Golden Gate Assembly of RMCE Integration plasmids

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I: Overview of RMCE integration vectors

Recombination-Mediated Cassette Exchange (RMCE) is a method for creating transgenic animals using recombinases (Nonet, 2020, 2023). The recombination events catalyze the exchange of DNA between two distinct FRT sites in a plasmid and similar FRT sites present in specialized landing sites in the genome. Two distinct approaches have been developed by my lab. The first approach (RMCE) uses a vector[2] containing a self-excising cassette (SEC) that permits both selection for the presence of the cassette as well as a heat shock controlled Cre recombinase gene to drive self-excision of the cassette after it has been integrated into the genome. The second approach, named rapid RMCE (rRMCE), uses vectors that contain a fluorescent protein gene and a selection marker (usually Hyg^R or Neo^R) to select for insertions. However, the selection marker is not excised during the process, but rather, if desired, can subsequently be excised by crossing through a germline Cre expressing line. Sequences to be integrated can be inserted into the vector by either traditional restriction enzyme cloning, Gibson assembly or Golden Gate assembly. The vectors for RMCE and rRMCE are distinct (Figure 1), and though both types are technically cross compatible, some of the features of each method are lost by mixing systems. Thus, choosing the correct vector is an important part of developing a transgenesis strategy. This manual concentrates on using Golden Gate assembly to create integration plasmid constructs.

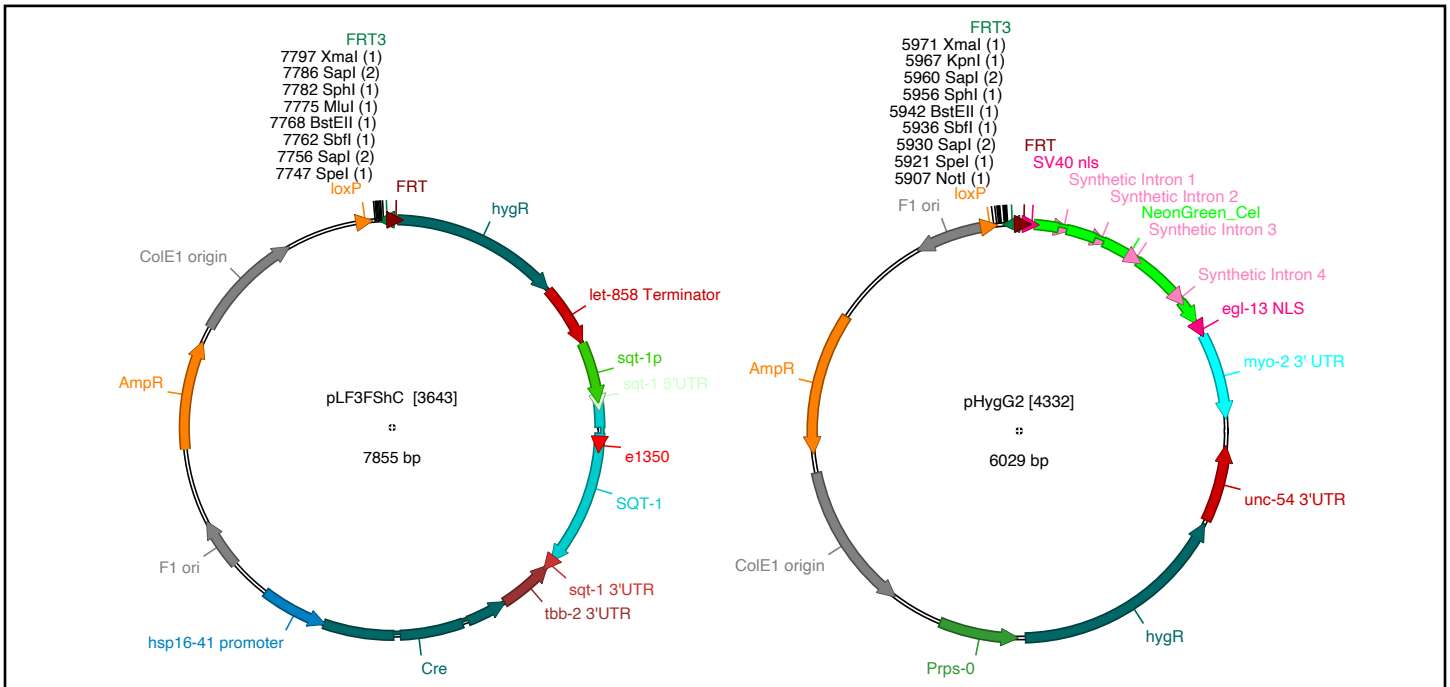

Figure 1 Organization of the pLF3FShC RMCE and pHygG2 rRMCE integration vectors.

A) The pLF3FShC integration vector contains partial Self Excision Cassette (SEC) lacking one *loxP* site. This consists of a promoter-less hygromycin (*hyg*^{R}) gene that permits selection of insertions into landing sites, a *sqt-1(e1350)* dominant mutation that permits visual screening for insertions, and a *hsp-16* heat shock promoter driven *Cre* recombinase which permits excision of the selection cassette. Sequences to be integrated are inserted into the multiple cloning site (MSC) between the *loxP* and *FRT3* site. B) The HygG2 vector contains an MCS, an *FRT* site, *mNG*, a *hyg*^{R} cassette and an *Amp*^{R} plasmid backbone, a *loxP* site and an *FRT3* site. Sequences to be integrated are inserted into the MSC between the *FRT3* and *FRT* sites. See https://sites.wustl.edu/nonetlab/rmce-integration/ for more details on how integration works.

II: Overview of Golden Gate plasmid assembly

The Golden Gate assembly (Engler et al. 2008) method facilitates simultaneously inserting multiple DNA fragments that each contain an element to be included in the final clone. Each element is flanked by restriction sites, which end up excluded from the final assembly. The method takes advantage of type IIS restriction enzymes, such as *SapI*, that cut at a non-palindromic recognition sequence and cleave the DNA outside its restriction selectivity site and leave a 5' overhang cohesive end. For example, *SapI* cleaves at 5' GCTCTTCN/NNN 3'. This it leaves a 3 base overhang that can be any sequence. By assembling fragments with different overhangs, one can assemble multiple fragments, and these will assemble only in a specific order based on base pairing (Figure 2).

Since the restriction enzyme sites are excluded from the final assembly, the digestion and ligation steps can be repeated numerous times to increase the efficiency of the assembly process. Using the Golden Gate method, it is possible to assemble over 10 fragments into a vector without too much difficulty using high quality DNA reagents. Although in theory any order of 3 base overhangs can be used to create plasmids, I use a specific set of pairs of overhangs as 'slots' and use a conventional order to facilitate the sharing of reagents. This permits development of libraries of clones which can be used interchangeably in the construction of new integration plasmids. An additional advantage of using sequenced plasmids as the source of inserts is that the final assembly does not require sequencing. Alternatively, the inserts can be supplied as PCR fragments. Very small inserts can be supplied as hybridized oligonucleotide pairs.

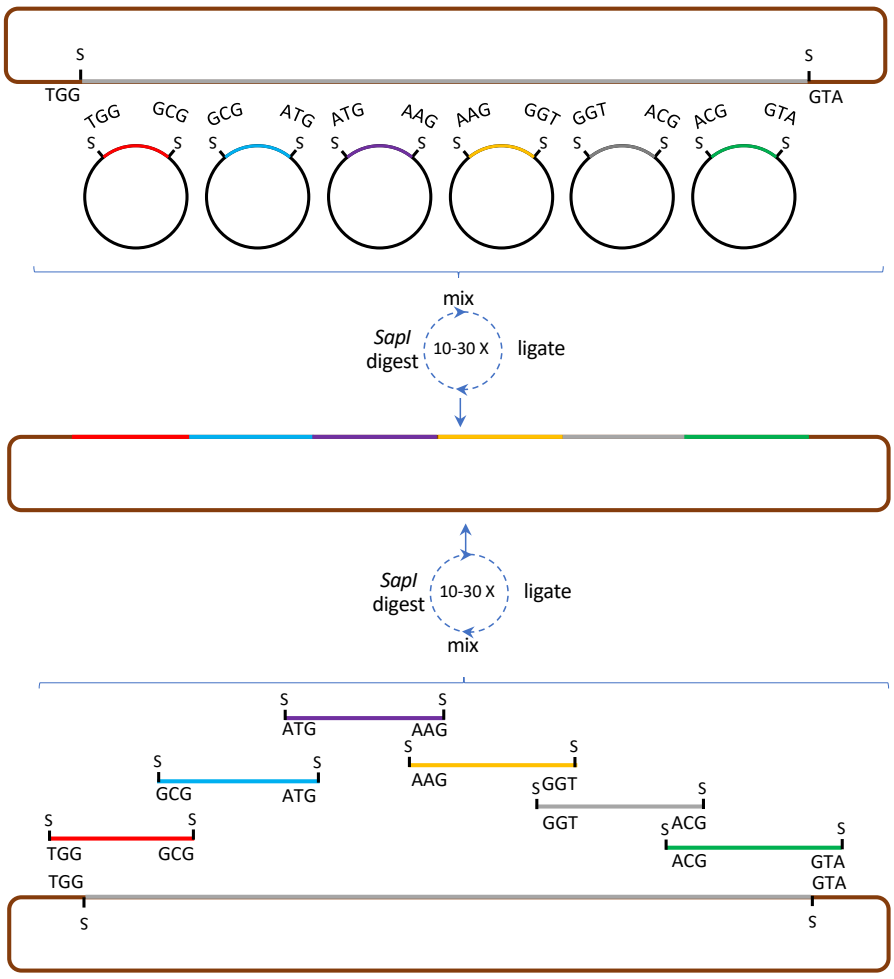

Figure 2. Golden Gate Cloning strategy using SapI 3 bp overlaps.

Examples of Golden Gate assembly using either plasmids or PCR fragments as the source of inserts. S presents a *SapI* restriction site. The plasmid backbone of the vector (brown) is Amp^R of the inserts (black) are Kan^R.

To assemble integration constructs I use a *SapI* Golden Gate strategy based on Dickinson's method for building CRISPR integration plasmids (Dickinson et al. 2018), which is a merging of two approaches, one developed by Dickinson et al. (2015) and one by Schwartz and Jorgensen (2016). The basic methodology (see below) involves introducing up to 8 distinct DNA fragments in order into an RMCE integration vector. Each element has distinct *SapI* sites at its ends forcing the ordered assembly (Figure 2).

For historical reasons these are often refer to these slots as the 'sgRNA', 'pU6', '5' arm', 'CT,' 'FP', 'SEC', 'NT' and '3' arm' slots because originally this approach was used to created SEC containing CRISPR/cas9 integration vectors (Dickinson et al., 2018). However, in our approach the SEC has been incorporated into the vector rather than being introduced as one of the inserts.

The approach is powerful for several reasons. First, many traditional cloning steps are combined into one using this approach. Second, as mentioned above, the approach permits one to use previously constructed plasmids as inserts which allows for developing "libraries" of inserts which facilities rapid construction of new integration constructs. The one major limitation is that these inserts cannot contain a *SapI* restriction site. If *SapI* sites are present in a potential insert they must be eliminated. The manual discusses how to remove sites if they are present.

In addition to pLF3FShC and pHygG2 (and related vectors) which are designed for six slots, pLF3FShC2 and pHygG12 (and related vectors) are designed for 8 slots with two additional slots on the 5' end (relative to pLF3FShC and pHygG2). Although I define these as 'six slot' and 'eight slot' vectors, I often merge slots when constructing simpler insertions (Figure 3). In theory one can also divide slots. The number of fragments one can insert is technically only limited by the number of different 3 bp overhangs. This approach provides great flexibility in creating new constructs. For example, a very common construct one would create is an N-terminal fluorescent protein (FP) fusion to a protein under a specific promoter. In this case, I place our promoters is a combined 5' arm-CT slot vector, use the FP slot for a fluorescent protein the SEC slot for a linker, the NT slot for our gene of interest, and the 3' arm slot for the 3' UTR of the construct (Figure 3). One can switch the promoter to create a construct that will express the fusion in a different cell type, the FP to express a different color fusion, or the gene of interest to make a distinct FP-protein fusion. As one has builds up libraries of promoters, FPs and cloned genes creating new derivatives is becomes simpler and simpler.

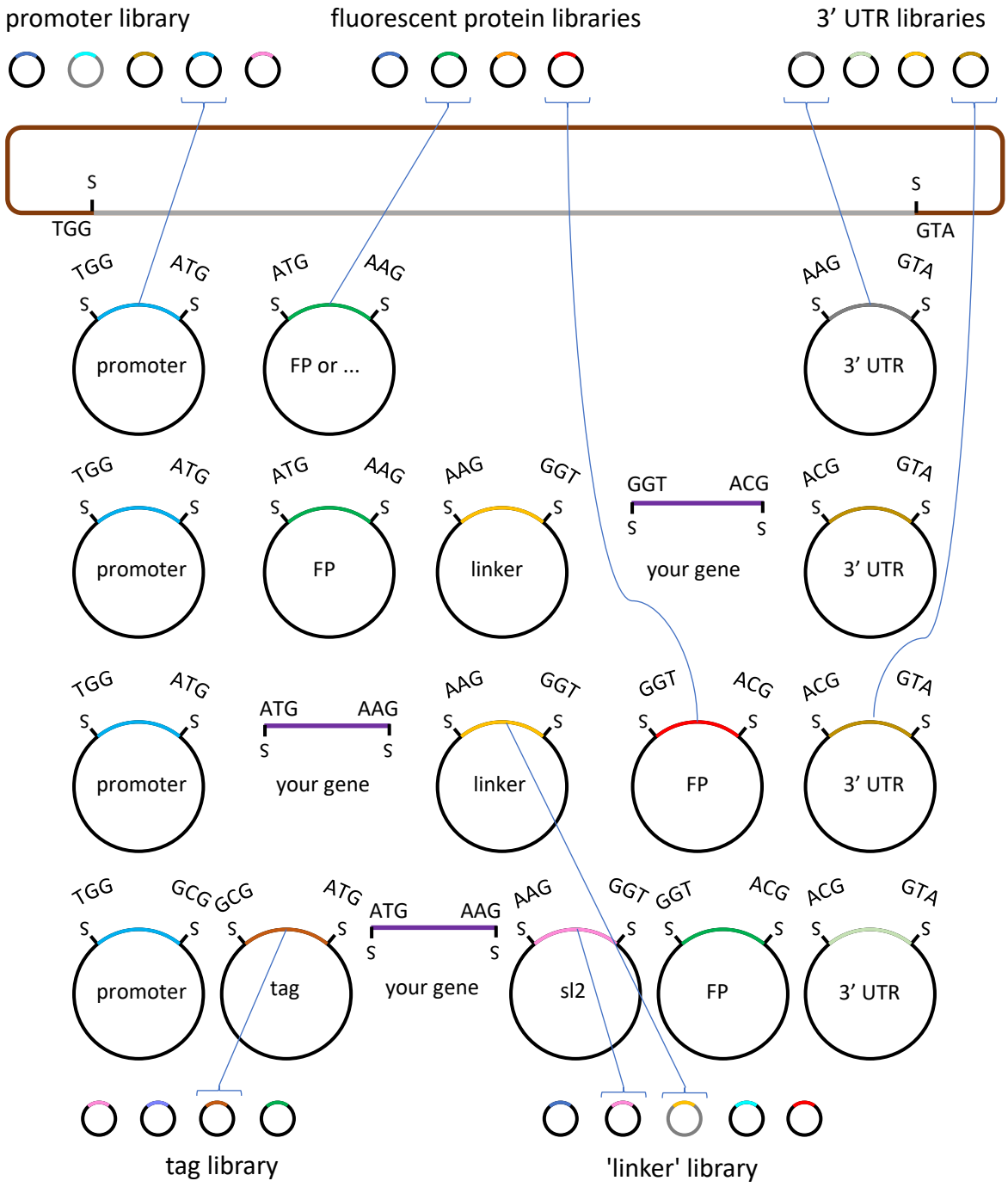

Figure 3. Cloned insert libraries provide the flexibility to quickly assemble novel constructs.

Examples of various integration clones that can be assembled from SapI entry clone libraries (Knoebel et al. 2023) in combination with a gene of interest PCR product. Common promoters, FPs, tags and 3' UTRs are supplied from the clone library, other small tags can also be supplied as hybridized oligonucleotide pairs (not shown) and the gene ORF is supplied as a PCR product flanked with *SapI* restriction sites. S presents a *SapI* restriction site. The plasmid backbone of the vector (brown) is Amp^R of the inserts (black) are Kan^R.

III: *In silico* assembly of plasmids

The plasmid editor ApE (Davis and Jorgensen, 2022) facilitates design and assembly of plasmids. It provides a tool to assemble plasmids using Golden Gate reactions i*n silico*. To use the tool, create ApE files representing each PCR product, insert plasmid, and the plasmid vector. Unfortunately, the program does not deal well with oligonucleotide pairs. One needs to create a file that represents the oligonucleotide pair with *SapI* sites on the ends. To create a plasmid (Figure 4), open the vector file and all the insert files. Select Tool/ Golden Gate Assembler, then select the enzyme being used for the reaction. Finally select all of the inserts and click OK. The program will create a new file of the fully assembled product. If 'No Circular Product Possible" appears then one of the inserts is missing or not properly designed. Using this program to test designs will save one from silly mistakes in choosing plasmids, oligonucleotides for amplifying products, etc.

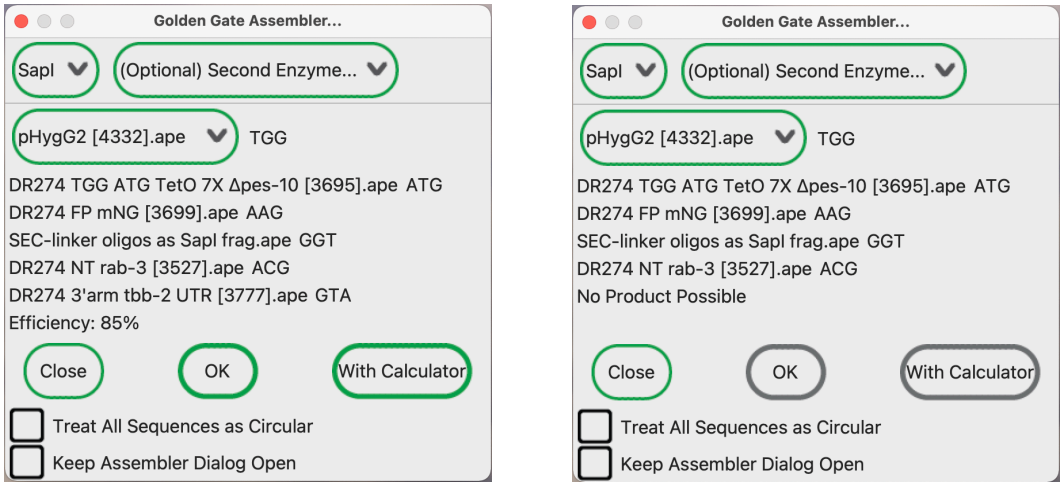

Figure 4. ApE in silico Golden Gate Reactions.
Assembly of a tetO 7Xp::mNG-linker-rab-3::tbb-2 3' UTR construct in pHygG2. Left) a functional and right) a non-functional assembly.

A second tool available for assembling RMCE plasmids that provides a better overview of the potential to create clones but provides less well annotated plasmids is an Excel based assembly tool called 'GG assembly Builder' available at https://sites.wustl.edu/nonetlab/golden-gate-cloning-resources/#GGABuilder. This excel document (Figure 5) has a series of dropdown menus which display the clones available from the Nonet lab (and some compatible plasmids published by other labs) which can be used to create assembles in either 6 or 8 slot vectors. One selects the vector, and the desired inserts for each slot, and the tool builds both a full sequence and an annotated sequence file. The contents can be pasted into a text document to create a sequence file or Genbank format annotated file.

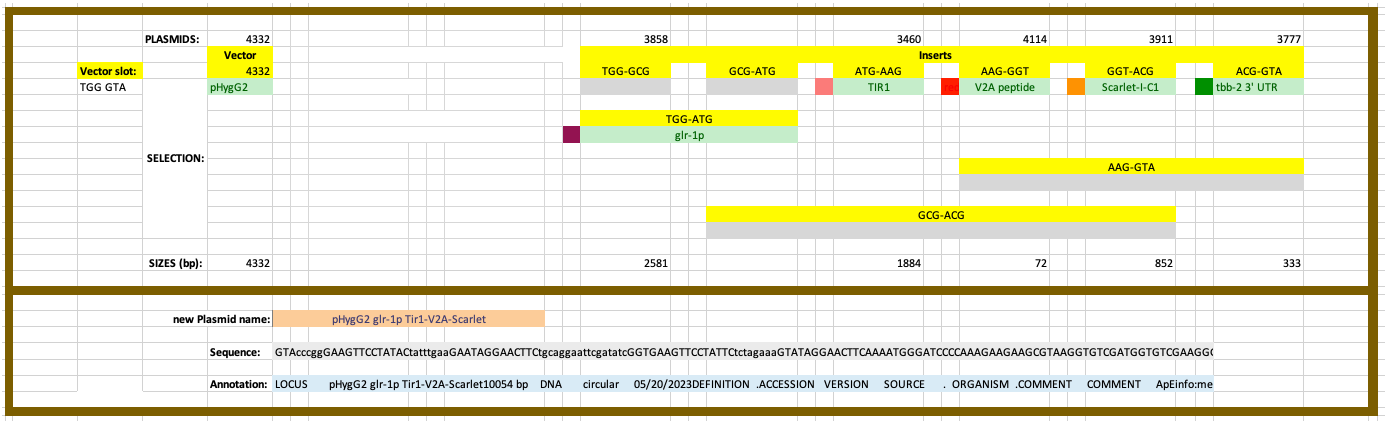

Figure 5. GG Assembly Builder *in silico* assembly tool
An example assembly of a pHygG2 *glr-1p::*TIR1-V2A-Scarlet::tbb-2 3' UTR rRMCE integration vector.

IV: RMCE integration plasmid assembly

Building a new RMCE integration vector involves

1. Designing the new construct.
2. Creating the novel PCR fragments or clones required for the final assembly.
3. Performing a *SapI* Golden Gate reaction to build the construct using the DNAs.

I address these steps in reverse order since it is possible to acquire many insert containing plasmids for assembling novel plasmids (from Addgene, the Nonet lab and by scouring publications) without performing any new cloning or PCR fragment amplification. I do not discuss design of integration plasmids as this is a complicated issue. A extensive discussion of clone design is found in Nance and Frøkjær-Jensen (2019).

Protocol 1: Golden Gate-based RMCE plasmid assembly

P1.1 Combining DNAs[3]

Clones, PCR products, and annealed oligonucleotides are mixed using the following ratios[4]

20 fmol of vector (~75 ng of the 6 kb pHygG2 vector)
20 fmol of each insert (e.g. 50 ng for a 4 kb plasmid, 12 ng of 1kb PCR product)
50 fmol of each oligonucleotide pair (e.g. 2 ng for a 60 bp oligonucleotide pair)

Dilute to 10 µl with TE if volume is less than 10 µl.

P1.2 *SapI* Golden Gate reaction

1 µl 10X Sap reaction buffer[5]
7 µl H_20
1 µl DNA mix
1/2 µl of SapI[6]
1/4 µl of T4 DNA ligase
1/4 µl of Polynucleotide Kinase (optional[7])

Perform the digestion/ligation in a PCR machine[8]. Typically, I run 37° for 10 min, followed by 10 cycles of (16°C for 5 min, 37° for 5 min), then a step of 65°C for 20 min. The reaction is even more efficient if one uses 30 cycles (which I do if I am running the reaction overnight). The last 37°C step is to allow *SapI* to do a last round of cutting of incomplete assemblies, so they are not circles. The 65°C step inactivates the enzymes.

P1.3 General *E. coli* transformation method

Thaw a tube of competent cells[9] on ice for 10-15 minutes.
Aliquot 50-100 µl to a pre-chilled tube on ice.
Add 1 µl of ligation to the competent cells and briefly vortex.
Incubate on ice 20-40 minutes (longer the better).
Heat shock 30 sec at 42°C.
Incubate 10-20 minutes on ice (longer the better).
Add 700 µl of SOC media.
Incubate 1 hr at 37 with shaking.
Plate 1/10th of the transformation on the appropriate drug[10] LB plate, and incubate o.n. at 37°C.

One should get hundreds of colonies using competent cells with 1 x 10^8 colonies/µg pBluescript DNA efficiency. A minimum of ~30-80% of colonies are correct in my experience. Often it is 100%.

P1.4 Clone analysis

Miniprep[11] 3-6 colonies and identify putative correct clones using a restriction digest (*EcoRI* is usually diagnostic). Perform additional restriction digests to confirm all inserts are present if small inserts (linkers, V2A tags) could be absent without significantly altering the digestion product pattern. If the inserts are derived from plasmids that have already been sequence verified, then I find sequencing is not needed. If the inserts are PCR products the error rate will depend on the quality of the template used and the error rate of the polymerase. It is rare to find errors using quality genomic DNA as template and NEB Q5 as the polymerase.

V: Creating insert PCR fragments

One approach for creating inserts is to PCR amplify the desired fragments from a high-quality source of DNA (genomic DNA, first strand cDNA, or a plasmid). However, if the fragment of interest contains *SapI* sites, they need to be 'removed' by introducing silent mutations. In such cases I find it is easier to remove the sites using a *BsaI* Golden Gate multi-insert strategy (see below) while cloning the fragment into a Kan^R plasmid. Alternatively, if multiple *SapI* sites are present, it may be simpler to have the fragment synthesized *in vitro*, or to perform the assembly using alternative methods such as Gibson assembly[12].

Protocol 2: Creating insert PCR fragments

P2.1 Design of oligonucleotides for PCR

Design appropriate oligonucleotides that will amplify the product of interest. Several things need to be checked. First the fragment cannot have any *SapI* sites in it. The 5' ends of each primer should contain a *SapI* site and the appropriate 3 bp overlap for the slot one is inserting into as shown in Figures 2 & 3. For example, for the fragment in the 5' arm slot they should be 5' GACTGCTCTTCgTGG and 5' CACTGCTCTTCgCGC. The first four bases are to ensure efficient cutting by *SapI*[13]. If the construct being assembled creates a protein fusion, remember to maintain frame at junctions between your insert sequences.

P2.2 General PCR method

Standard 25 ul PCR reaction
In one PCR tube **on ice**:

16 µl H20
5 µl of 5X Q5 polymerase buffer
2 µl 2.5 mM dNTPs
1 µl template DNA (25 ng N2 genomic DNA or 1ng of plasmid)[14]
1/2 µl 10-25 uM forward oligonucleotide[15]
1/2 µl 10-25 uM reverse oligonucleotide

To reaction mix add 1/4 µl of Q5 polymerase[16]. Mix well by pipetting and return to ice.
Start PCR machine using an appropriate program and add tube(s) when machine hits 72°C[17].

Typical PCR conditions for oligonucleotides designed with a 55-58°C annealing temperature[18]:

98°C 0:30, 30 cycles (98°C for 0:10, 58-62°C for 0:30, 72°C for 1:00/kb).

Optional: (required if the template is an Amp^R plasmid) Add 1/2 µl of *DpnI* and digest 15 minutes at 37°C.

P2.3 General column DNA purification method

Add 125 µl (5 volumes) of diluted PBI[19] to the PCR reaction.
Load the reaction on a Qiagen or Monarch mini column.[20]
Spin 30 seconds to bind to column (optional - remove binding liquid).
Add 150 µl PE buffer, spin 30 sec. (optional- remove PE).
Add 200 µl PE buffer, spin 30 sec. Rotate tube 180 degrees, Spin 1 minute.
Transfer column to a 1.5 ml Eppendorf tube.
Add 10 µl of TE. Spin at 100 RCF, 1 minute to drive TE into column.
Spin full speed 1 minute.
(optional) To increase yield, but lower concentration, add 10 µl TE and repeat elution.

A typical yield will be ~1-2 µg of DNA for a 25 µl PCR reaction[21].

Quantify using a NanoDrop spectrophotometer, or more crudely by running a 1 µl aliquot on a gel with a known concentration of a DNA ladder.

VI: Annealed oligonucleotide pairs as inserts

In cases where one would like to insert a very small fragment in one of the slots (for example adding a FLAG or HA tag to the end of a FP insert, one can use a pair of oligonucleotides that have been annealed as a fragment in the *SapI* assembly reaction. The key here is to make sure that one does not add too much fragment into the reaction by accounting for the small size of the oligonucleotide fragment. For the typical 60 bp annealed insert, 1 ng of each oligonucleotide would be ~ 50 fmol.

Protocol 3: Annealing oligonucleotides

P3.1 Annealing oligonucleotides

Mix 1 µl of each 100 uM oligonucleotide stock (100 pmol/µl) in 10 µl of water
Add 1 µl of that dilution to 100 µl 1X anneal buffer
Heat to 95°C for 2 minutes and slow cool to 37 at -6°C per minute in a PCR machine.

This creates a working stock at 100 fmol/µl of the annealed oligonucleotides. Remember to add PNK to the Golden Gate reaction when using oligonucleotides as these are not phosphorylated on the 5' end.

VII: Creating insert clones

Another option is to create a *SapI* entry clones for the fragment(s) instead of using a PCR product. This may save time if one is going to use the insert for multiple different integration constructs. In particular, once the plasmid has been sequenced, one can be confident the final product will not have an error, while if the insert is a PCR product errors are more likely. The general approach is to PCR amplify the product of interest, digest it with restriction enzymes, and ligate the fragment into a Kan^R vector. Several approaches can be used which are outlined in Figure 6.

Common insert cloning approaches:

There are many approaches that one can use to create *SapI* insert clones. The ones that the Nonet lab uses most often are:

A) *BsaI* Golden Gate method

In this approach the insert fragment is introduced into a plasmid that has the *SapI* sites using a *BsaI* Golden Gate cloning strategy. This is the simplest approach if your insert does not contain *BsaI* sites. It is virtually 100% reliable, rapid and requires very little DNA. Specifically, I have created Kan^R vectors for each slot (e.g. the 'FP' ATG AAG slot), that permits inserting the fragment by adding *BsaI* sites on the end of the fragment. This 'only'[22] works if the fragment does not contain *BsaI* sites, but it is very easy to perform.

B) *SapI* ligation method

In this approach the *SapI* fragment is introduced into the same vector described in A) but using a *SapI* digestion and ligation. This approach is used for inserts that contain *BsaI* sites or to clone a fragment that originally was inserted as a PCR fragment.

C) Gibson assembly method

Gibson assembly (Gibson, 2011) is a popular approach of cloning DNA fragments and utilizes 15-20 bp of homology between the insert and the vector to drive the specificity of the reaction. This is alternative method to insert fragments that contain *BsaI* sites.

D) Double sticky-end cloning method

Another well-vetted approach to clone fragments which relies on double sticky-end restrictions sites to drive orientation and specificity of the cloning reaction into a Kan^R (or other non-Amp^R vector).

E) Multi-fragment *BsaI* Golden Gate method

In cases where there are *SapI* (or *BsaI*) sites in the fragment of interest and one wishes to clone the fragment, one can use a multi-insert *BsaI* Golden Gate strategy to lesion all the sites (using synonymous changes if in coding sequence) and assemble a *BsaI* and *SapI* lacking version of the insert.

F) Multi-fragment Gibson assembly method

A similar strategy to E) can also be implemented using Gibson assembly driving proper assembly by using homology rather than restriction endonucleases. This is particularly useful for fragments that contain many *BsaI* sites in addition to *SapI* sites.

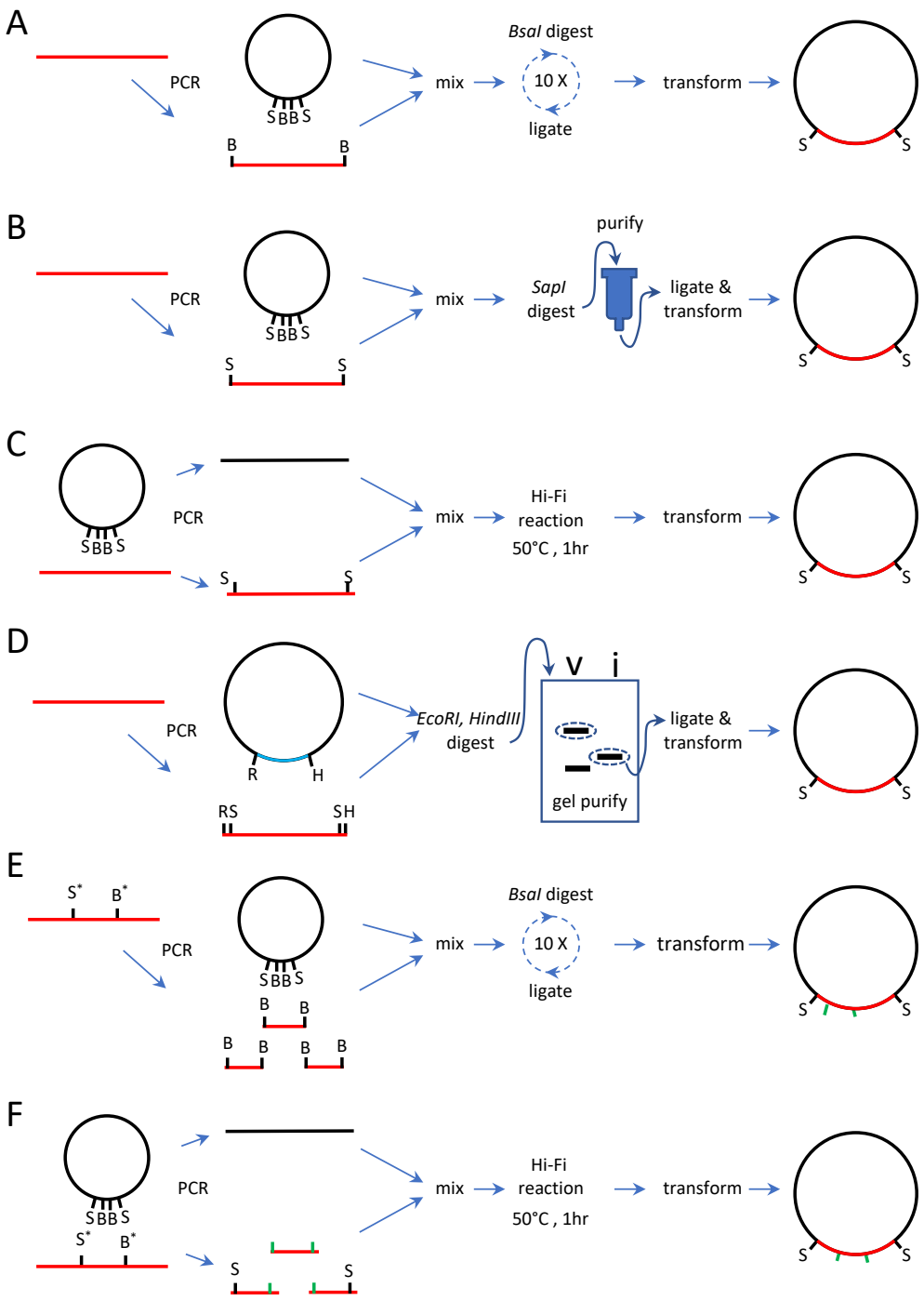

Figure 6. Alternatives for cloning inserts into Golden Gate 'entry' vectors.

Six alternative methods for creating Golden Gate entry vectors. A) a *BsaI* Golden Gate approach, B) a *SapI* cloning approach, C) a Gibson assembly cloning approach, D) a traditional double sticky-end cloning approach E) a multi-fragment *BsaI* Golden Gate approach, and F) a multi-fragment Gibson assembly cloning approach. Restrictions sites: B= *BsaI,* R=*EcoRI*, H=*HindIII*, S= *SapI*. Inserts are shown in red and the vector in black.

A) *BsaI* Golden Gate method

This simple method works highly efficiently and is our favorite approach for inserts that contain neither a *BsaI* or a *SapI* site. One amplifies the insert of interest with oligonucleotides that append *BsaI* sites on each end of the product and insert the fragments into the appropriate DR274 slot vector. Vectors are available for all eight slots and some common combined slots (Table 1). An example is shown in Figure 7.

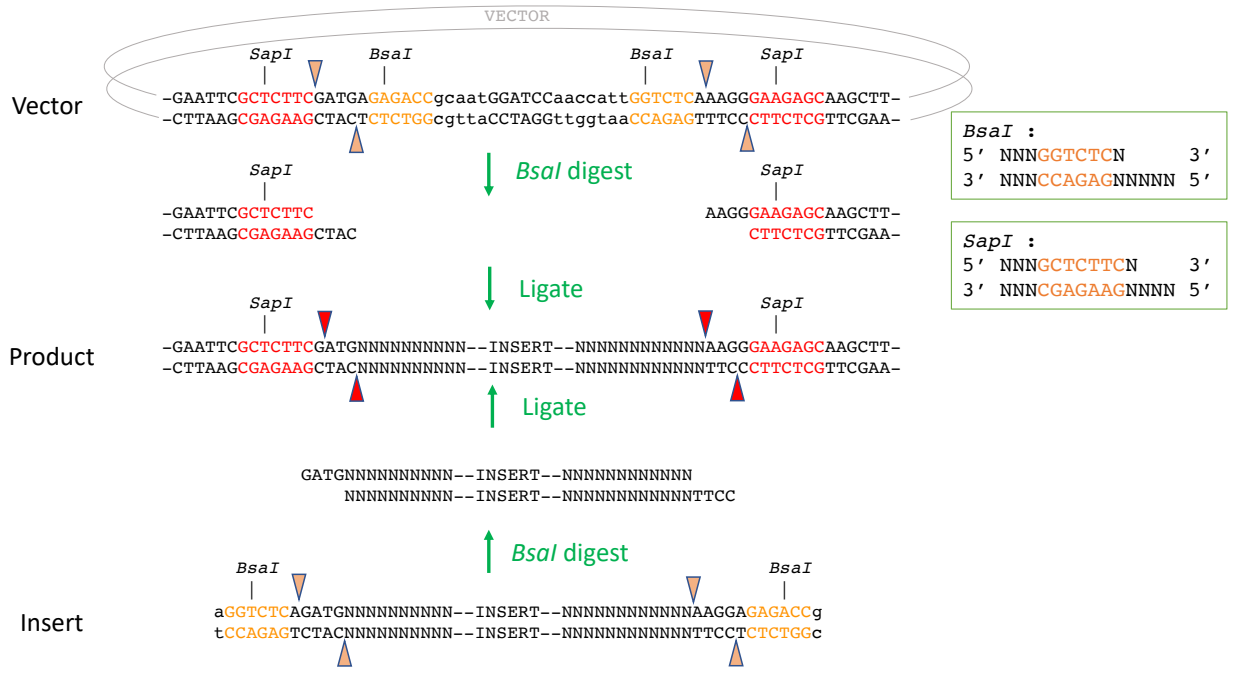

***Figure 7. BsaI* Golden Gate insert cloning into DR274 slot entry vectors.**
Schematic diagram of *BsaI* Golden Gate cloning to create *SapI* Golden Gate entry clones. Shown is an insert PCR product with *BsaI* sites on each end. The insert and vector are mixed and co-assembled using a *BsaI* Golden Gate reaction. The limitation of this method is that the insert cannot contain either a *BsaI* site or a *SapI* site. Red arrowheads represent the cut sites for *SapI* and orange arrowheads represent the cut sites for *BsaI.*

Protocol 4: *BsaI* Golden Gate Method

P4.1 Oligonucleotide design

Design oligonucleotides with *BsaI* sites each designed to match the overlap of the appropriate DR274 slot- *BsaI* vector (Table 1). For example, for the FP vector, the overlaps are GATG on the 5' side and AAGG for the 3' side. Thus, the oligonucleotides should start with 5' AGGTCTCAGATG 3' and 5' AGGTCTCACCTT 3'. A single base 5' of the *BsaI* site is added to ensure this PCR fragments cut effectively with *BsaI*. Remember to make sure that the correct reading frame is maintained if one is cloning an ORF or functional domain.

P4.2 PCR Amplification of insert

Amplify and purify the PCR product using the general PCR method and purify using the general column purification method.

P4.3 Combining DNAs

Mix
50 fmol of plasmid DR274 BsaI-Slot (50 ng vector)
60 fmol Insert PCR (25 ng of 1 kb insert)
Add TE to 10 µl of TE total

P4.4 *BsaI* Golden Gate reaction

1 µl of 10 Sap reaction buffer
1/2 µl of DNA mix
7 µl H20
1/2 µl *BsaI*
1/4 µl T4 DNA ligase

Run reaction: 37°C, 10 min; 16°C, 5 min; 10X (37°C, 2 min; 16°C, 2 min); 37°C,10 min; 65°C 10 min.[23]

P4.5 Transformation into *E. coli* and analysis

Transform using the general *E. coli* transformation method with 1/2 µl and plate 1/10th of the transformation. This should yield thousands of colonies and virtually all will be correct. Analysis of 3 clones should be sufficient to get a clone and a backup (in case of PCR induced error). Sequencing of clones is wise for those encoding ORFs and recommended for those encoding promoters, intergenic regions, or 3' UTRs.

B) *SapI* cloning method

I use this approach if there are internal *BsaI* sites in the fragment I am attempting to clone and I have chosen not to remove them. An example is shown in Figure 8.

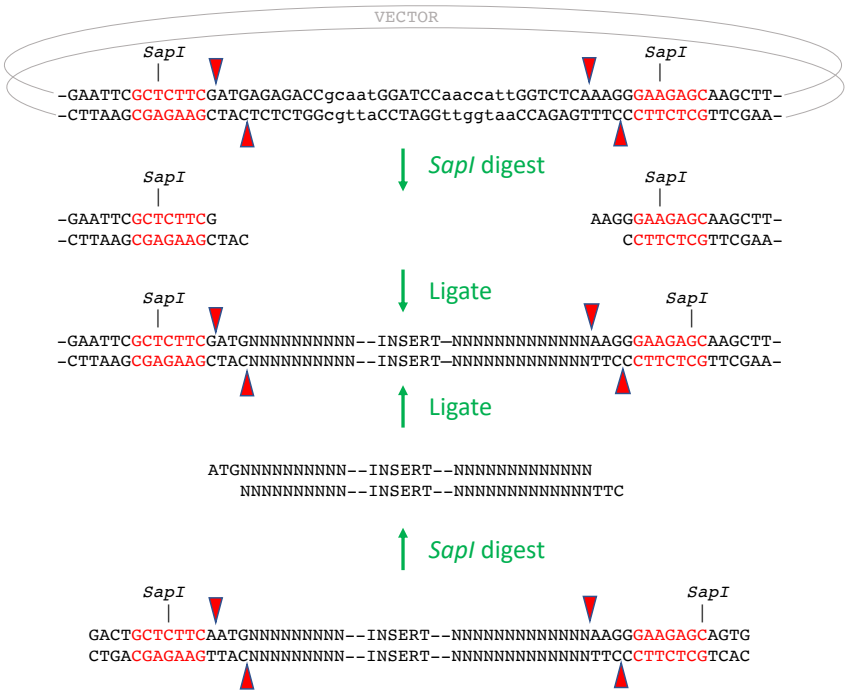

Figure 8. *SapI* insert cloning into DR274 slot entry vectors.

Schematic diagram of *SapI* restriction enzyme cloning to create *SapI* GG entry clones. Shown is an insert PCR product with *SapI* sites appended on each end. The insert and vector are mixed, digested with *SapI*, column purified and assembled using a ligation reaction. Red arrowheads represent the cut sites for *SapI.*

Protocol 5: *SapI* ligation cloning

P5.1 Oligonucleotide design

Design appropriate oligonucleotides that will amplify the product of interest. The 5' ends of each primer should contain a *SapI* site and the appropriate 3 bp overlap for the slot vector one is utilizing. For example, for the fragment being introduced in the 5' arm slot they should be 5' GACTGCTCTTCgTGG and 5' CACTGCTCTTCgCGC. Add four bases 5' of the *SapI* recognition site to ensure efficient cutting by *SapI*. If the construct being assembled creates a protein fusion, remember to maintain frame at junctions between your insert sequences.

P5.2 PCR amplification of insert

Amplify the fragment using the general PCR method and purify using the general column purification method.

P5.3 Combining DNAs

Mix
50 fmol of plasmid DR274 *BsaI* slot (50 ng vector)
60 fmol insert PCR (25 ng of 1 kb insert)
dilute to 10 µl with TE

P5.4 Digest DNA

7 µl H2O
1 µl 10X NEB Smart buffer
2 µl DNA mix
1/3 µl of *SapI*
Incubate 30 min to 1 hr.

P5.5 Column purify digested DNA

Dilute the digestion to 25 ul and column purify as outlined in purification of PCR product**.** This step is performed to remove the small *SapI* insert of the vector which greatly increases the efficiency of the ligation by eliminating competition for reinsertion that fragment into the vector. It is important to use diluted PBI otherwise the insert will stay on the column and the purification will accomplish little.

P5.6 Ligate DNA

To the eluted DNA (20 ul), add 2ul 10X T4 DNA ligase buffer and 1/4 µl of T4 DNA ligase. Ligate 30 minutes.

P5.7 Transformation into *E. coli* and analysis

Transform using the general *E. coli* transformation method with 1/2 µl of ligation mix and plate 1/10th of transformation. You should get thousands of colonies and the majority will be correct insert clones. Analysis of 3 clones should be sufficient to get a clone and a backup (in case of PCR induced error). Sequencing of clones is necessary for those encoding ORFs and recommended for those encoding promoters, intergenic regions or 3' UTRs.

C) Gibson assembly method

DR274 slot vectors do not exist for all combinations of *SapI* sites. In some cases, one may use an unusually combination because one is adding a slot or deleting a slot to create an unusual plasmid. In these cases, cloning the PCR fragments to create an entry vector is easiest done by either Gibson assembly or standard double sticky-end cloning. In either case, the *SapI* sites with appropriate ends and either 15 bp of homologous sequences or restriction sites are appended to the 5' end of the oligonucleotides to create appropriate PCR products. This can be used both to create clones using single inserts or more complex assemblies using multiple fragments (to alter internal *BsaI* or *SapI* sites). A major benefit of Gibson assembly over Golden Gate cloning is that there is footprint left at the junction while in our Golden Gate strategy there is a 3 bp footprint. Gibson assembly is also relatively insensitive to the presence of primer dimers unlike the Golden Gate approach. Gibson Assembly uses homology between 15-30 bp overlaps between DNA fragments (usually created by PCR) to drive the proper assembly. Any source or DNA (gel purified restriction digestion

products, synthetic DNA, or PCR products can be used as the substrate for a Gibson assembly reaction). However, we have found in the past that PCR is the most efficient approach as restriction digested vectors sometimes contain a very small amount of partially cut product that can cause issues with background. We use the Hi-Fi 2X master mix from NEB to perform our reactions. To create a novel clone using this strategy oligonucleotides are designed to create vector and insert fragments that have 15-20 bp overlaps - longer overlaps work better. The vector and insert fragments are PCR amplified, *DpnI* digested to remove template DNA, and purified. The fragments are then mixed with 2X NEB Hi-Fi master mix for 1 hr. and transformed. The approach is efficient but requires more DNA that Golden Gate cloning. An example is shown in Figure 9. A note of caution about this protocol. The Nonet lab no longer uses Gibson assembly very often and thus I

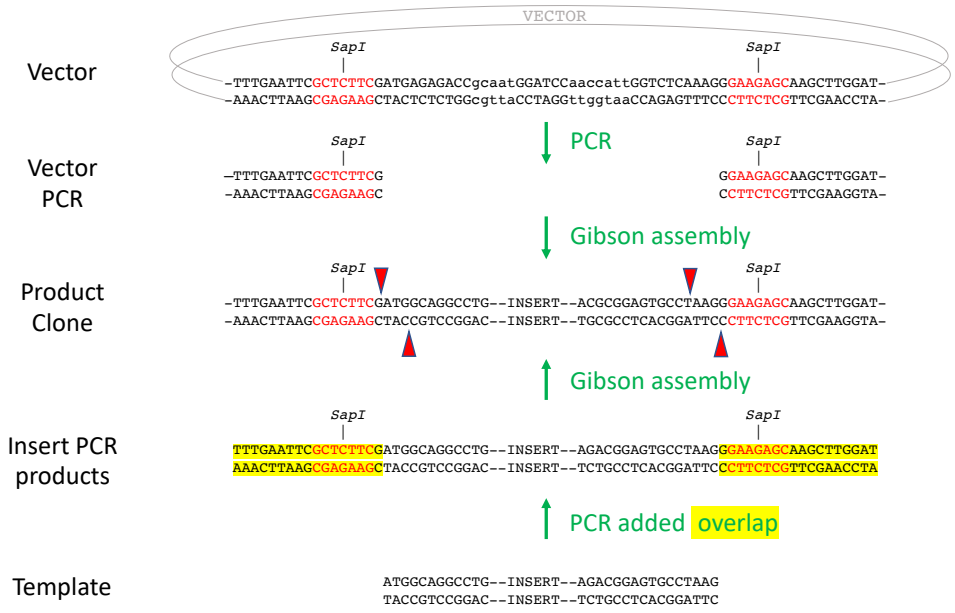

Figure 9 Gibson Assembly.

Schematic diagram of Gibson assembly cloning to create *SapI* GG entry clones. Shown is an insert PCR product with *SapI* sites and a small homology arm appended on each end. The insert and vector are mixed and co-assembled using a HiFi Gibson assembly reaction. Red arrowheads represent the cut sites for *SapI.*

have not optimized this protocol and I do not keep up on 'advances' in Gibson assembly strategies.

Protocol 6: Gibson Assembly

P6.1 Oligonucleotide design

To create a clone by Gibson assembly I typically create the clone *in silico* by cutting and pasting the insert(s) into the vector of choice to create an *in silico* version which marks all fragment junctions. I then decide which oligonucleotide should be appended to add the overlap. This determines which purified PCR fragments are easily re-usable for other Gibson assembly reactions. A 55°C T_m overlap is our typical choice. The desktop program ApE and the NEBuilder website both have tools to facilitate the design of primers for Gibson assembly. An example is shown in Figure 9.

P6.2 PCR amplification of vector and insert

Amplify the vector and insert using the general PCR method and purify using the general column purification method.

P6.3 Gibson assembly reaction

Mix the following

25 fmol of vector PCR

50 fmol of insert PCR[24]
TE to 5 ul
5 ul of 2X HiFi Assembly Master Mix

Incubate at 50°C for 1 hr.

P6.4 Transformation into *E. coli* and analysis

Transform using the general *E. coli* transformation method with 1/2 µl of ligation mix and plate 1/10th of transformation. The number of colonies obtained is highly dependent on the length of the overlap, but a few hundred is common for 55°C T_m overlaps. The majority will be correct insert clones. Analysis of 3 clones should be sufficient to get a clone and a backup (in case of PCR induced error).

D) Double sticky-end cloning

This is the traditional approach to cloning my lab used to create most single insert clone for most of the 30 years my lab had been doing molecular biology before switch to using Golden Gate cloning for most applications. It can be more efficient to use that the *SapI* method to clone fragments that contain *BsaI* sites if one if performing lots of different reactions with different *SapI* overhangs. This is because the *SapI* sites are incorporated into the PCR insert and thus in theory all the different fragments can be cloned into the same double cut vector.

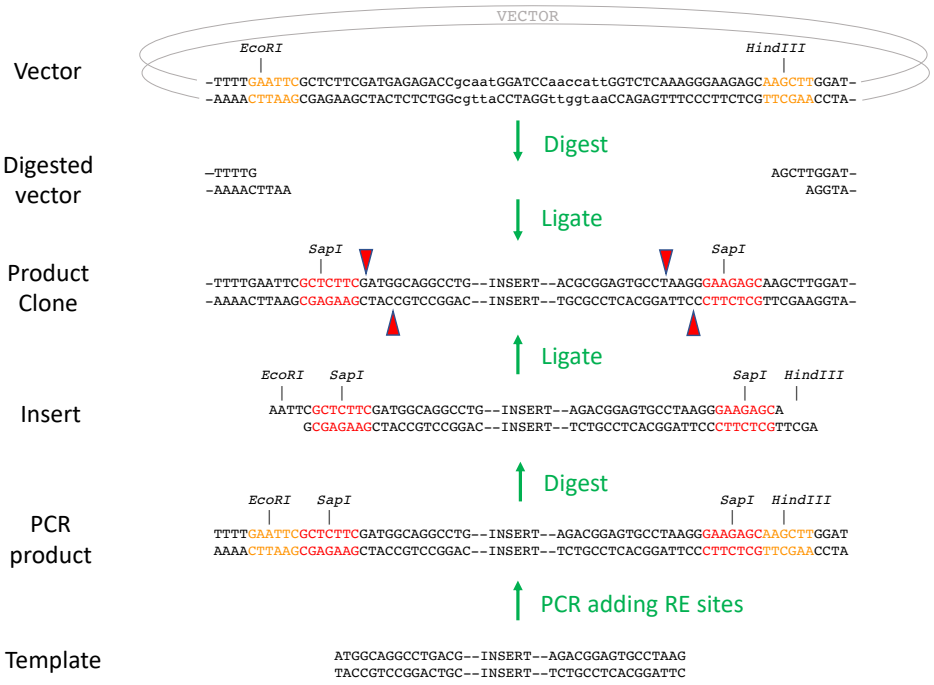

Figure 10 Double sticky-end cloning.

In this approach a DNA fragment is amplified with oligonucleotides which append both the *SapI* site and another restriction site on each end of the fragment. The PCR products are then digested, gel purified and ligated into gel purified digested vector. This approach can simplify the parallel cloning of many inserts with different SapI overhangs by using common other sites (*EcoRI* and *HindIII* in this example) to perform all the cloning steps. Red arrowheads represent cut sites for *SapI.*

My lab typically uses typically use the plasmid DR274 TbLCTb. It contains the following sites.

HindIII / *BspHI* / *XbaI* / *SphI* — [1.5 Kb insert w/ *HindIII* and *BsrGI* sites] — *EagI* / *BsrGI* / *EcoRI*

Thus, using any combination of 1 of the 4 sites on the left and 1 of the 3 sites on the right of the insertion site will allow you insert a clone. An example of double sticky-end cloning is shown in Figure 10.

Protocol 7: Double sticky-end cloning

P7.1 Oligonucleotide design

Design a pair of oligonucleotides that append the proper overlap (a *SapI* site and an appropriate cloning site) to the product you are wanting to insert. For example, CAGTTGAATTCGCTCTTCaATG for the forward oligonucleotide for cloning into the FP site, and GCAAGCTTGCTCTTCtCTT for the reverse oligonucleotide.

P7.2 PCR amplification of insert

Amplify the fragment using the general PCR method and purify using the general column purification method.

P7.3 Restriction digestions

Digest ~ 150 ng of both the PCR product and vector with appropriate enzymes.

Set up two restriction digestion reactions (one for vector and one for insert)

- 8-12 µl H20 (for a total of 14 µl before addition of enzyme)
- 1.5 µl of 10X restriction buffer
- 1-4 µl of DNA (~150 ng total)
- 0.75 µl Enzyme A
- 0.75 µl Enzyme B

Incubate 1-2 hr at 37°C.

P7.4 Gel purification of DNA fragments

Load the samples on a wide lane 0.8 % low melt agarose gel[25].
Run gel 20-30 minutes.
View the gel under UV (with low iron glass or UV transparent Plexiglas between the gel and UV source to protect the DNA from damage), and "punch out" each DNA with a 100 µl capillary pipette in ~10 µl.

P7.5 Ligation

Melt the agarose vector and insert punches at 65°C for 2 minutes.
mix in order

- 14 µl H20
- 2 µl 10X ligase buffer
- 2 µl melted vector
- 2 µl melted insert
- 1/4 µl T4 DNA ligase.

Incubate 0.5 hr. to o.n. at 15°C

P7.6 Transformation into *E. coli* and analysis

Melt the ligation at 65°C and transform using the general *E. coli* transformation method using 100 µl of competent cells and 1 µl of the melted ligation. Colony yield depends greatly on the amount of DNA obtained from the punch. Our general rule of thumb is that if you can see the DNA, it is enough to get the clone using this approach. Miniprep 2-4 colonies. Digest DNA with diagnostic enzymes to confirm clone identity. Sequence if appropriate.

E) Multi-fragment BsaI cloning method

This approach is used either to create more complicated inserts that contain multiple independent fragments and/or to eliminate *BsaI* or *SapI* sites from templates.

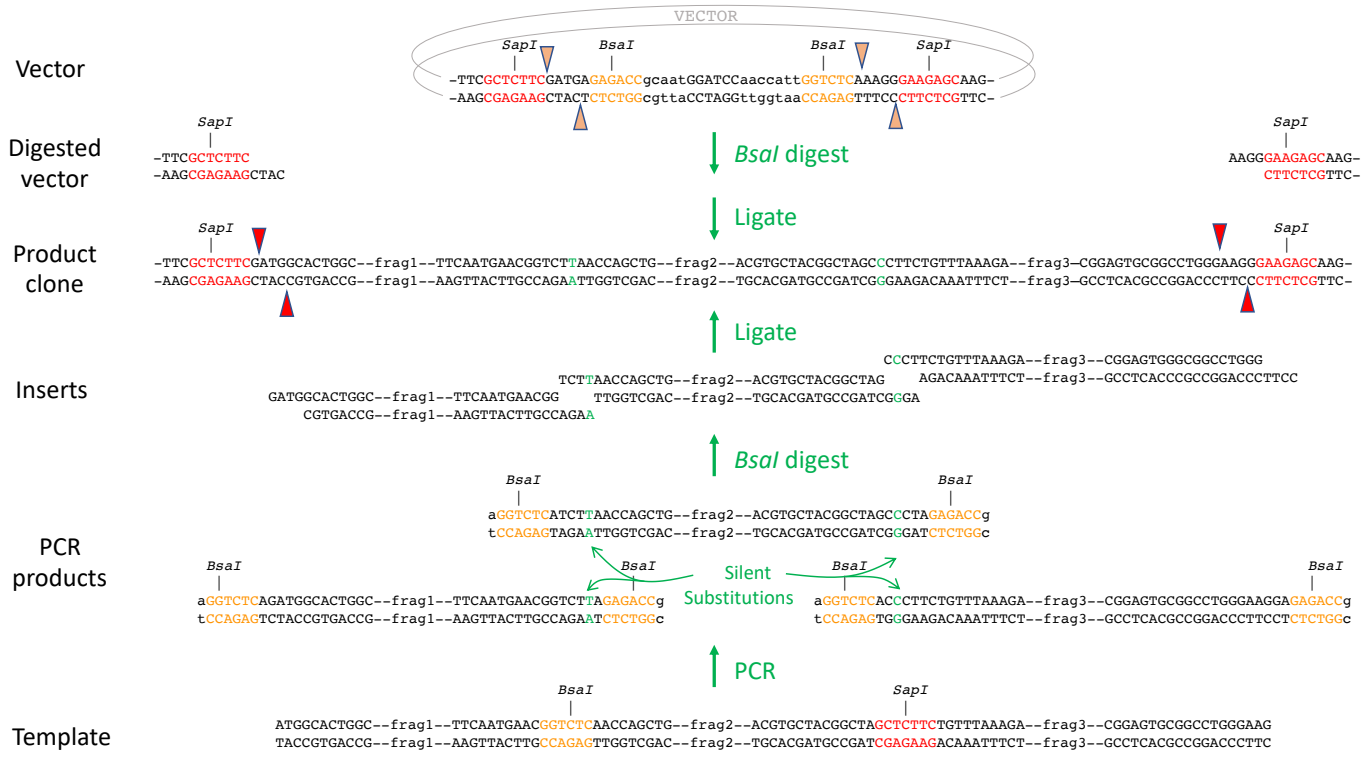

Figure 11 Multi-fragment BsaI Golden Gate cloning.

An example eliminating both a *BsaI* and a *SapI* site from a template by lesioning each site and co-assembling the insert from three PCR fragments. Oligonucleotides are designed that both lesion the *SapI* or *BsaI* site and appended a unique *BsaI* site on the 5' end of oligonucleotide. All products are amplified and purified, the co-assembled in a *BsaI* Golden Gate reaction. Red arrowheads represent the cut sites for *SapI* and orange arrowheads represent the cut sites for *BsaI.*

Protocol 8 Multi-fragment *BsaI* Golden Gate cloning

P8.1 Oligonucleotide design

Design a set of oligonucleotide primers to amplify fragments which each have a *BsaI* site on each end of PCR product and no *BsaI* sites or *SapI* site in the fragment. Each *BsaI* overlap must be designed to properly match the next fragment in the assembly, and the sites must be distinct in sequence so that only one assembly is possible. *BsaI* digest yields a 4 bp overhang. Potapov et al (2018) showed that not all 4 bp overhangs are equal. Of course, palindromes must be avoided since the can be used in either orientation. In addition, TAAA and TTTA are exceedingly inefficient at ligation and the general recommendation is to avoid TNNA overhangs. Furthermore, certain overhangs are susceptible to mismatches. In, particular G:T mismatches during ligation in the N_1 and N_4 position of the $N_1N_2N_3N_4$. Thus, it is best to avoid having two overhangs such as GCGG and TCGG that can base pair with a single N_1 or N_4 G:T mis-pairing especially if creating large assemblies.

If a fragment you want to insert contains a *BsaI* or *SapI* site you can eliminate it by turning the one fragment into two fragments with the site mutagenized (silent change if in coding), with *BsaI* sites on the end as shown in Figure 11.

P8.2 Amplification of inserts
Amplify each of the fragments from a plasmid or genomic DNA using the general PCR method. Try not to use Kan^R plasmids as templates, but if that is the only choice, digest the reaction with *DpnI* to cleave the template before purification of the PCR fragment. Purify the fragments using the general column purification method.

P8.3 Combining DNAs

Mix
50 pMol of the appropriate DR274-BsaI plasmid (50 ng vector)
60 pMol of each Insert PCR (25 ng of 1 kb insert)
Dilute to 10 µl with TE.

P8.4 *BsaI* Golden Gate reaction

1 µl of 10x SAP reaction buffer
7 µl H_20
1 µl DNA mix
1/2 µl BsaI
1/4 µl of T4 DNA ligase

Perform digestion/ligation in a PCR machine. 37°C , 10 min; 16° C, 5 min; 10 X (37°C, 5 min; 16°C, 5 min); 37°C, 10 min; 65°C, 20 min.[26]

P8.5 Transformation into *E. coli and Analysis*
Transform using the general *E. coli* transformation method with 1/2 µl of ligation mix and plate 1/10th of transformation. One should get thousands of colonies >90% of which should be correct (for a 3 insert construct).

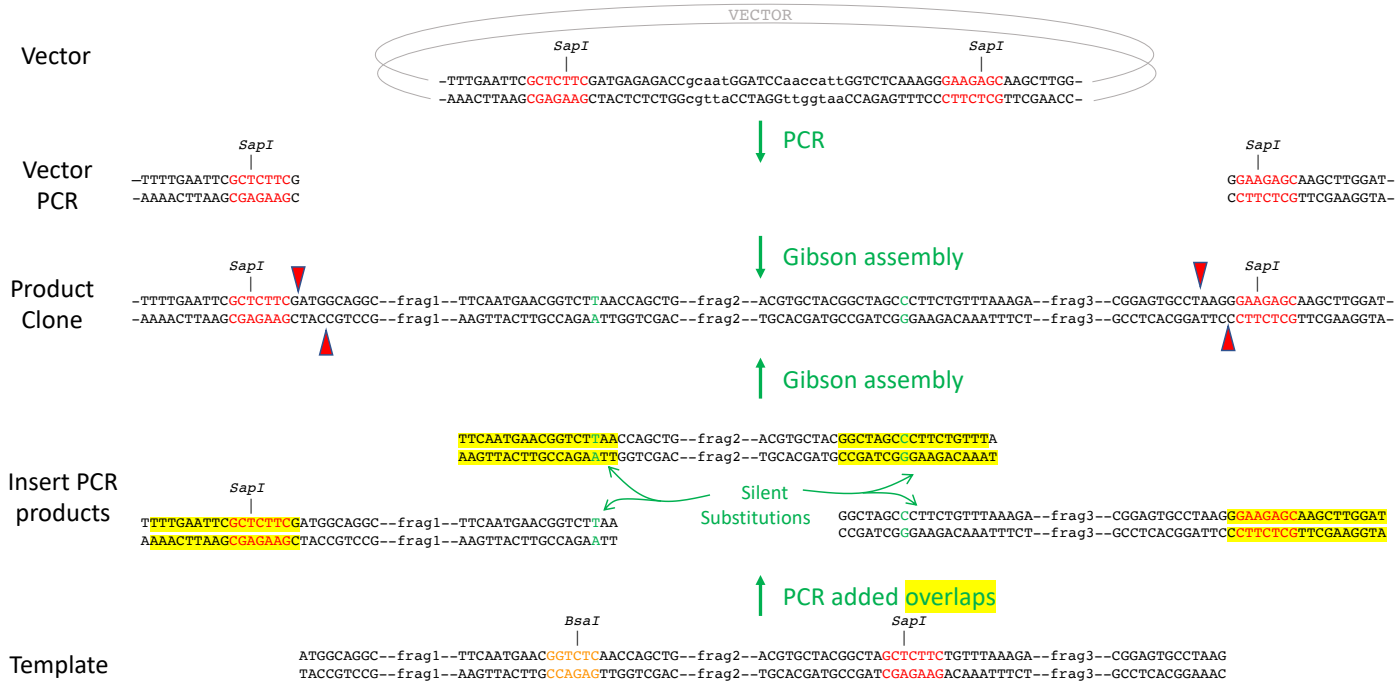

Figure 12 Multi-fragment Gibson assembly.
An example eliminating both a *BsaI* and a *SapI* site from a template by lesioning each site and co-assembling the insert from three PCR fragments. Oligonucleotides are designed which contain at least a 15bp overlap with the adjacent PCR fragment and lesion the *SapI* or *BsaI* site. The vector and all products are amplified and purified, the co-assembled in a Gibson Assembly reaction.

F) Multi-Fragment Gibson assembly

This is another method which can be used to create complicated entry clones that stitch together multiple inserts or to eliminate *SapI* or *BsaI* sites in a DNA template of interest.

Protocol 9 Multi-fragment Gibson assembly

P9.1 Oligonucleotide design

To create a clone by Gibson assembly I typically create the clone *in silico* by cutting a pasting the insert(s) into the vector of choice to create an *in silico* version which marks all the junctions. I then decide which PCR fragments should contain the overlap. A 55°C T_m overlap is our typical choice (~20 bp), but longer overlaps are more efficient for multi-insert Gibson reactions. The desktop program ApE and the NEBuilder website both have tools to facilitate the design of primers for Gibson assembly. An example is shown in Figure 12.

P9.2 PCR amplification of vector and inserts

Amplify the fragments using the general PCR method and purify using the general column purification method.

P9.3 Gibson Assembly reaction

Mix the following

25 fmol of vector PCR
50 fmol of each insert PCR[27]
TE to 5 µl
5 ul of 2X HiFi Assembly Master Mix

Incubate at 50°C for 1 hr.

P9.4 Transformation into *E. coli* and analysis

Transform using the general *E. coli* transformation method with 1/2 µl of ligation mix and plate 1/10th of transformation. The number of colonies obtained is highly dependent on the number of fragments and the length of the overlap. The majority will be correct insert clones. Analysis of 6 clones should be sufficient to get a clone and a backup (in case of PCR induced error).

VIII: Troubleshooting *BsaI* and *SapI* Golden Gate cloning

If you are having trouble getting the correct insert into a Golden Gate reaction, the most common problems arise from:

1) a restriction site (*BsaI* or *SapI*) is present in one of the inserts. Confirm that none of your fragments happens to have an unexpected site. One can accidentally include a site if one is modifying the sequence (for example lesioning a sgRNA target site), or one can simply forget to check if there is a site in the fragment you are inserting. Remember that if you are working with mouse or zebrafish DNA the "reference sequence" may not be exactly the same as the sequence of the genome you are PCR amplifying from.

2) an oligonucleotide design issue with the restriction site overlap sequences not being correct. I recommend building the clone you plan to make *in silico* before you order the oligonucleotides you have designed for the task. This usually catches both 1) and 2) errors.

3) Partial assemblies that lack one of the inserts are usually the result of presence of primer dimers in purified PCR inserts. If one of your PCR reactions efficiently forms a primer dimer from the forward and reverse oligonucleotides, this primer dimer will be used as template for assembly. Even a small amount barely visible on the gel will be a high molar concentration of this fragment due to its small mass. I recommend using diluted PBI to purify PCR fragments to minimize retention of primer dimers during purification of the PCR product.

4) Poor cleavage of the *SapI* or *BsaI* restriction sites at the end of the PCR fragment. Remember to add one (*BsaI*) to four (*SapI*) bases 5' of the restriction site in the oligonucleotides. Cloning the PCR fragment using the Gibson assembly method will also solve this problem.

5) Incomplete *SapI* or *BsaI* digestion. If the *BsaI* or *SapI* digestion does not go to completion the transformation will include a high background of parent vector. This can occur because of poor mixing of the reaction or if the enzyme has stopped performing optimally. In particular, NEB states that their *SapI* tends to 'settle' and recommend pipetting before removing enzyme from the stock.

6) A poorly designed overlap. This should not be an issue unless many fragments are being inserted. Potapov et al. (2018) demonstrated that not all 4 bp *BsaI* overlaps ligate precisely and that mis-match hybridization occurs and that the occurrence of mismatches is sequence dependent. For Example, G:T mis-matches in the N^1 N^2 N^3 N^4/$N^{4'}$ $N^{3'}$ $N^{2'}$ $N^{1'}$ occur at 3% while C:C mis matches occur at 0.04% with ligation performed at 25°C. Thus certain overlaps are more likely to form errors in assembly by mis-match hybridization. This mismatching is temperature dependent occurring at higher frequency if ligation occurs at a lower temperature. Performing the *BsaI* digestion/ligation experiment at 37°C can mitigate this problem. In addition, avoid single mismatches in the outer position N^1 and N^4 and positions, if possible. A set of compatible overlaps that have high fidelity are listed in Appendix III.

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Table 1. DR274 *BsaI* GG SapI entry cloning vectors

Plasmid #	Name	*SapI* slot (caps) & *BsaI* overlap
NMp3855	DR274 sgRNA - BsaI	gAAC-CAAc
NMp3854	DR274 U6p -BsaI	gCAA-TGGc
NMp3467	DR274 5’ arm - BsaI	gTGG-GCGa
NMp3468	DR274 CT - BsaI	gGCG-ATGa
NMp3469	DR274 FP - BsaI	gATG-AAGg
NMp3707	DR274 SEC- BsaI	gAAG-GGTc
NMp3456	DR274 NT - BsaI	gGGT-ACGc
NMp3470	DR274 3’ arm - BsaI	gACG-GTAg
NMp3967†	pET28- FP -BsaI	gATG-AAGg
NMp3844	DR274 sgRNA-U6p - BsaI	gAAC-TGGc
NMp3698	DR274 5’ arm-CT - BsaI	gTGG-ATGa
NMp3701	DR274 5' arm- SEC -BsaI	gTGG-GGTa
NMp3773	DR274 CT-NT - BsaI	gGCG-ACGc
NMp4617	DR274 FP-SEC -BsaI	gATG-GGTa
NMp3869	DR274 FP-NT - BsaI	gATG-ACGc
NMp3843	DR274 SEC-NT - BsaI	gAAG-ACGc
NMp3702	DR274 SEC-3’ arm - BsaI	gAAG-GTAg
† low copy number vector for toxic inserts		

Appendix I Reagents

TE buffer

10 mM Tris pH 8.0
0.1 mM EDTA

PE wash buffer

10 mM Tris-HCl pH 7.5
80% ethanol

PB buffer

5 M guanidine hydrochloride
30% isopropanol

PB is Qiagen's name for the binding buffer for column purification of DNA using QIAquick silica columns. PBI includes a pH indicator that turns from yellow to purple as the pH increases above 7.5. Diluted PBI is (PBI diluted 1:4 with 15 mM NaAc pH 5.2). The indicator is not required but comes with Qiagen QIAquick column kits. Diluting the PBI makes binding to the column more stringent and prevents small double strand DNA fragments (below approximately 50 bp) from binding to the column.

10X SapI buffer

96 µl 10X T4 DNA ligase buffer
4 µl 2.5 M KAc

10X Anneal buffer

500 mM KAc
50 mM Tris pH 7.5

SOC media

20g Bacto Tryptone,
5g Bacto Yeast Extract,
2 ml 5M NaCl,
2.5 ml of 1M KCl
fill to 1 liter

Sterilize and supplement with
10ml of 1M sterile $MgCl_2$,
10ml of 1M sterile $MgSO_4$
20ml of sterile 1M glucose

Aliquot and freeze to reduce odds of contamination.

Appendix II High Fidelity overlap sets from Potapov et al. (2018)

Set 1 (15 overhangs [a])
TGCC, GCAA, ACTA, TTAC, CAGA, TGTG, GAGC, AGGA, ATTC, CGAA, ATAG, AAGG, AACT, AAAA, ACCG

Set 2 (20 overhangs [a])
AGTG, CAGG, ACTC, AAAA, AGAC, CGAA, ATAG, AACC, TACA, TAGA, ATGC, GATA, CTCC, GTAA, CTGA, ACAA, AGGA, ATTA, ACCG, GCGA

Set 3 (25 overhang [a])
GCCC, CCAA, ATCC, GGTA, ACGG, AAAT, ATAG, CTTA, AGGA, AGTC, ACAC, ATGA, GCGA, CATA, CTGC, AACG, CGCC, AGTG, CCTC, GAAA, CAGA, ACCA, AAGT, CGAA, CAAC

[a] Only one sequence for each overhang pair is shown.

Footnotes

[1] Version history
1.5 5/22/2021 Changed text to suggest 4 bp before a *SapI* site. And also modified the figures to show the same. Three bases is usually OK, 2 bases pairs is sometimes problematic for efficient digestion. 4 bp is a safer bet for a manual.
1.5.1 11/12/2021 Added comment about using PB wash for miniprep DNA.
1.5.2. 2/10/2022 Minor formatting changes to remove large blank spaces.
2.0 5/19/2023 Added rRMCE to the introduction.
2.1.0 6/12/2023 Added Gibson Assembly
2.1.1 6/15/2023 Updated figures

[2] I use the term vector for the parent plasmid used to create an integration plasmid.

[3] This step is not technically needed as the inserts could all be added to the reaction below. However, we find it easier to mix all the DNAs then perform a dilution of the mix rather than individually diluting the vector and each insert.

[4] I typically just use 100 ng of the vector, and 50 ng of each insert plasmid. The more complicated the assembly the more important is keeping ~ 1:1 ratios.

[5] 10X *SapI* reaction buffer: 96 ul of 10X ligase buffer, 4 µl of 2.5 M KAc.

[6] According to NEB *SapI* will 'settle' in the tube, so one should consider mixing the *SapI* by gentle pipetting before pull out an aliquot. *LguI* from Invitrogen (Anza version) also works under these conditions, and I favor this enzyme over *SapI*.

[7] Polynucleotide kinase will phosphorylate the 5' OH of oligonucleotides. Without adding PNK the oligonucleotide linkages will only have one of the two phosphoester bonds formed. These nicked circles will transform, but likely reduce the efficiency of the reaction. If two adjoining inserts are oligonucleotides PNK is essential. For multi-insert assemblies I recommend using PNK even if a single oligonucleotide pair is used.

[8] Conditions for effective ligation vary:
For 10 inserts, the Voytas TALEN protocol (Dalhem et al., 2012) reaction recommends:
10X (37°C for 5 min, 16°C 10 min), 55°C for 5 min, 80°C for 5 min
NEB recommends:
1 to 4 inserts 37°C for 1hr, followed by 55°C for 5 min.
5 to 10 inserts 30X (37°C for 1 min, 16°C 1 min), 55°C for 5 min.
11 to 20 inserts 30X (37°C for 5 min, 16°C 5 min), 55°C for 5 min.

[9] Competent cells can be purchased or made in house much more economically using this protocol.

[10] 100 ug/ml Ampicilin or 50 mg/ml Kanamycin.

[11] If you are going to inject the clone into *C. elegans*, consider including a PB wash in your miniprep procedure as this likely improves transformation frequencies (Huang et al., 2021). Although the Huang micropublication uses PureLink HQ column, Dickinson has since posted that he gets the same improvement using Qiagen columns. https://community.alliancegenome.org/t/miniprep-kits-for-plasmid-dnas-for-microinjection/6299/5

[12] This is the major limitation of the Golden Gate approach.

[13] NEB provides a table in their catalog that show how effectively most enzymes cut sites at positions 1-6 bp from the end of the fragment.

[14] Genomic DNA or first strand cDNA are ideal templates. Plasmids can be used (use < 1ng of template), but if they are Amp^R they can cause contamination issues in the subsequent *SapI* assembly reaction. If you do use an Amp^R plasmid, include a 15 minute incubation with 1/2 µl of *DpnI* (cleaves methylated plasmid DNA, but not unmethylated PCR DNA) to destroy the template.

[15] Oligonucleotides are resuspended in water.

[16] I use Q5 polymerase because it is very efficient and has a very low error rate. I use about 1/3rd the Q5 recommended by NEB. 1/3 ul per 100 ul of master mix cocktail for multiple reactions. Order their 5X Q5 buffer pack if you run out of their "proprietary buffer". A 25 µl reaction is usually sufficient to obtain 1 µg of PCR product.

[17] The critical factor is to keep the reaction on ice and then heat as rapidly as possible to 98°C to prevent primer dimer formation. We call this approach of starting the reaction on ice a 'pseudo' hot start. At low temperatures, primers can anneal to each other, and then form double stranded short DNAs. These compete with the real PCR amplification reaction. By reducing the time when polymerase can be active before the first 98°C denature step, this issue is mitigated. In a true hot start reaction, polymerase is added after heating to 98°C. But this is annoying to perform. Another option is using Q5 hot start polymerase, which contains an aptamer-based inhibitor that block polymerase activity at low temperature.

[18] NEB has a T_m calculator that can be used to determine the T_m of an oligonucleotide. Remember to only include the portion of the oligonucleotide that matches the template, not the additional sequences that adds the *SapI* when using this calculator.

[19] PBI diluted 1:4 with 15 mM NaAc pH 5.2. Using 1:4 diluted PBI will prevent dsDNA under ~50 bp from binding to the column. Using 1:7 PBI will prevent dsDNA under ~ 80 bp from binding. This binding data was obtained with Qiagen columns.

[20] I purify the DNA on micro-elution columns. I use either those from Qiagen or NEB. Note these are different from the standard PCR purification columns that require 30 or 40 µl of elution volume.

[21] PCR products can be stored at 4°C. However, the volume is so small that evaporation/condensation cycles can damage the DNA reasonably rapidly (~ a month). Freezing works better for long term storage. I store all my plasmid DNAs at 4°C in TE and they are stable (and remain functional in Golden Gate reactions) for years.

[22] One can get the *BsaI* strategy to work relatively efficiently (50% correct clones) even if there is a *BsaI* site in the PCR product as long as 1) the ends do not match either site of the vector, and 2) one adds a ligation step after the Golden Gate reaction (including the 65° C inactivation). Simply add 10 ul of 1X ligase buffer and 1/4 ul of ligase and incubate at 15°C for 30 minutes. The Golden Gate reaction will have yielded lots of linear DNAs that are the clone one is seeking but cut at the internal *BsaI* site. Adding a ligation step after heat inactivation of the *BsaI* allows one to recover these as circular products.

[23] The last 37° C step is to allow the *BsaI* enzyme to do a last round of cutting of incomplete assemblies so they are not circles. The 65°C step inactivates the ligase.

[24] NEB recommends using a 1:2 vector to insert molar ratio for 1-3 inserts and a 1:1 vector to insert molar ratio for 4 or more inserts. Very small inserts are recommended to be used in a 5-fold molar excess. This is because the method relies on a 5' endonuclease to create the single stranded regions that hybridize. Very small fragments are quickly chewed back until the two strands no longer overlap and thus reduces the efficiency of the reaction.

[25] More detail about this method is available on my website at https://sites.wustl.edu/nonetlab/protocols-2/

[26] The last 37° C step is to allow the *BsaI* enzyme to do a last round of cutting of incomplete assemblies so they are not circles. The 65°C step inactivates the ligase.

[27] NEB recommends using a 1:2 vector to insert molar ratio for 1-3 inserts and a 1:1 vector to insert molar ratio for 4 or more inserts. Very small inserts are recommended to be used in a 5-fold molar excess. This is because the method relies on a 5' endonuclease to create the single stranded regions that hybridize. Very small fragments are quickly chewed back until the two strands no longer overlap and thus reduces the efficiency of the reaction.