Chapter 9

Golden Gate Cloning

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Abstract

DNA assembly methods are essential tools for biological research and biotechnology. Therefore various methods have been developed to clone DNA fragments of interest. Conventional methods usually require several cloning steps to generate a construct of interest. At each step, a single DNA fragment is transferred from a donor plasmid or PCR product to a recipient vector. In the past few years, a number of methods have been developed to facilitate and speed up this process. One of these methods, Golden Gate cloning, allows assembling up to nine fragments at a time in a recipient plasmid. Cloning is performed by pipetting in a single tube all plasmid donors, the recipient vector, a type IIS restriction enzyme and ligase, and incubating the mix in a thermal cycler. Despite the simplicity of the cloning procedure, the majority of clones obtained after transformation contain the expected construct. Using Golden Gate cloning however requires the use of carefully designed donor and recipient plasmids. We provide here a protocol describing how to design these plasmids and also describe the conditions necessary to perform the assembly reaction.

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

1 Introduction

In the past few years several methods have been developed to allow assembly of multiple DNA fragments in a single cloning step [1–7]. Most of these methods are based on recombination between homologous sequences present at the ends of the DNA fragments to assemble. These methods have the advantage of allowing seamless assembly of any sequence of choice irrespective of the presence of restriction enzyme recognition sites. A limitation is however the need for overlapping sequences of at least 15 nucleotides at the ends of the fragments. Assembly of non-overlapping DNA fragments therefore requires adding terminal extensions or using bridging oligonucleotides [7]. This is a limiting factor for combinatorial assembly of multiple DNA fragments of interest since a correspondingly large number of bridging oligonucleotides will be required.

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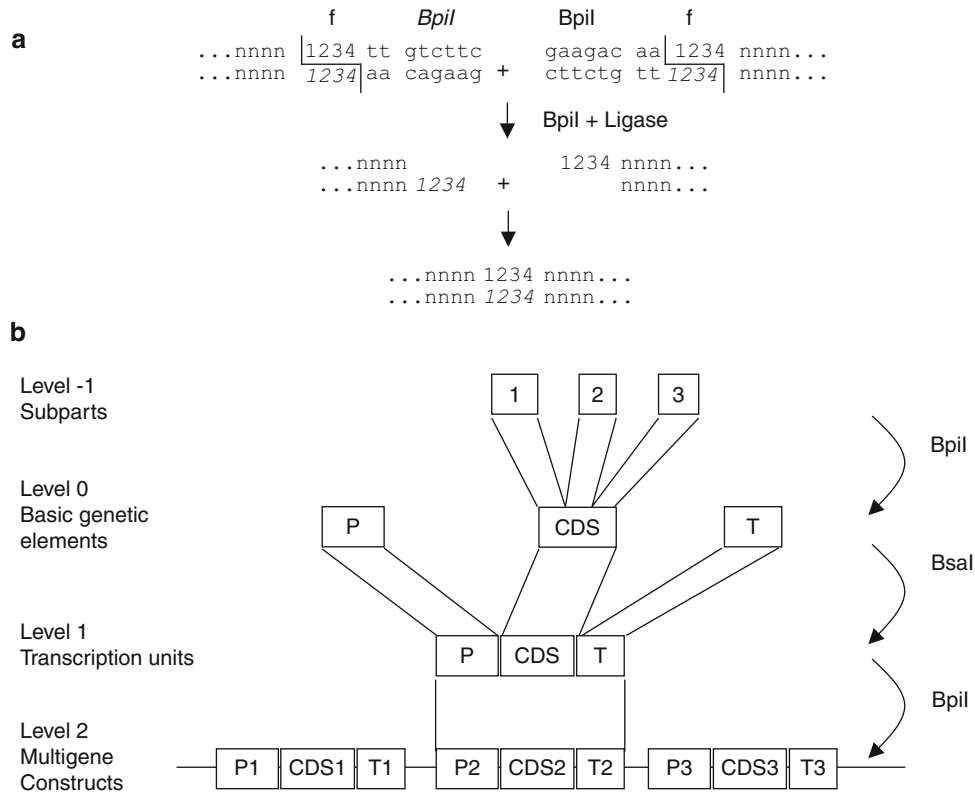

Fig. 1 Golden Gate cloning principle and use for the MoClo system. (**a**) Principle of Golden Gate cloning. Digestion of two DNA fragments containing the same four nucleotide sequence (f, standing for fusion site) flanked by a type IIS restriction site such as BpiI leads to generation of complementary overhangs. Ligation of these two fragments leads to a sequence lacking the original type IIS restriction site. The sequence of the cleavage site can be any four nucleotide sequence of choice. Opposite orientations of the BpiI recognition sites are indicated with non-italics and italics. (**b**) Golden Gate cloning is used for all steps of construct assembly with the MoClo system. Each cloning 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 a construct that lacks restriction sites for the type IIS enzyme used. Cloning from level −1 to level 0 can be used for gene or promoter shuffling, to make various gene fusions, and to remove internal type IIS restriction sites from native sequences for cloning of level 0 modules. *P* promoter, *CDS* coding sequence, *T* terminator

We have previously developed a method that allows assembly of up to nine DNA fragments in a single cloning step, but that does not require homology at the ends of the fragments to assemble, except for three or four nucleotides of sequence that consist of a restriction enzyme cleavage site [4, 8]. The principle of this method, called Golden Gate cloning, is based on the ability of type IIS enzymes to cleave outside of their recognition sites, allowing two DNA fragments flanked by compatible restriction sites to be digested and ligated seamlessly [1, 9] (Fig. 1a). Since the ligated product of interest no longer contains 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. This leads to increasing formation of the desired product with increasing time of incubation, and results in a high cloning efficiency. Since the sequence of the overhangs at the ends of the digested fragments can be chosen to be any four nucleotide sequence of choice, multiple compatible DNA fragments can be assembled in a defined linear order in a single restriction–ligation step.

Golden Gate cloning can be used for gene shuffling and for assembly of any construct of interest. To facilitate construct engineering, a modular cloning system (MoClo) that uses Golden Gate cloning for all assembly steps was developed [10]. The base of the MoClo system consists of libraries of standard level 0 modules (for details, please refer to the Fig. 1 legend) that each contains a defined genetic element such as a promoter, a 5′ untranslated region, and a coding sequence or a terminator (Fig. 1b). Transcription units are assembled from level 0 modules using a first assembly reaction and multigene constructs assembled from transcription units using a second cloning step. The process can be repeated to generate constructs containing larger numbers of transcription units. To be suitable for Golden Gate cloning for all assembly steps, level 0 modules internal sequences should not contain restriction sites for any of the type IIS enzymes used for MoClo. Level 0 modules lacking type IIS restriction sites can be cloned from native sequences using Golden Gate cloning.

We provide here a protocol for Golden Gate cloning, using as example the construction of MoClo level 0 modules. The protocol requires amplifying several fragments by PCR using primers designed to mutate internal restriction sites, cloning the amplified fragments and sequencing them, and finally assembling the cloned fragments in a compatible destination vector. The protocol provided here can also be used for gene shuffling or for combinatorial assembly of various sequences of interest [11].

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, or any other high fidelity DNA polymerase.
2. Custom-made primers can be ordered from many commercial vendors.
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. Restriction endonuclease BpiI (10 U/μL) (Fermentas GmbH, St. Leon-Rot), supplied with 10× Buffer G (100 mM Tris–HCl pH 7.5, 100 mM $MgCl_2$, 500 mM NaCl, 1 mg/mL BSA).
4. 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).
5. For measuring of DNA concentration, we use the NanoDrop ND2000 (Peqlab, Erlangen).
6. Lysogeny Broth (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.
7. 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).
8. 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 Screening of Colonies

1. NucleoSpin® Plasmid Quick Pure (Macherey Nagel, Düren) for preparation of miniprep DNA.
2. Restriction endonucleases (NEB or Fermentas), 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, 57.1 mL acetic acid, and 100 mL 0.5 M EDTA, pH 8.0, in 1 L of deionized water.
5. For preparation of gels for electrophoresis, 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.
6. Running buffer for 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).

2.4 Sequencing

1. DNA constructs that need to be sequenced are sent to an external contractor. 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

Level 0 modules of the MoClo system must conform to a standard structure. All modules are flanked by two BsaI restriction sites in inverted orientation (Figs. 2 and 3) and should not contain restriction sites for the enzymes BsaI, BpiI, and Esp3I in internal

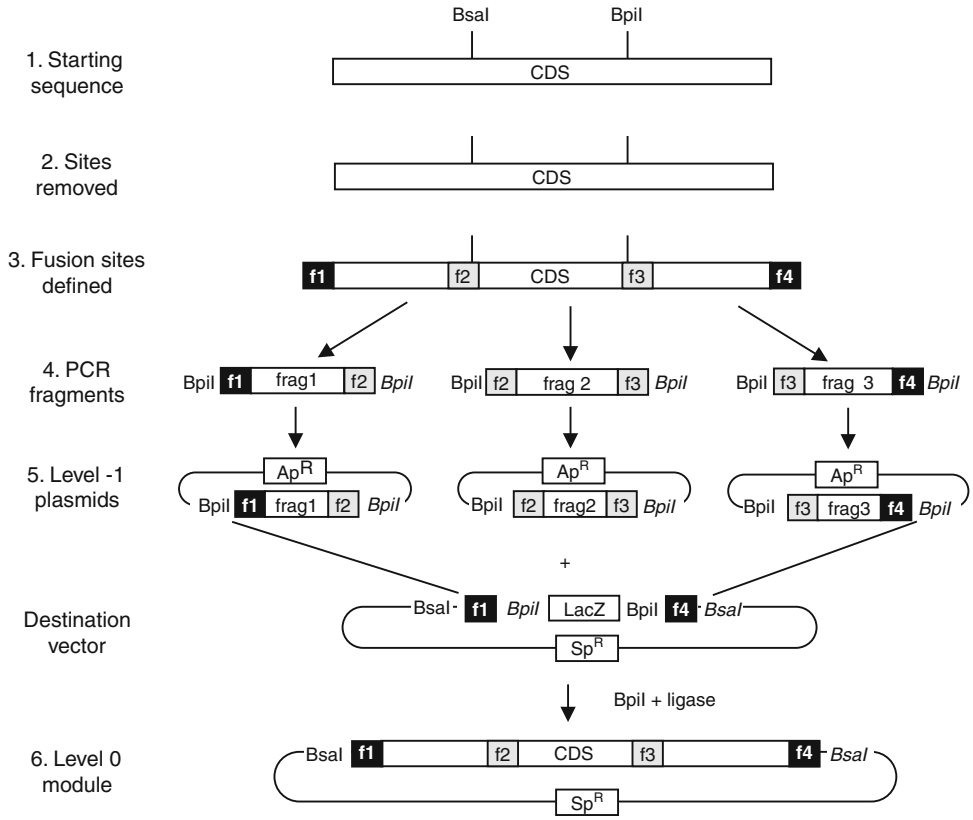

Fig. 2 Overview of intermediate steps and sequences for construction of level 0 modules. An example of a starting native sequence containing two type IIS restriction sites (for BpiI and BsaI) is depicted in [1]. The two restriction sites are mutated by introducing two single nucleotide substitutions. Fusion sites (shown in *grey*, f2 and f3) are then defined overlapping or near the mutated sites. Two fusion sites of standard sequence (shown in *black*, f1 and f4) are added at the beginning and the end of the sequence. These two sites must be compatible with two fusion sites in the destination vector. **Steps 2** and **3** are performed in silico to design the sequence that needs to be assembled. All following steps describe the physical construction of the module. Ap^R, Sp^R: ampicillin and spectinomycin resistance markers. Opposite orientations of the BpiI or BsaI recognition sites are indicated with *non-italics* and *italics*

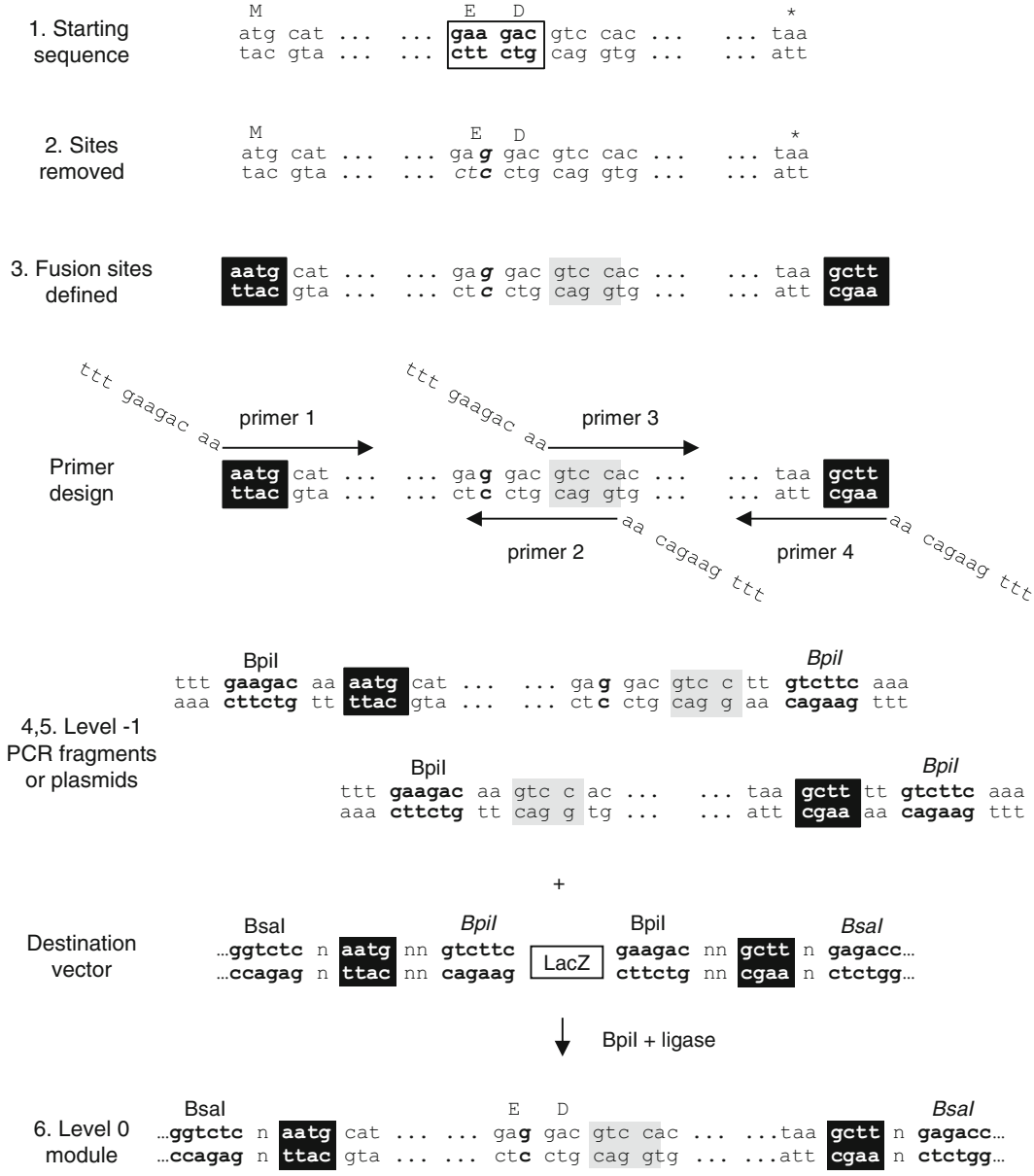

Fig. 3 Depiction of sequence details of the DNA fragments and vectors shown in Fig. 2. For simplicity, removal of a single BpiI site is shown. In **step 3**, the fusion site gtcc does not overlap with the introduced mutation (nucleotide in *italics*), but could be selected on this sequence as well

sequences. Generation of such modules using Golden Gate cloning requires the following steps.

3.1 Domestication of the Target Sequence

The first step consists of modifying the sequence of interest to remove any unwanted restriction site (a process called domestication [12]). This step is performed in silico using a program such as Vector NTI, or any other program of choice. A single nucleotide

substitution is sufficient to remove any type IIS restriction site (an example is shown in **step 2** in Fig. 3). For removal of sites within coding sequences, it is always possible to make a silent mutation that will not change the protein sequence. However, for noncoding sequences such as promoters where regulatory sequence motifs may not necessarily be known, it may be necessary to later check that the introduced mutation has not affected the genetic function encoded by the sequence of interest.

3.2 Selection of Fusion Sites and Primer Design

Fusion sites are selected at each point where the sequence needs to be split in several sub-fragments. This includes locations where restriction sites need to be removed, and any position where several variant sequences may need to be recombined. Finally, fusion sites may also be defined at several locations to split a large sequence into several smaller ones. This may be useful for cloning of large level 0 modules, since it is sometimes more efficient to sequence and screen several small fragments at −1 level than sequencing a larger fragment at level 0.

Restriction sites are eliminated by amplification with primers containing a mismatch in the sequence that binds the target sequence (Figs. 2 and 3). $n+1$ fragments are usually amplified by PCR to remove n restriction sites (*see* **Note 1**). To be able to reconstitute the entire sequence from the amplified fragments, all primers have an extension that contains a BpiI restriction site. The sequence at the cleavage site must correspond exactly to a sequence from the target sequence (the fusion site) to avoid introducing unwanted mutations to the sequence of interest. Cleavage of the amplified fragments using BpiI will release a fragment containing only target sequence, flanked on each side by four nucleotide DNA overhangs derived from the fusion site. The fusion site may be designed to overlap the mutated site but may also be selected near the mutated site (as shown in Fig. 3).

Two fusion sites are also needed at the beginning and the end of the sequence of interest. These two fusion sites must be compatible with two fusion sites in the destination vector. These sites have a standard sequence (AATG and GCTT for MoClo level 0 modules for coding sequences, Fig. 3), and are therefore not necessarily found in native sequences. For example, AATG overlaps with the ATG start codon but an A that is not necessarily native must be added upstream of the ATG; GCTT is usually not part of the sequence of interest and is added after the stop codon (fusion sites of standard sequence are shown in black in Figs. 2 and 3).

Fusion sites must be carefully selected to all have a different sequence to avoid assembly of the amplified fragments in the wrong order. It is important to check that all fusion sites also do not match the complementary sequence of the other fusion sites, since this would sometimes lead to ligation of two inappropriate fragments, one in the inverse orientation. For example, choice of the sequence

ATTC will preclude the choice of the sequence GAAT for any of the other sites. Another requirement is to avoid the 16 palindromic sequences (for four nucleotide fusion sites), since palindromic DNA ends are compatible with themselves in the other orientation. For enzymes that cleave on a four nucleotide sequence, 240 possible sequences are therefore available.

3.3 PCR Amplification of the Modules

1. A PCR mix is set up following the manufacturer's instructions. For example, using KOD polymerase (*see* **Note 2**), the following conditions are used: 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 most primers, (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 34 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 thermal cycler.
3. Of the PCR product obtained, 2 μL is 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.

3.4 Blunt-End Cloning of the Modules

Cloning of the modules before assembly is optional since PCR fragments can be assembled directly in a level 0 cloning vector (*see* **Note 3**). However, as mentioned above, cloning of level −1 fragments may be preferable to facilitate cloning of large level 0 modules. Cloning fragments before assembly may also be useful if one wants to assemble several sequence fragment variants combinatorially.

Cloning of the level −1 fragments can be performed using commercial kits such as the pGEM-T (Promega), pJET (Fermentas), and the TOPO® TA (Invitrogen) kits. PCR products can also be cloned efficiently using blunt-end cloning with a protocol that uses restriction–ligation [13, 14]. The vector chosen for cloning of level −1 fragments should fulfill two requirements: (1) it should preferably not contain any restriction site for the type IIS enzyme that will be used for the following assembly step, i.e., BpiI in the present example (*see* **Note 4**), and (2) the vector backbone should have a different selection marker from the destination vector used for the next step of assembly (level 0 modules have a spectinomycin resistance marker; therefore, level −1 cloning vectors can have an ampicillin resistance marker). Since many cloning vectors have a BsaI restriction site in the ampicillin resistance gene (for example, pGEM-T or pJET or pUC19), we have made a modified pUC19 vector that lacks this site (*see* **Note 5**).

1. Add 0.5 μL of vector (50 ng), 1 μL of PCR product (50–100 ng), 2 μL of 10× ligation buffer, 1 μL of SmaI enzyme (10 U), 1 μL of T4 DNA ligase (3 U), 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 (*see* **Note 6**).
2. The entire ligation mix is transformed into DH10B chemically competent cells and plated on LB plates containing X-gal and the appropriate antibiotic.
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 following the manufacturer's instructions.
5. Plasmid DNA can be checked by restriction enzyme digestion using BpiI, followed by analysis of the digested DNA by agarose gel electrophoresis.
6. DNA from two minipreps is sent for sequencing using primers M13RP and/or M13FP.
7. When a correct sequence has been identified, DNA concentration of the plasmid prep is measured using the NanoDrop ND2000.

3.5 Construction of the Destination Vector

A destination vector compatible with the entry modules needs to be made. The vector should contain two BpiI sites compatible with the two fusion sites present at the beginning of the first level −1 fragment and the end of the last fragment (*see* Figs. 2 and 3). The vector backbone should not contain any additional BpiI restriction site and should have an antibiotic resistance gene different from

the one used for cloning of level −1 modules. Additionally, the vector may contain a lacZα fragment to allow blue-white selection of the resulting clones. For MoClo vectors, two restriction sites for a second type IIS enzyme (BsaI) are placed flanking the two fusion sites to allow the level 0 modules to be further subcloned (for assembly of transcription units, for example). If the assembled modules do not need to be subcloned, restriction sites for this second enzyme may be omitted.

3.6 Golden Gate Assembly

Once entry constructs and the recipient vector are made and sequenced, assembling the fragments only requires pipetting all components into a reaction mix and incubating the mix in a thermal cycler.

1. A restriction–ligation is set up by pipetting into a tube 40 fmol (approximately 100 ng, *see* **Note 7**) of each level −1 module (or PCR fragment) and of the vector, 2 μL 10× ligation buffer, 10 U (1 μL) of BpiI, and either 3 U (1 μL) of ligase for assembly of 2–4 modules or 20 U (1 μL) HC ligase for assembly of more than 4 modules (final volume of 20 μL).
2. The restriction–ligation mix is incubated in a thermal cycler. For assembly of 2–4 level −1 modules, incubation for 60–120 min at 37 °C is sufficient. If more modules 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 30–50 times (*see* **Note 8**).
3. Restriction–ligation is followed by a digestion step (5 min at 37 °C for BpiI or 50 °C if BsaI is used for cloning) and then by heat inactivation for 5 min at 80 °C. The final incubation step at 80 °C is very important and is needed 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 when the reaction vessel is taken out of the thermal cycler and would lead to a higher proportion of colonies containing incorrect constructs.

3.7 Transformation of the Constructs into Competent Cells

The entire ligation is transformed into chemically competent DH10B cells (*see* **Note 9**).

1. Thaw frozen chemically competent cells (100 μL per tube) on ice.
2. Add the entire ligation to the cells, and incubate on ice for 30 min.
3. Incubate 90 s at 42 °C in a water bath.
4. Let the cells recover on ice for 5 min.
5. Add 1 mL of LB medium to the cells, and incubate the tube at 37 °C in a shaker-incubator (150 rpm) for 45 min to 1 h.
6. After incubation, plate 25–100 μL of the transformation on LB agar plates containing antibiotic and X-gal.

7. Incubate the plates overnight at 37 °C. Many white and very few blue colonies should be obtained.
8. Pick a few white colonies from the plate (*see* **Note 10**).
9. Check by restriction digest using BsaI (for MoClo level 0 modules) or any other suitable enzyme.

Level 0 modules that have been made from sequenced level −1 constructs do not need to be sequenced again. Modules that have been directly assembled from PCR products of course need to be sequenced. Level 0 modules are then ready for further assembly using BsaI and ligase and MoClo vectors (described in ref. 10).

4 Notes

1. Two restriction sites located next to each other can be mutated using a single long primer. Also, the fusion site may be selected between the two sites than need to be mutated, and the two mutations introduced by a single mismatch in each of the two primers required at this fusion site. Therefore, the number of PCR fragments that needs to be amplified to remove n restriction sites may sometimes be lower than $n+1$.
2. KOD polymerase is a useful enzyme as it has proofreading activity and does not add any nucleotide at the end of the amplified fragments (unlike Taq polymerase). Fragments amplified with KOD polymerase therefore have blunt ends, which is a prerequisite for blunt-end cloning using SmaI.
3. Direct assembly of PCR products in a destination vector is possible [15]. It is however recommended to purify the PCR products using a column to remove DNA polymerase and primer dimers. 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 and therefore need to be sequenced.
4. The presence of a BpiI site in the vector backbone of level −1 modules would not prevent assembling them using Golden Gate cloning, as the final construct will not contain this vector backbone. However, the presence of a BpiI site in all level −1 vector backbones would lead to continuous digestion and religation at this site, which would unnecessarily consume some ATP from the ligation mix.
5. A simple strategy, enzymatic inverse PCR [16], can be used to eliminate the internal BsaI site in pUC19. The entire plasmid is amplified with two primers designed to introduce a mutation in the BsaI site: primers bsarem1 (ttt ggtctc a ggtt ctcgcggtatcattgcagc) and bsarem2 (ttt ggtctc a aacc acgctcaccggctccag).

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 amplified fragment is subjected to restriction–ligation with BsaI and ligase and is then transformed in *E. coli*.

6. SmaI cannot be used for cloning of fragments containing an SmaI restriction site. However, another restriction enzyme that produces blunt ends, such as EcoRV, could be used as well (a cloning vector containing a unique EcoRV site in the polylinker is however required). Alternatively, cloning of such products using a commercial kit might be simpler.
7. 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, when plasmids or PCR fragments with widely different sizes are used, calculating an equimolar amount should provide a higher cloning efficiency. The following relation (from the NEB catalog) can be used: 1 μg of a 1,000 bp DNA fragment corresponds to 1.52 pmol. Therefore, the volume of DNA to pipet (in μL) to have 40 fmol is given by the equation: 40 (fmol) × size (bp) of the DNA fragment/(concentration (ng/μL) × 1,520).
8. For cloning of level 0 modules, which usually does not require assembling too many fragments, and where only a few positive clones are needed (for screening by sequencing), any program should work efficiently, including with continuous incubation at 37 °C. Optimization of the assembly conditions is more important for construction of libraries by DNA shuffling where cloning efficiency needs to be higher.
9. Any other *E. coli* strain can also be used. If higher transformation efficiency is required, for DNA shuffling for example, 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.
10. Two colonies should be sufficient for constructs that are assembled from sequenced level −1 modules, since most colonies should contain the correct fragment, and clones with a correct restriction pattern do not need to be sequenced.

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