so as to obtain individual colonies. The plate is incubated overnight at 37 °C.

2. Inoculate 5 mL of LB from a single colony and incubate the flask overnight in a shaker-incubator (37 °C, shaking 150 rpm).
3. The following day, transfer 2 mL of this culture to a flask containing 200 mL of LB and incubate for around 2 h until $OD_{600}$ reaches 0.6.
4. Cool down the cells on ice for 10 min. The cells are pelleted in a centrifuge for 5 min at 4,500 rpm (4,000 × *g*) at 4 °C. The cells are resuspended in 0.4 volume of ice-cold TFB1.
5. Repeat the centrifugation. Resuspend the pellet in 1/25 volume of ice-cold TFB2.
6. The cells are aliquoted 100 μL per tube and shock-frozen in liquid nitrogen. The aliquots are stored at −80 °C.

## 4 Notes

1. Several different type IIS enzymes can be used for construct assembly. We have, for example, tested the enzymes BsaI, BpiI, and Esp3I. For all three, restriction-ligation can be performed efficiently in ligase buffer from Promega. All three have a 6-bp recognition sequence and a 4-nucleotide cleavage site located 1 (BsaI and Esp3I) or 2 nucleotides (BpiI) away from the recognition sequence. Enzymes of the type of SapI such as LguI can also be used efficiently in a restriction-ligation [21]. These enzymes have a 7-bp recognition sequence, and, therefore, fewer sites will have to be removed from sequences of interest to clone (discussed in Subheading 3.2). However, these enzymes have a 3-nucleotide cleavage site, meaning that only 64 different sequences are available to choose from for use as fusion sites.
2. To standardize DNA assembly reactions, each basic module type (such as promoter, coding sequence, or terminator), is flanked by two defined fusion sites. This allows any module to be cloned interchangeably with any other module of the same type using the same assembly reaction. We and others have earlier published sets of fusion sites that can be used for assembly of transcription units [11, 22]. These sequences may be used by other groups, although they may still be changed before they are universally adopted. For example, we have selected 4-nucleotide-long fusion sites for assembly of transcription units, but the use of 3-nucleotide sequences (using SapI or LguI for assembly) would allow to have fewer nucleotides fixed in a transcription unit.

3. The minimal size of modules that we have tested is 38 bp (including the fusion sites, but excluding the flanking BsaI recognition sites). In theory, a module needs to be long enough for the two strands to remain annealed under restriction-ligation conditions, usually 37 °C. Therefore, smaller modules could theoretically be made, which would be useful if a user wants to focus his efforts on a very small region of a protein of interest. Another example of small modules would consist of modules containing protein purification or detection tags.
4. We have successfully assembled up to 17 fragments in one step, but with seriously reduced efficiency (0–3 positive clones out of 12 colonies analyzed [17]). Ligation of nine fragments can be quite efficient, but efficiency seems to decrease rapidly with a higher number of fragments. Assembly of more than nine fragments can be performed efficiently using two successive cloning steps rather than one [17].
5. Assembly of PCR products directly in the destination vector without first cloning them as intermediate constructs is possible. It is however recommended to purify the PCR products using a column to remove any DNA polymerase left in the PCR product and to remove primer dimers that may be produced during PCR amplification. Indeed some of the primer dimers are flanked by two fusion sites (these are part of the primers) and can therefore be cloned, resulting in incorrect constructs. The final constructs may also contain PCR-induced mutations.
6. For generation of entry clones, the steps consisting of PCR amplification, cloning, and sequencing can be replaced by ordering the desired sequence from a gene synthesis company. The fragment to be synthesized should be ordered directly with the appropriate type IIS restriction sites flanking the sequence of interest. Also, it is useful to make sure upon ordering that the cloning vector in which the ordered DNA fragment will be cloned does not contain additional sites for the type IIS enzyme chosen, and has an antibiotic resistance gene different from the one in the vector that will be used for assembly.
7. Basically, two primers overlapping an internal BsaI site are made, one in each orientation, with a mismatch designed to introduce a silent mutation in the type IIS restriction site. Two separate PCRs are performed with primers designed to amplify the two halves of the module. The PCR products are purified on a column, and a mix of both is used as a template for a second PCR performed using both flanking primers only (the two primers flanking the given module). This PCR is purified on a column, cloned, and sequenced.
8. For small modules of up to 80 nucleotides, PCR amplification does not necessarily require a DNA template. For example, two

complementary primers can be ordered covering the entire sequence of the module (including the flanking type IIS restriction sites). Both primers are annealed in water and directly used for blunt-end cloning in the cloning vector. For larger but still small modules, two overlapping primers can be ordered that are complementary at their 3′ end on a length of 20–25 nucleotides. A double-stranded DNA fragment can be obtained by performing a PCR with both primers without a template. In theory, one single PCR cycle should be sufficient, but using 35 cycles as for normal PCR also works well.

9. One restriction for blunt-end cloning is that the ends of the primer should not recreate a SmaI site (or any other blunt-end restriction site used for cloning) after ligation of the PCR product (i.e., the DNA fragment to be cloned should not start with the sequence GGG or finish with CCC). A second restriction is that the fragment to be cloned should not contain an internal restriction site for the enzyme used for cloning. If this is the case, another enzyme should be chosen for cloning, for example, EcoRV (a cloning vector containing a unique EcoRV site in the polylinker should be used).
10. The presence of a BsaI site in the vector backbone of the entry modules does not prevent from using them for Golden Gate shuffling, since plasmids containing the final shuffled sequences should not contain this vector backbone. However, the presence of such a site in all entry constructs would lead to continuous ligation and redigestion at this site, and would therefore unnecessarily consume some ATP from the ligation mix, at the expense of the desired ligation events.
11. The widely used pUC19 vector also contains a BsaI site in the ampicillin resistance gene. A simple strategy, enzymatic inverse PCR [23], can be used to eliminate the internal BsaI site in pUC19. The entire plasmid can be amplified with two primers overlapping with the BsaI site: primers bsarem1 (ttt ggtctc a ggtt ctcgcggtatcattgcagc) and bsarem2 (ttt ggtctc a aacc acgctcaccggctccag). These primers are designed to introduce a single silent nucleotide mutation in the BsaI recognition site in the vector. The primers are themselves flanked by two BsaI restriction sites that form two compatible overhangs after BsaI enzyme digestion. After amplification of the entire plasmid with both primers, the PCR is purified with a column (to remove remaining polymerase and nucleotides). The linear fragment is subjected to restriction-ligation using BsaI and ligase, and transformed in *E. coli*.
12. In practice, if all module plasmids and the vector have approximately the same size (4–5 kb), simply adding 100 ng of DNA of each module set and of the vector will work relatively well. However, if plasmids with widely different sizes are used,

calculating an equimolar amount should provide a higher cloning efficiency. The following formula (from the NEB catalog) can be used: 1 μg of a 1,000-bp DNA fragment corresponds to 1.52 pmol.

13. We have found that both types of programs work well when high concentration ligase is used, but both programs can be tested in parallel by the users to optimize ligation efficiency.
14. Any other *E. coli* strain can also be used. If higher transformation efficiency is required, the restriction-ligation mix can be transformed in electrocompetent *E. coli* cells. In this case, DNA from the restriction-ligation mix should first be ethanol-precipitated and resuspended in 10 μL of water.

## Acknowledgments

The authors would like to thank Dr. Stefan Werner and Dr. Ernst Weber for critical reading of this manuscript.

### References

1. Ellis T, Adie T, Baldwin GS (2010) DNA assembly for synthetic biology: from parts to pathways and beyond. Integr Biol (Camb) 3:109–118
2. Li MZ, Elledge SJ (2007) Harnessing homologous recombination in vitro to generate recombinant DNA via SLIC. Nat Methods 4:251–256
3. Gibson DG, Benders GA et al (2008) One-step assembly in yeast of 25 overlapping DNA fragments to form a complete synthetic Mycoplasma genitalium genome. Proc Natl Acad Sci U S A 105:20404–20409
4. Shao Z, Zhao H, Zhao H (2009) DNA assembler, an in vivo genetic method for rapid construction of biochemical pathways. Nucleic Acids Res 37:e16
5. Engler C, Kandzia R, Marillonnet S (2008) A one pot, one step, precision cloning method with high throughput capability. PLoS One 3: e3647
6. Engler C, Gruetzner R, Kandzia R, Marillonnet S (2009) Golden gate shuffling: a one-pot DNA shuffling method based on type IIs restriction enzymes. PLoS One 4:e5553
7. Lebedenko EN, Birikh KR, Plutalov OV, Berlin YA (1991) Method of artificial DNA splicing by directed ligation (SDL). Nucleic Acids Res 19:6757–6761
8. Szybalski W, Kim SC, Hasan N, Podhajska AJ (1991) Class-IIS restriction enzymes—a review. Gene 100:13–26
9. Berlin YA (1999) DNA splicing by directed ligation (SDL). Curr Issues Mol Biol 1:21–30
10. Lu Q (2005) Seamless cloning and gene fusion. Trends Biotechnol 23:199–207
11. Weber E, Engler C, Gruetzner R, Werner S, Marillonnet S (2011) A modular cloning system for standardized assembly of multigene constructs. PLoS One 6:e16765
12. Werner S, Engler C, Weber E, Gruetzner R, Marillonnet S (2012) Fast track assembly of multigene constructs using golden gate cloning and the MoClo system. Bioeng Bugs 3:38–43
13. Sanjana NE, Cong L et al (2012) A transcription activator-like effector toolbox for genome engineering. Nat Protoc 7:171–192
14. Morbitzer R, Elsaesser J, Hausner J, Lahaye T (2011) Assembly of custom TALE-type DNA binding domains by modular cloning. Nucleic Acids Res 39:5790–5799
15. Cermak T, Doyle EL et al (2011) Efficient design and assembly of custom TALEN and other TAL effector-based constructs for DNA targeting. Nucleic Acids Res 39:e82
16. Geißler R, Scholze H et al (2011) Transcriptional activators of human genes with programmable DNA-specificity. PLoS One 6:e19509
17. Weber E, Gruetzner R et al (2011) Assembly of designer TAL effectors by golden gate cloning. PLoS One 6:e19722

18. Horton RM, Ho SN et al (1990) Gene splicing by overlap extension. Biotechniques 8:528–535
19. Bolchi A, Ottonello S, Petrucco S (2005) A general one-step method for the cloning of PCR products. Biotechnol Appl Biochem 42:205–209
20. Liu ZG, Schwartz LM (1992) An efficient method for blunt-end ligation of PCR products. Biotechniques 12:28–30
21. Kotera I, Nagai T (2008) A high-throughput and single-tube recombination of crude PCR products using a DNA polymerase inhibitor and type IIS restriction enzyme. J Biotechnol 137:1–7
22. Sarrion-Perdigones A, Falconi EE et al (2011) GoldenBraid: an iterative cloning system for standardized assembly of reusable genetic modules. PLoS One 6:e21622
23. Stemmer WP, Morris SK (1992) Enzymatic inverse PCR: a restriction site independent, single-fragment method for high-efficiency, site-directed mutagenesis. Biotechniques 13:214–220