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Corresponding Author:	Zhen Wang University at Buffalo - The State University of New York Amherst, NY UNITED STATES
Corresponding Author's Institution:	University at Buffalo - The State University of New York
Corresponding Author E-Mail:	zhenw@buffalo.edu
Order of Authors:	Zhen Wang
	Minakshi Mukherjee
	Emily Caroll
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TITLE:

Rapid Assembly of Multi-Gene Constructs Using Modular Golden Gate Cloning

AUTHORS AND AFFILIATIONS:

Minakshi Mukherjee[1]*, Emily Caroll[1]*, Zhen Q. Wang[1]

[1]Department of Biological Sciences, University at Buffalo, State University of New York, Buffalo, NY, USA

*These authors contributed equally

Corresponding author:
Zhen Wang (zhenw@buffalo.edu)

Email addresses of co-authors:
Minakshi Mukherjee (mmukherj@buffalo.edu)
Emily Carroll (carroll3@buffalo.edu)

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SUMMARY:

The goal of this protocol is to provide a detailed, step-by-step guide for assembling multi-gene constructs using the modular cloning system based on Golden Gate cloning. It also gives recommendations on critical steps to ensure optimal assembly based on our experiences.

ABSTRACT:

The Golden Gate cloning method enables the rapid assembly of multiple genes in any user-defined arrangement. It utilizes type IIS restriction enzymes that cut outside of their recognition sites and create a short overhang. This modular cloning (MoClo) system uses a hierarchical workflow in which different DNA parts, such as promoters, coding sequences (CDS), and terminators, are first cloned into an entry vector. Multiple entry vectors then assemble into transcription units. Several transcription units then connect into a multi-gene plasmid. The Golden Gate cloning strategy is of tremendous advantage because it allows scar-less, directional, and modular assembly in a one-pot reaction. The hierarchical workflow typically enables the facile cloning of a large variety of multi-gene constructs with no need for sequencing beyond entry vectors. The use of fluorescent protein dropouts enables easy visual screening. This work provides a detailed, step-by-step protocol for assembling multi-gene plasmids using the yeast modular cloning (MoClo) kit. We show optimal and suboptimal results of multi-gene plasmid assembly and provide a guide for screening for colonies. This cloning strategy is highly applicable for yeast metabolic engineering and other situations in which multi-gene plasmid cloning is required.

INTRODUCTION:

Synthetic biology aims to engineer biological systems with new functionalities useful for pharmaceutical, agricultural, and chemical industries. Assembling large numbers of DNA fragments in a high-throughput manner is a foundational technology in synthetic biology. Such a complicated process can break down into multiple levels with decreasing complexity, a concept borrowed from basic engineering sciences[1,2]. In synthetic biology, DNA fragments usually assemble hierarchically based on functionality: (i) Part level: "parts" refers to DNA fragments with a specific function, such as a promoter, a coding sequence, a terminator, an origin of replication; (ii) Transcription units (TU) level: a TU consists of a promoter, a coding sequence, and a terminator capable of transcribing a single gene; (iii) Multi-gene level: a multi-gene plasmid contains multiple TUs frequently comprised of an entire metabolic pathway. This hierarchical assembly pioneered by the BioBrick community is the foundational concept for the assembly of large sets of DNAs in synthetic biology[3].

In the past decade[4-7], the Golden Gate cloning technique has significantly facilitated hierarchical DNA assembly[2]. Many other multi-part cloning methods, such as Gibson cloning[8], ligation-independent cloning (SLIC)[9], uracil excision-based cloning (USER)[10], the ligase cycling reaction (LCR)[11], and *in vivo* recombination (DNA Assembler)[12,13], have also been developed so far. But Golden Gate cloning is an ideal DNA assembly method because it is independent of gene-specific sequences, allowing scar-less, directional, and modular assembly in a one-pot reaction. Golden Gate cloning takes advantage of type IIS restriction enzymes that recognize a non-palindromic sequence to create staggered overhangs outside of the recognition site[2]. A ligase then joins the annealed DNA fragments to obtain a multi-part assembly. Applying this cloning strategy to the modular cloning (MoClo) system has enabled the assemble of up to 10 DNA fragments with over 90% transformants screened containing the correctly assembled construct[4].

The MoClo system offers tremendous advantages that have accelerated the design-build-test cycle of synthetic biology. Firstly, the interchangeable parts enable combinatorial cloning to test a large space of parameters rapidly. For example, optimizing a metabolic pathway usually requires cycling through many promoters for each gene to balance the pathway flux. The MoClo system can easily handle such demanding cloning tasks. Secondly, one needs to sequence the part plasmid but typically not the TU or the multi-gene plasmids. In most cases, screening by colony PCR or restriction digestion is sufficient for verification at the TU and multi-gene plasmid level. This is because cloning the part plasmid is the only step requiring PCR, which frequently introduces mutations. Thirdly, the MoClo system is ideal for building multi-gene complex metabolic pathways. Lastly, because of the universal overhangs, the part plasmids can be reused and shared with the entire bioengineering community. Currently, MoClo kits are available for plants[14,15,5,16,17], fungi[6,18-22], bacteria[7,23-27], and animals[28,29]. A multi-kingdom MoClo platform has also been introduced recently[30].

For *Saccharomyces cerevisiae*, Lee et al.[6] have developed a versatile MoClo toolkit, an excellent resource for the yeast synthetic biology community. This kit comes in a convenient 96-well format and defines eight types of interchangeable DNA parts with a diverse collection of well-characterized promoters, fluorescent proteins, terminators, peptide tags, selection markers,

origin of replication, and genome editing tools. This toolkit allows the assembly of up to five transcription units into a multi-gene plasmid. These features are valuable for yeast metabolic engineering, in which partial or entire pathways are over-expressed to produce targeted chemicals. Using this kit, researchers have optimized the production of geraniol, linalool[31], penicillin[32], muconic acid[33], indigo[34], and betalain[35] in yeast.

Here we provide a detailed, step-by-step protocol to guide the use of the MoClo toolkit to generate multi-gene pathways for either episomal or genomic expression. Through extensive use of this kit, we have found that the accurate measurement of DNA concentrations is key to ensuring the equimolar distribution of each part in the Golden Gate reaction. We also recommend the T4 DNA ligase over the T7 DNA ligase because the former works better with larger numbers of overhangs[36]. Lastly, any internal recognition sites of BsmBI and BsaI must be removed or domesticated prior to assembly. Alternatively, one may consider synthesizing parts to remove multiple internal sites and to achieve simultaneous codon optimization. We demonstrate how to use this toolkit by expressing a five-gene pathway for β-carotene and lycopene production in *S. cerevisiae.* We further show how to knock out the *ADE2* locus using the genome-editing tools from this kit. These color-based experiments were selected for easy visualization. We also demonstrate how to generate fusion proteins and to create amino acid mutations using Golden Gate cloning.

PROTOCOL:

NOTE: The hierarchical cloning protocol offered in this toolkit can be divided into three major steps: 1. Cloning part plasmids; 2. Cloning transcription units (TUs); 3. Cloning multi-gene plasmids (**Figure 1**). This protocol starts from the primer design and ends with applications of the cloned multi-gene plasmid.

1. Primer design for cloning the part plasmid (pYTK001):

1.1. Design the forward and reverse primers containing flanking nucleotides TTT at the 5' end, a BsmBI recognition site with an additional nucleotide (CGTCTCN), a 4-nucleotides (nt) overhang (TCGG) complementary to that of the entry vector, a BsaI recognition site with an additional nucleotide (GGTCTCN), and a 4-nt part-specific overhang, in addition to the template-specific sequence (**Figure 2A**). GoldenBraid 4.0[37] and GoldenMutagenesis[38] are some of the online software that can be used for Golden Gate specific primer design.

1.2. If BsaI or BsmBI recognition sites are present in any part, use a domestication step to mutate these sites prior to Golden Gate assembly[39]. For integrative plasmids (see step 4), domesticate any NotI recognition site. To domesticate a part with one undesirable recognition site, divide the part into two subparts near the undesirable site (**Figure 2A**):

1.2.1. Design the forward primer of the first subpart in the same manner as in step 1.1. but design the reverse primer with the BsmBI site and a 4-nt gene-specific overhang only.

1.2.2. Design the second sub-part forward primer with the BsmBI site and the 4-nt gene-specific overhang only that overlaps with the reverse primer of the first sub-part. Design the reverse primer of the second sub-part in the same manner as in step 1.1.

1.2.3. Introduce the desired mutation(s) in either the reverse (for step 1.2.1) or forward primer (step 1.2.2) at the gene-specific region of the primer.

NOTE: Alternatively, a BsmBI- and BsaI-free and codon-optimized part can be synthesized commercially. For coding sequences (CDS), a synonymous mutation can be readily incorporated at the third nucleotide of an amino acid codon. For promoters and terminators, however, checking the mutated promoter or terminator activity using a reporter assay is recommended[39]. If there is an undesirable restriction site toward the end of the sequence, it can be mutated using a longer reverse primer. If multiple undesirable sites are present, site-directed mutagenesis allowing mutating multiple sites may be performed[40].

1.3. Occasionally, fusing two proteins as a single part with a linker in between is desirable (**Figure 2A**). The linker helps to ensure the structural integrity of the two individual proteins[41].

1.3.1. The forward primer for the first gene is the same as in step 1.1. In the reverse primer, include a BsmBI site, a 4-nt gene-specific overhang, and a linker sequence. The 4-nt overhang can be either the linker or second gene's first few nucleotides.

1.3.2. For the second gene, design the forward primer such that it has a BsmBI recognition site and a 4-nt overhang complementary to that of the reverse primer of the first gene. Design the reverse primer of the second gene as in step 1.1.

1.4. To amplify the parts by polymerase chain reaction (PCR)[42], use a high-fidelity DNA polymerase to amplify the parts from either a genomic DNA, a cDNA, or a plasmid. Check the PCR product on a 1% agarose gel followed by gel-purification. Using purified DNA is strongly recommended, if gel purification is laborious, use at least a spin column to purify the PCR product.

2. Cloning parts into the entry vector (pYTK001) to create part plasmids (Figure 2B)

2.1. To set up the Golden Gate reaction mix, add 20 fmol of each PCR product and the entry vector (pYTK001), 1 μL of 10X T4 ligase buffer, 0.5 μL of Esp3I (a highly efficient isoschizomer of BsmBI), and 0.5 μL of T4 ligase. Add ddH_2O to bring the total volume to 10 μL.

2.2. To set up the cloning reaction, run the following program in a thermocycler: 25-35 cycles of 37 °C for 5 min (digestion) and 16 °C for 5 min (ligation), followed by a final digestion at 50 °C for 10 min and enzyme inactivation at 80 °C for 10 min.

NOTE: 35 cycles of digestion/ligation are recommended when cloning multiple DNA pieces into the entry vector simultaneously, for example, during the cloning of fusion genes or domesticating a gene.

2.3. Transform the entire reaction mix into the DH5α strain or equivalent *Escherichia coli* chemically competent cells by heat shock. Transforming the entire 10 μL cloned product into 35 μL chemically competent *E. coli* cells (2 X 10^5 cfu/mL, cfu is calculated from transforming 5 ng pYTK001 into 100 μL of the competent cells) is recommended. Spread on a lysogeny Broth (LB) plate with 35 μg/mL chloramphenicol (Cm). Incubate at 37 °C overnight.

2.4. After 16-18 h, take the plate out from the incubator and keep the plate at 4 °C for about 5 h to let the super folder green fluorescent protein (sfGFP) develop for a more intense green color.

2.5. For easier screening, place the plate on an ultraviolet (UV) or a blue light transilluminator. The sfGFP containing colonies will fluoresce under the UV light.

2.6. The green colonies are negative because they contain the uncut pYTK001. The white colonies are likely positive. The cloning is usually successful if there are ~30-100% white colonies. Perform further screening of a few white colonies by either colony PCR or restriction digestions (suggested enzyme: BsaI-HFv2).

2.7. Purify plasmids from a few of the potentially correct colonies and confirm the sequences by Sanger sequencing.

3. Assembling part plasmids into "cassette" plasmids:

NOTE: A cassette plasmid contains a user-defined transcription unit (TU) consisting of a promoter, a CDS, and a terminator. A cassette plasmid allows the expression of a single gene. If the cassette plasmids will be assembled into a multi-gene plasmid, then the first step is to determine the number and the order of TUs in the multi-gene plasmid. These will determine which connectors to use in the cassette plasmids since connectors link TUs in the multi-gene plasmid. The first TU's left connector should be ConLS, and the right connector of the last TU should be ConRE. They will overlap with ConLS' and ConRE' of multi-gene plasmids. The rest of the connectors should be in the increasing numerical order. For example, if the multi-gene plasmid contains four TUs, the connector combinations would be ConLS-TU1-ConR1, ConL1-TU2-ConR2, ConL2-TU3-ConR3, and ConL3-TU4-ConRE (**Figure 1**).

3.1. Before assembling transcription units, assembling an intermediate vector with the following six parts is recommended: the left connector, the sfGFP dropout (pYTK047), the right connector, a yeast selection marker, a yeast origin of replication and the part plasmid with an mRFP1, an *E. coli* origin and the ampicillin-resistant gene (pYTK083) (**Figure 3**).

3.1.1. Purify the above six plasmids. Record their concentrations using a UV-Vis spectrophotometer or a fluorescence-based assay and dilute each plasmid with ddH_2O so that 1 μL has 20 fmol of DNA. Calculate the DNA molar concentration by using an online calculator.

NOTE: It is very important to measure the DNA concentrations accurately and to pipet precisely for the assembly to work, especially for assemblies with five to seven part plasmids. Small errors in the DNA concentration of each plasmid can cause a significant decrease in cloning efficiency.

3.1.2. Add 1 μL of each plasmid, 1 μL of 10X T4 ligase buffer, 0.5 μL of BsaI-HFv2 (a highly efficient version of BsaI), and 0.5 μL of T4 ligase. Make up the volume to 10 μL by adding ddH_2O.

3.1.3. To set up the cloning reaction, run the following program in the thermocycler: 25-35 cycles of 37 °C for 5 min (digestion) and 16 °C for 5 min (ligation). Omit the final digestion and heat inactivation steps as the BsaI sites need to be retained in the intermediate vector (**Figure 3**).

3.1.4. Transform the entire reaction mix into the DH5 α strain or other *E. coli* competent cells. Spread on an LB plate with 50 μg/mL carbenicillin (Cb) or ampicillin. Incubate at 37 °C overnight.

NOTE: Carbenicillin is a stable analog of ampicillin.

3.1.5. After 16-18 h, take the plate out of the incubator. The plate will contain both pale red and pale green colonies (**Figures 6C and 6D**). Keep the plate at 4 °C for about 5 h to let the mRFP1 and sfGFP mature. Use a UV or a blue light transilluminator to identify the green colonies, which contain the potentially correct intermediate vector.

3.1.6. Streak out the green colonies on an LB + Cb plate and incubate at 37 °C overnight. The next day, streak out again on an LB + Cm plate and incubate at 37 °C overnight. The colonies growing on LB + Cm plates contain misassembled plasmids because Cm resistant part vectors are retained.

3.1.7. Pick the colonies that do not grow on the LB + Cm plate and perform restriction digestions (suggested enzymes: BsaI-HFv2, Esp3I) to confirm the correctly assembled plasmid. Alternatively, use colony PCR for screening.

3.2. Once the intermediate vector has been successfully assembled, the next step is to assemble transcription units. This a 4-piece assembly with the following parts: the intermediate vector, a promoter, a CDS, and a terminator.

3.2.1. Purify the four part plasmids. Record their concentrations and dilute each of plasmid so that 1 μL has 20 fmol of DNA.

3.2.2. To set up the reaction mix, follow Step 3.1.2.

3.2.3. To set up the cloning reaction, follow Step 2.2.

3.2.4. Transform the entire cloning reaction mix into the DH5α or equivalent *E. coli* competent cells and plate on LB + Cb. Incubate at 37 °C overnight.

3.2.5. After 16-18 h, take the plate out from the incubator. White and pale green colonies will appear (**Figures 6E and 6F**). Keep the plate at 4 °C for about 5 h to let the sfGFP mature. Use a UV or a blue light transilluminator to identify the non-fluorescent white colonies. These contain the potentially correct transcription units.

3.2.6. Streak out and grow 8-10 white colonies and perform a colony PCR. Purify plasmids from the colonies that test positive from colony PCR. Carry out restriction digestion (suggested enzyme: Esp3I) to further confirm the assembly.

Note: Sequencing transcription units is typically not necessary because the cloning involves only restriction digestion and ligation. All sequences of interest have been confirmed at the part plasmid level.

4. Assembling cassette plasmids into "multi-gene" plasmids:

NOTE: Multi-gene plasmids allow the expression of more than one gene. Depending on the downstream application, multi-gene plasmids could be replicative or integrative. Replicative plasmids have the yeast origin of replication; therefore, it can be stably maintained when yeast cell divides. Integrative plasmids do not have the yeast origin of replication. Instead, they have 5' and 3' homology arms allowing the integration of multiple genes into specific loci of the genome through homologous recombination.

4.1. For multi-gene plasmids, assemble an intermediate vector first.

4.1.1. To assemble replicative intermediate vectors (**Figure 4A**), assemble the following six parts: the left connector (ConLS'-pYTK008), the sfGFP dropout (pYTK047), the right connector (ConRE'-pYTK072), a yeast selection marker, a yeast origin of replication, and the part plasmid with mRFP1, an *E. coli* origin of replication, and the kanamycin-resistant gene (pYTK084).

4.1.1.1. For assembly, follow the steps from 3.1.1 to 3.1.3.

4.1.1.2. Transform the entire cloning reaction mix into DH5α or equivalent *E. coli* competent cells, and plate on LB plus 50 μg/mL kanamycin (Km). Incubate at 37 °C overnight.

4.1.1.3. For red/green color-based screening, follow step 3.1.5.

4.1.1.4. For screening of misassemblies, streak and grow the green colonies on an LB + Km plate. Then follow step 3.1.6.

4.1.2. For integrative multi-gene vectors (**Figure 4B**), determine the genomic locus of interest first, then design approximately 500 base pairs of 5' and 3' homology arms for integrating to that

locus.

4.1.2.1. Clone the 5' and 3' homology arms from yeast genomic DNA into the entry vector-pYTK001. Follow steps 1 and 2.

4.1.2.2. Assemble the following seven plasmids: the left connector (ConLS'-pYTK008), the sfGFP dropout (pYTK047), the right connector (ConRE'-pYTK072), a yeast selection marker, the 3' homology arm, the part plasmid with mRFP1*, E. coli* origin of replication, and the kanamycin-resistant gene (pYTK090), and the 5' homology arm.

4.1.2.3. For assembly and screening, follow step 4.1.1.

4.2. Assembly of the multi-gene plasmid

4.2.1. Purify plasmids of the intermediate vector obtained in step 4.1 and the cassette plasmids from step 3. Record their concentrations using a UV-Vis spectrophotometer or a fluorescence-based assay. Dilute each in ddH_2O so that 1 μL has 20 fmol DNA.

4.2.2. Add 1 μL of intermediate vector, 1 μL of each transcription unit, 1 μL of 10x T4 ligase buffer, 0.5 μL of Esp3I, and 0.5 μL of T4 ligase. Bring the volume to 10 μL of using ddH_2O.

4.2.3. To set up the cloning reaction, follow step 2.2.

4.2.4. Transform the entire cloning reaction mix into DH5α or equivalent *E. coli* competent cells, and plate on LB + Km. Incubate at 37 °C overnight.

4.2.5. Perform the green/white screening as in step 3.2.5.

4.2.6. Purify plasmids from a few white colonies and perform restriction digestions. Using the NotI-HF enzyme is recommended because there are two NotI sites at the *E. coli* origin and Km selection marker part, respectively (**Figure 4B**). If the assembled plasmid is very large, then another restriction site can be chosen for further confirmation. Alternatively, screen with colony PCR before proceeding to restriction digestion.

5. Applying multi-gene plasmids for chromosomal or plasmid-based gene expression

5.1. Integrating multi-gene plasmid into the yeast genome for chromosomal gene expression (**Figure 5**)

5.1.1. Design a guide RNA (gRNA) for the desired locus. Using multiple online resources, such as Benchling, CRISPRdirect[43], and CHOPCHOP[44], are recommended to determine the maximum on-target specificity.

5.1.2. Clone the synthesized 20-nt gRNA into the pCAS plasmid[45] by Gibson cloning. Linearize

the pCAS plasmid by PCR using a reversed primer pair binding to the 3' of the HDV ribozyme and the 5' of the tracrRNA respectively (**Figure 5**). Alternatively, clone the gRNA into the sgRNA dropout plasmid (pYTK050) and assemble the dropout plasmid into a cassette plasmid with linkers. Then assemble the Cas9 TU with the Cas9 part plasmid (pYTK036). Lastly, assemble the Cas9 TU and the sgRNA TU into a replicative multi-gene plasmid.

5.1.3. Linearize 5-15 μg of integrative multi-gene plasmid with 1 μL of NotI-HF enzyme overnight. Transform 1 μg of pCAS-gRNA and the linearized integrative multi-gene plasmid into *S. cerevisiae*. Prepare competent cells using either a commercially available yeast transformation kit or following the protocol by Geitz and Schiestl, 2007[46].

NOTE: It is unnecessary to purify the linearized multi-gene plasmid after the NotI digestion.

5.1.4. Pellet the cells after recovery, discard the supernatant, wash with an equal volume of water. Plate yeast cells on the complete synthetic medium (CSM) dropout plate or the yeast extract peptone dextrose medium (YPD) plate with antibiotics, depending on the yeast selective marker. Incubate at 37 °C for two days for colonies to form. In case no colony is observed, incubate for an additional one to two days at 30°C.

5.1.5. Screen yeast colonies for integration by colony PCR[47].

5.1.6. To cure the pCAS, streak out the colony with the correct integration onto a non-selective YPD plate. Grow at 30 °C overnight. Streak one colony from the YPD plate onto a fresh YPD plate. Again, grow at 30 °C overnight. Streak a colony from the second YPD plate to a YPD plus 100 μg/mL nourseothricin, the selection marker of pCAS. Successful curing occurs when yeast cells fail to grow on the selective plate.

NOTE: If the pCAS plasmid is not cured in two rounds of non-selective YPD, streak again onto a fresh YPD for another 1-2 rounds.

5.2. Transforming replicative multi-gene plasmid for plasmid-based gene expression

5.2.1. Transform 100 ng-1 μg pure multi-gene plasmid into *S. cerevisiae* competent cells.

5.2.2. Plate yeast cells immediately after transformation onto the CSM dropout plate or YPD plus antibiotic plate, depending on the yeast selection marker used. Incubate at 30°C for 2-3 days for colonies to form.

REPRESENTATIVE RESULTS:

Here the results of four replicative multi-gene plasmids for β-carotene (yellow) and lycopene (red) production. One integrative multi-gene plasmid for disrupting the *ADE2* locus was constructed, the colonies of which are red.

Cloning CDSs into the entry vector (pYTK001)

ERG20 was amplified from the yeast genome and the three carotenoid genes *crtE*, *crtYB*, *crtI* from the plasmid pLM494[48] into the entry vector pYTK001 as described. Yeast promoters *pENO2, pTIP1, pPYK1* and *pPDC1* and terminators *tTDH2, tHSP26, tADH2, tACS2* were cloned as part plasmids. A point mutation was introduced into CrtYB (G247A) for producing lycopene and a *BTS1-ERG20* fusion construct was created to better channel the prenyl intermediate to carotenoids. **Figures 6A and 6B** show a representative plate of the successful cloning of a part plasmid and provide an example of the green/white screening with 90.35 ± 4.22% total colonies being potentially correct (white).

Assembly of transcription units (cassette plasmids)

Before assembling the cassette plasmid, the design of the multi-gene plasmid was finalized. For the four carotenoid TUs, four intermediate vectors with different connectors were cloned first following step 3.1. **Figures 6C and 6D** show a representative plate from a successful assembly of the intermediate vector and provide an example of the red/green screening, with 17.56 ± 3.32% total colonies being positive (green). Although this ratio is relatively low, the screening is greatly facilitated by the green fluorescence.

Next, the TUs for *BTS1-ERG20, ERG20*, *crtE, crtYB, crtYB(G247A)*, and *crtI* were assembled following step 3.2 (**Table 1**). **Figures 6E and 6F** show a representative plate of a successful assembly of the TUs and provide an example of a green/white screening method, with 65.02 ± 4.99% total colonies being positive (white).

Assembly of multi-gene plasmids

Four replicative and one integrative multi-gene plasmids were assembled. For replicative multi-gene plasmids, *ERG20*, *BTS1-ERG20*, *crtE*, *crtYB*, *crtYB (G247A)* and *crtI* were assembled in four different combinations, creating four plasmids: two for β-carotene and two for lycopene production (**Table 2**). The ratio of potentially correct colonies (green) for the intermediate vector cloning was 1.83 ± 0.15% (**Figures 7A and 7B**). Although this number seems low, the screening was made easy by detecting the green fluorescence (**Figure 7B**). Once the intermediate plasmid was cloned, the success rate of assembling multi-gene plasmids (white) from the intermediate was 93.77± 1.65% (**Figures 7C and 7D**). **Figures 7E and 7F** show a suboptimal assembly of multi-gene plasmids, as the numbers of positive colonies (white) were negligible. After transforming into yeast, colonies producing β-carotene (yellow) and lycopene (red) grew on day three. Four colonies from each plate were streaked out onto fresh plates and grown for two more days. **Figures 8A and 8B** show that fusing the *BTS1-ERG20* directs more geranylgeranyl-pyrophosphate toward the carotenoid production, as seen by darker colors than using the *ERG20* alone. Upon extraction[49] and quantification of the carotenoids by UV-Vis spectrophotometry with authentic standards, it is seen that fusion of *BTS1-ERG20* leads to the production of 0.729 μg/mg β-carotene, which is ~35 fold higher than 0.021 μg/mg β-carotene produced by the strain with *ERG20* alone. Likewise, the production of lycopene is ~16.5 fold higher in the strain with *BTS1-ERG*20 (1.126 μg/mg) compared to *ERG20* (0.068 μg/mg) alone (**Figure 8C**).

Replicative multi-gene plasmids transformed into yeast with (**A**) *BTS1-ERG20* fusion TU and (**B**) *ERG20* TU. On each plate, yeast on the left side has plasmids containing crtE TU, crtYB TU and crtI

TU for the production of β-carotene and yeast on the right side has plasmids containing crtE TU, crtYB (G247A)TU, and crtI TU for the production of lycopene.

For the integrative plasmid, the ConLS', sfGFP dropout, ConRE', *HIS*3 (yeast selection marker), *ADE2* 5' and 3' homology arms were assembled following step 4.1.2. The 5' and 3' homology arms were 500 bp apart, deleting ~180 amino acids after integration. Additionally, the 5' homology arm had six stop codons towards its 3' end. The mutated *ADE2* resulted in red colonies[50]. The *ADE2* gRNA 5'-ATTGGGAC GTATGATTGTTGAGG-3'[51] were used and followed step 5.1 for genomic integration. After 3-4 days, red colonies were observed on the YPD plate + 100 μg/mL nourseothricin, indicating that *ADE2* had been successfully disrupted (**Figures 8D and 8E**).

FIGURE AND TABLE LEGENDS

Figure 1: Overview of multi-gene assembly. Assembly takes place in three levels. In level 1, the CDS, or any other part, is amplified from the genome or synthesized, and then cloned into the pYTK001 entry vector using the BsmBI (or Esp3I) enzyme. ColE1*: E. coli* origin of replication; Cm^R: Chloramphenicol resistant gene. In level 2, the transcription unit (TU) containing a promoter, a CDS, and a terminator is assembled using the BsaI enzyme. In level 3, up to five transcription units are assembled into a multi-gene plasmid using the BsmBI (or Esp3I) enzyme. The multi-gene plasmid can be either replicative or integrative.

Figure 2: Primer design and cloning of part plasmids. (**A**) Primer design for amplifying individual parts, domesticating genes or creating point mutations, and assembling fusion proteins. Primers include BsmBI and BsaI recognition and cut sites and the MoClo overhang for proper assembly. MoClo overhangs are represented as 1, 2, 3, 4, and 5, 6, 7, 8. Internal primers for domestication or creating fusion protein contains the BsmBI but not the BsaI sites. The overhangs for these are customized internal sequences (NNNN and N'N'N'N' in purple). Terminal "ttt" are included for optimal enzyme digestion. GOI: gene of interest. (**B**) Cloning amplified parts into the pYTK001 entry vector using BsmBI (or Esp3I). Complementary overhangs lead to the integration of the part and the removal of the BsmBI recognition site. ColE1*: E. coli* origin of replication; Cm^R: Chloramphenicol resistant gene.

Figure 3: Transcription unit assembly. To assemble the TU plasmids, the assembly of an intermediate vector is recommended to facilitate combinatorial TU assembly. To assemble the intermediate vector, clone the Con LX (X: one of the five left connectors), the sfGFP dropout, the Con RY (Y: one of the five right connectors), a yeast ORI (origin of replication), and a yeast marker part into the mRFP1 dropout vector using the BsaI enzyme. The intermediate plasmid is resistant to ampicillin. The BsaI recognition sites are retained for TU plasmid cloning. To clone the TU plasmid, a promoter, a CDS, and a terminator are assembled into the intermediate vector using BsaI. The cloned TU will have BsmBI sites at the ConLX and ConRY regions for the next step multi-gene assembly. The cloned TU is also resistant to ampicillin.

Table 1: Transcription units used in this study. Promoters and terminators were amplified from *S. cerevisiae*. *BTS1* (geranylgeranyl diphosphate synthase) and *ERG20* (farnesyl pyrophosphate

synthetase) were amplified from *S. cerevisiae*. The genes *crtE* (geranylgeranyl diphosphate synthase), *crtYB* (bifunctional lycopene cyclase/phytoene synthase), and *crtI* (phytoene desaturase) were from *Xanthophyllomyces dendrorhous*.

(Please place table 1 next to figure 3)

Figure 4: Multi-gene plasmid assembly. (**A**) Replicative plasmid assembly. Assembly of the replicative intermediate vector includes cloning the Con LS', the sfGFP dropout, the Con RE', a yeast ORI, a yeast marker, and an *E. coli* origin and marker on the mRFP1 dropout vector using the BsaI enzyme. The ConLS' and ConRE' sites introduce BsmBI recognition sites to the vector. Potentially correct assemblies can be screened by looking for green colonies on a selective plate with kanamycin. The previously assembled TUs can then be cloned into the intermediate vector using the BsmBI enzyme. This plasmid contains a yeast ORI allowing it to replicate in a yeast host. (**B**) Integrative plasmid assembly. Assembly of the integrative intermediate vector includes cloning the Con LS', the sfGFP dropout, the Con RE', a 5' homology arm, a 3' homology arm, a yeast marker, and the *E. coli* origin and marker into the RFP dropout vector using the BsaI enzyme. Correct assemblies should appear green on a selective plate with kanamycin. Transcription units previously made can be cloned into the replicative intermediate vector using the BsmBI enzyme. This vector does not have a yeast ORI and will be integrated into the target locus through CRISPR and homologous recombination.

Table 2: Multi-gene Plasmids used in this study.

(Please place table 2 next to figure 4)

Figure 5: CRISPR integration. (**A**) CRISPR and helper plasmid cloning. The pCAS plasmid contains the Cas9 endonuclease and components (tRNA promoter, SNR52 terminator, HDV ribozyme, and tracrRNA) for optimal expression of a gRNA. Clone the pCAS+gRNA plasmid by assembling the synthetic gRNA with the linearized pCAS using Gibson cloning. (**B**) CRISPR aided genetic integration. Step 1: Cotransformation: pCAS +gRNA was co-transformed into yeast with the integrative plasmid containing the gene(s) of interest (GOI), a yeast selective marker, and 5' and 3' homology region targeting the genomic locus. For optimal integration, linearize the integrative plasmid with NotI. Step 2: Integration at target locus: Growing the transformed yeast on a plate selective for the yeast marker, either antibiotic or auxotrophic. Perform genotyping to confirm the integration. Step 3: Cure the pCAS + gRNA plasmid by streaking yeast on a non-selective plate.

Figure 6: Representative plates of entry vector and transcription unit level cloning in *E. coli.* Representative plates of the successful cloning of a gene into the entry vector pYTK001 under (**A**) visible light and (**B**) UV light. Positive colonies are white and negative colonies are green. The ratio of positive colonies overall colonies is 90.35 ± 4.22 %. Successful assembly of the intermediate vector for transcription unit level assembly and green/red selection under (**C**) visible light and (**D**) UV light. Positive colonies are green. The ratio of positive colonies overall colonies is 17.56 ± 3.32 %. Successful assembly of transcription unit from the intermediate vector and green/white screening under (**E**) visible light and (**F**) UV light. Positive colonies are white and

negative colonies are green. The ratio of positive colonies overall colonies is: 65.02 ± 3.32 %. Data are from three biological replicates.

Figure 7: Representative plates of multi-gene plasmid cloning in *E. coli*. Assembly of the intermediate vector for multi-gene level assembly and red/green selection under (**A**) visible light and (**B**) UV light. Positive colonies are green and negative colonies are red. The ratio of positive colonies overall colonies is 1.83 ± 0.15%. Successful assembly of multiple TUs from the intermediate vector and green/white selection (**C**) under visible light and (**D**) UV light. Positive colonies are white. The ratio of positive colonies overall colonies is 93.77 ± 1.65%. Suboptimal assembly of multi-gene plasmid under (**E**) visible light and (**F**) UV light. The number of positive colonies is negligible. Data are from three biological replicates.

Figure 8: Representative plates of integrative and replicative plasmids in yeast. Replicative multi-gene plasmids transformed into yeast with (**A**) *BTS1-ERG20* fusion TU and (**B**) *ERG20* TU. On each plate, yeast on the left side has plasmids containing crtE TU, crtYB TU and crtI TU for the production of β-carotene and yeast on the right side has plasmids containing crtE TU, crtYB (G247A)TU, and crtI TU for the production of lycopene. (**C**) quantifying β-carotene and lycopene from yeast extract with four multi-gene plasmids using a UV-vis spectrometer. The maximal absorbance was recorded at 450 nm and 470 nm for β-carotene and lycopene respectively. The absolute quantification was performed using authentic standards (**Supplementary Figure S3**). Fusion of BTS1-ERG20 leads to the production of ~35-fold higher β-carotene and ~16.5-fold higher lycopene compared to ERG20 alone. Representative plates for the disruption of the *ADE2* locus by the integration of a multi-gene integrative plasmid with a gRNA and no helper DNA (**D**) and with gRNA and a multi-gene integrative plasmid as helper DNA (**E**).

DISCUSSION

The MoClo based cloning kit developed by Lee et al. provides an excellent resource for quick assembly of one to five transcription units into a multi-gene plasmid either for replication or integration into the yeast genome. The use of this kit eliminates the time-consuming cloning bottleneck that frequently exists for expressing multiple genes in yeast.

We tested five different conditions for the digestion/ligation cycles of Golden Gate cloning with T4 DNA ligase. We found that 30 cycles of digestion at 37 °C for 5 min and ligation at 16 °C for 5 min followed by a final digestion step at 50 °C for 10 min and a protein inactivation step at 80 °C for 10 min resulted in 99.5% colonies that were potentially correct in a 4-piece assembly (**Supplementary Figure S1 and Table S3**). Ligation at 16 °C ensures optimal ligase activity and overhang annealing. Final digestion at 50 °C prevents digested products from re-ligation. This cycle was followed for all of the assembly reactions unless otherwise specified.

We have also found some critical steps that need special attention for optimal results. We strongly recommend assembling intermediate vectors with the sfGFP dropout before assembling transcription units. In theory, all seven parts can be cloned into the *E. coli* vector with the mRFP1, followed by red/white screening. However, in practice, mRFP1 develops color very slowly and it is challenging to distinguish between pale red and pale-yellow *E. coli* colonies under visible light.

As sfGFP absorbs at the UV range[52], using a UV or a blue light transilluminator facilitates the screening for bright green colonies. Also, cloning an intermediate vector allows more facile assemble of the various promoter, CDS, and terminator combinations, enabling an easier combinatorial library creation, since an assembly with four parts usually results in more positive colonies than an assembly with more parts. **Supplementary Figure S2** shows a progressive decrease in the ratio of potentially correct colonies from a 6-piece to an 8-piece assembly.

The concentrations of all of the parts must be measured meticulously to ensure the equimolar concentration of each part. Quantifying parts using a UV-Vis spectrometer is usually sufficient but more sensitive methods, such as fluorescence-based assays, may give better results. Failure to accurately quantify all of the parts often results in a low assembly efficiency. One should select the highly efficient Esp3I and BsaI-HFv2, respectively. We have observed that T4 works well for both overhangs in the kit and customized overhangs are necessary to fuse two parts, which is consistent with literature[36], although the original paper recommended T7 ligase[6]. Increasing the number of cycles can increase the number of potentially correct colonies to some extent. For example, 25 cycles of digestion and ligation are enough to assemble three to four parts but 30-35 cycles give better results for more parts.

The MolClo strategy is advantageous over alternative multi-part assembly methods[8-12] because it allows modular and highly versatile cloning. However, the primary limitation is the domestication step since parts sometimes have multiple BsmBI, BsaI, or NotI sites. Mutating all of the parts can be time-consuming. In this case, one may consider synthesizing the CDS without these restriction sites. However, for promoters and terminators, mutating even a single nucleotide may change their functionality. Therefore, validating the activity of these parts using a reporter assay is recommended[39]. Another limitation is that this kit only allows up to five transcription units in a multi-gene plasmid. If more transcription units are desired, additional connectors may be constructed by selecting a set of highly compatible overhangs[36].

The kit provides 27 promoters, six terminators, seven yeast selection markers, and two yeast origin of replications. Customized parts, such as promoters and terminators, either native or synthetic, can be cloned into the entry vector following this protocol. The yeast MoClo kit has been used primarily for overexpressing multi-gene metabolic pathways to produce high-value chemicals in yeast. This protocol can also be used when different switches for biological circuits are desired in yeast. There is also tremendous potential to apply this kit for the investigation of basic biological questions about protein-protein interactions, protein localization, and enzyme activities. Overall, this protocol is extremely flexible and reliable and can support any demanding cloning in yeast biology.

ACKNOWLEDGMENTS

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DISCLOSURES:

The authors have nothing to disclose.

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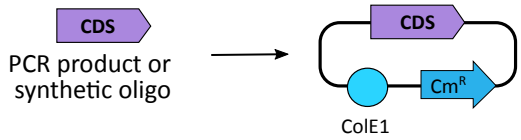
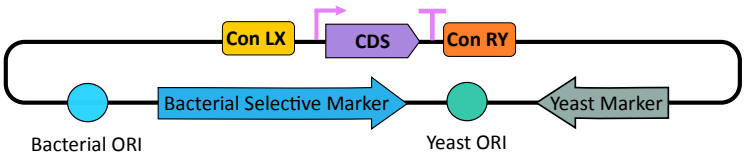
Level 1: Cloning DNA parts into entry vector using BsmBI (Esp3I) enzyme.



Level 2: Assembly of transcription units (cassette plasmids) using BsaI enzyme.



Level 3: Assembly of multi-gene plasmids using BsmBI (Esp3I) enzyme.

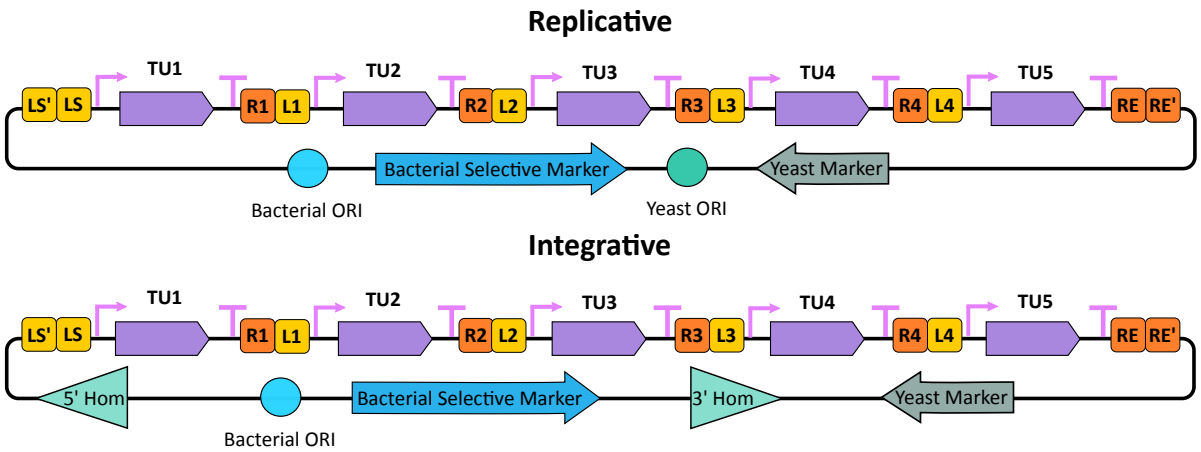


Figure 2

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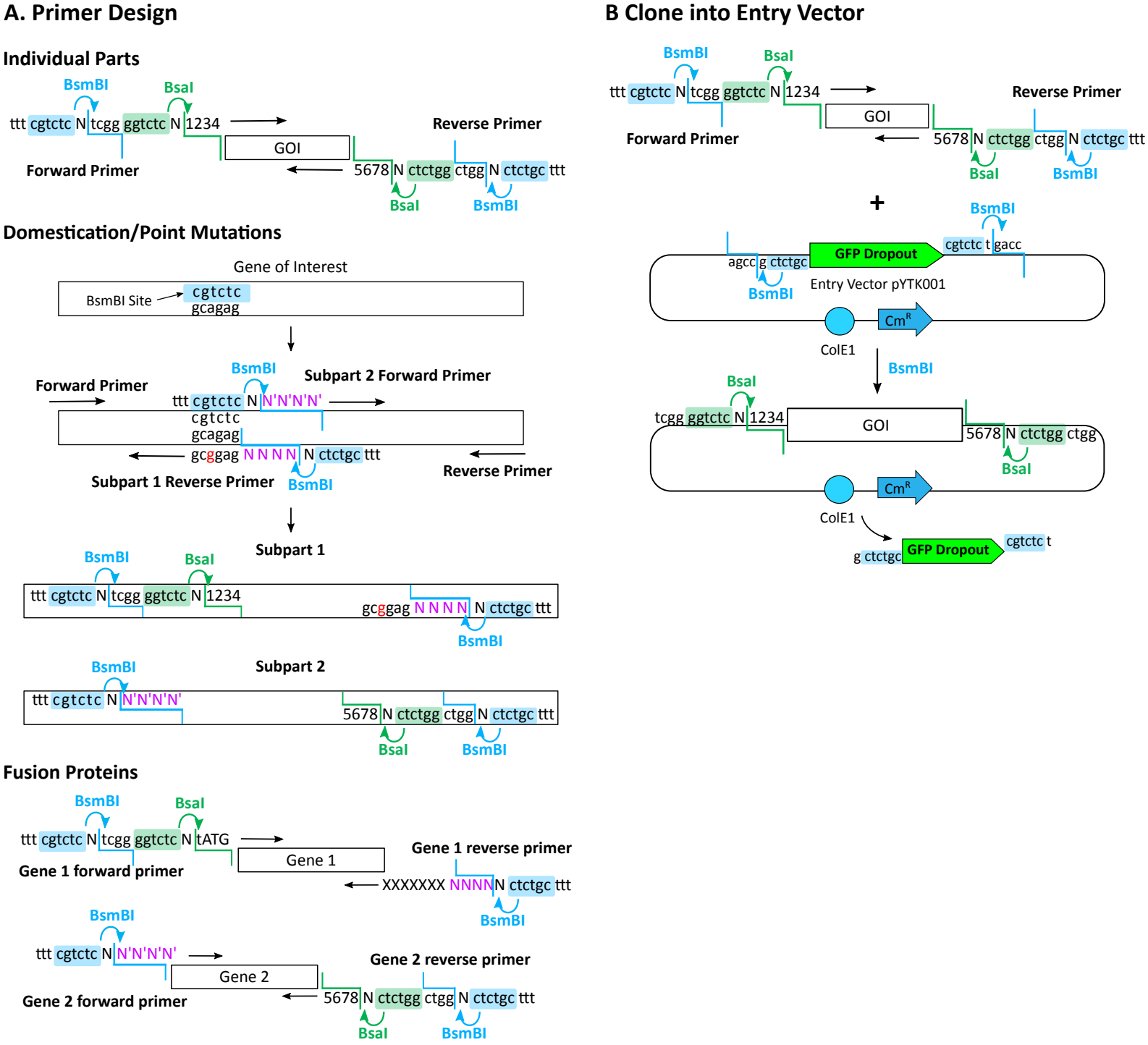

Figure 3

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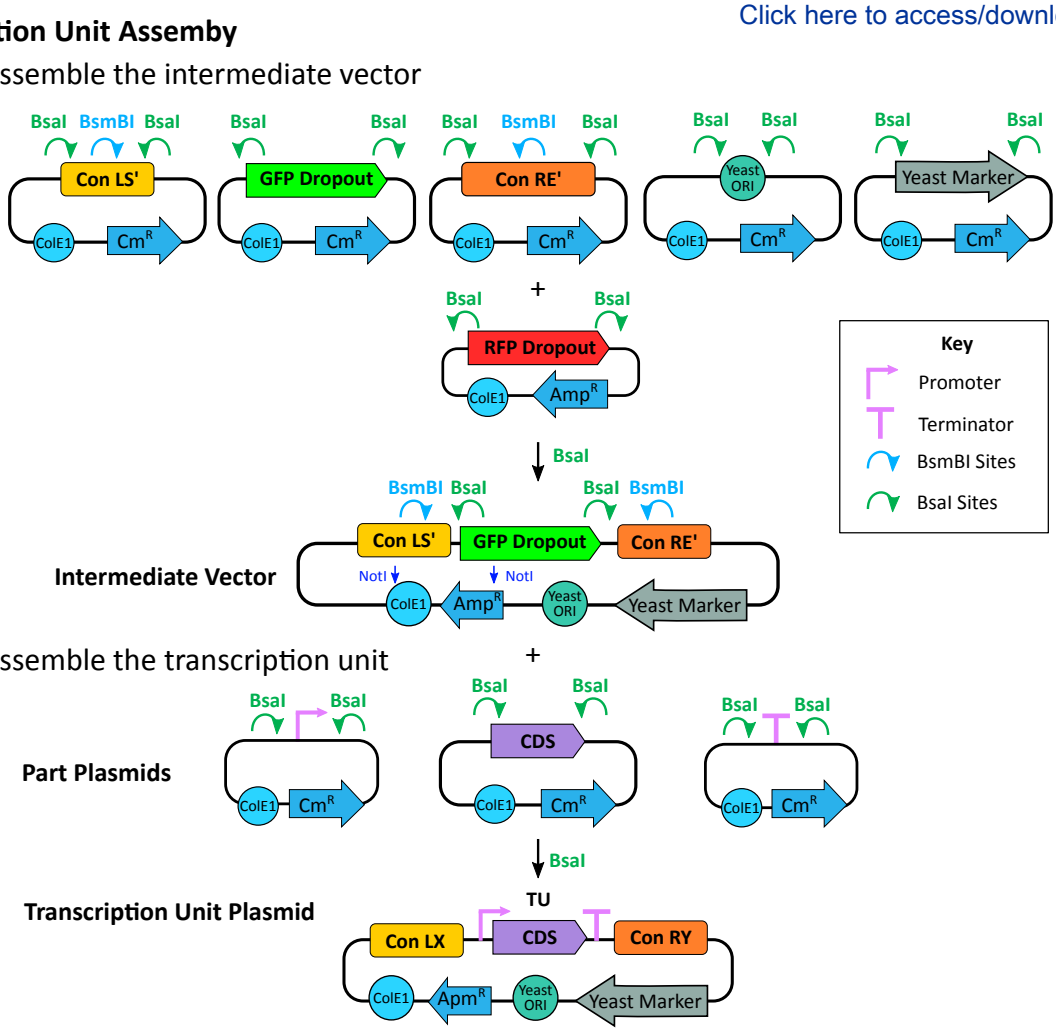

Figure 4

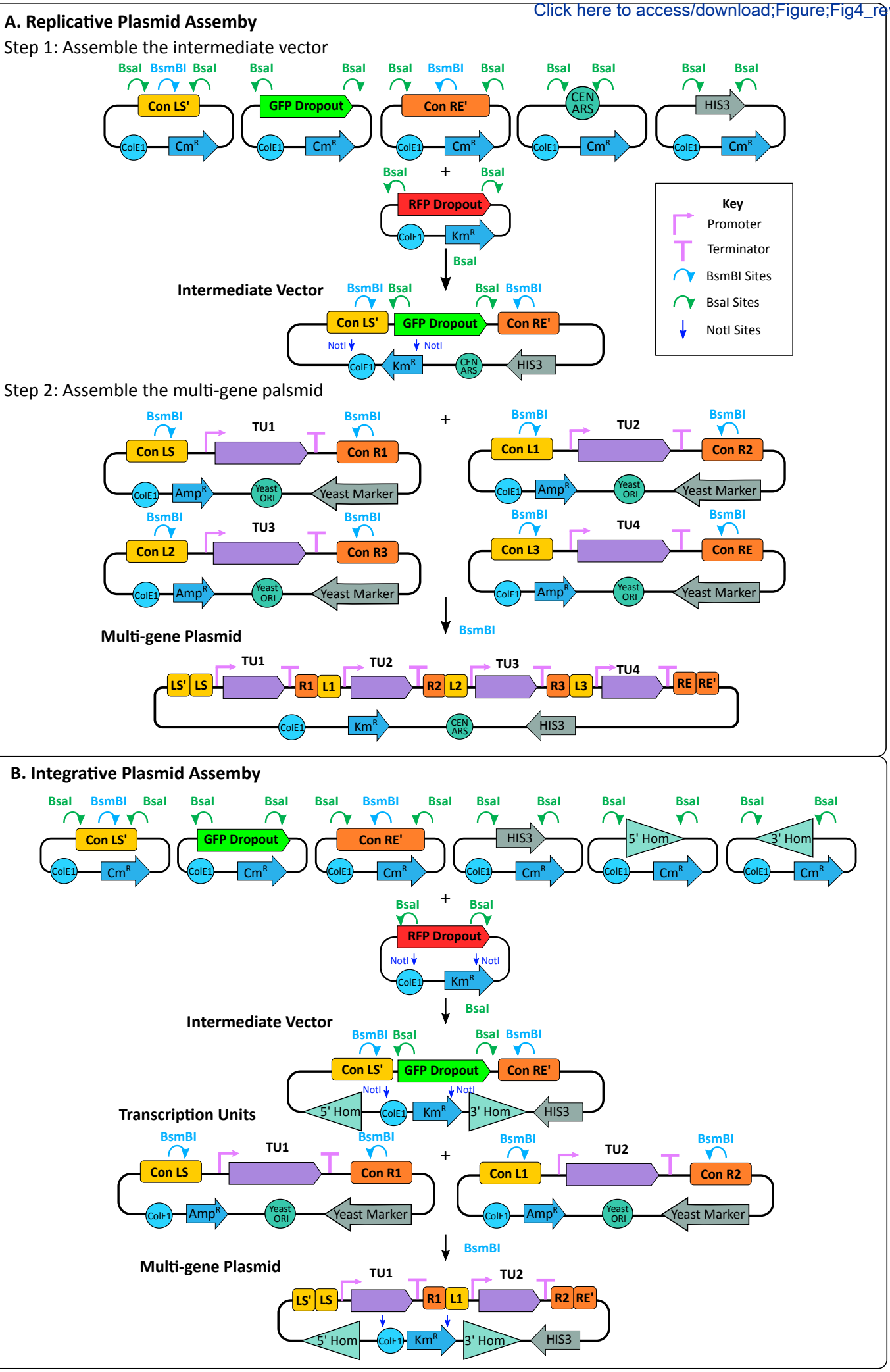

Figure 5

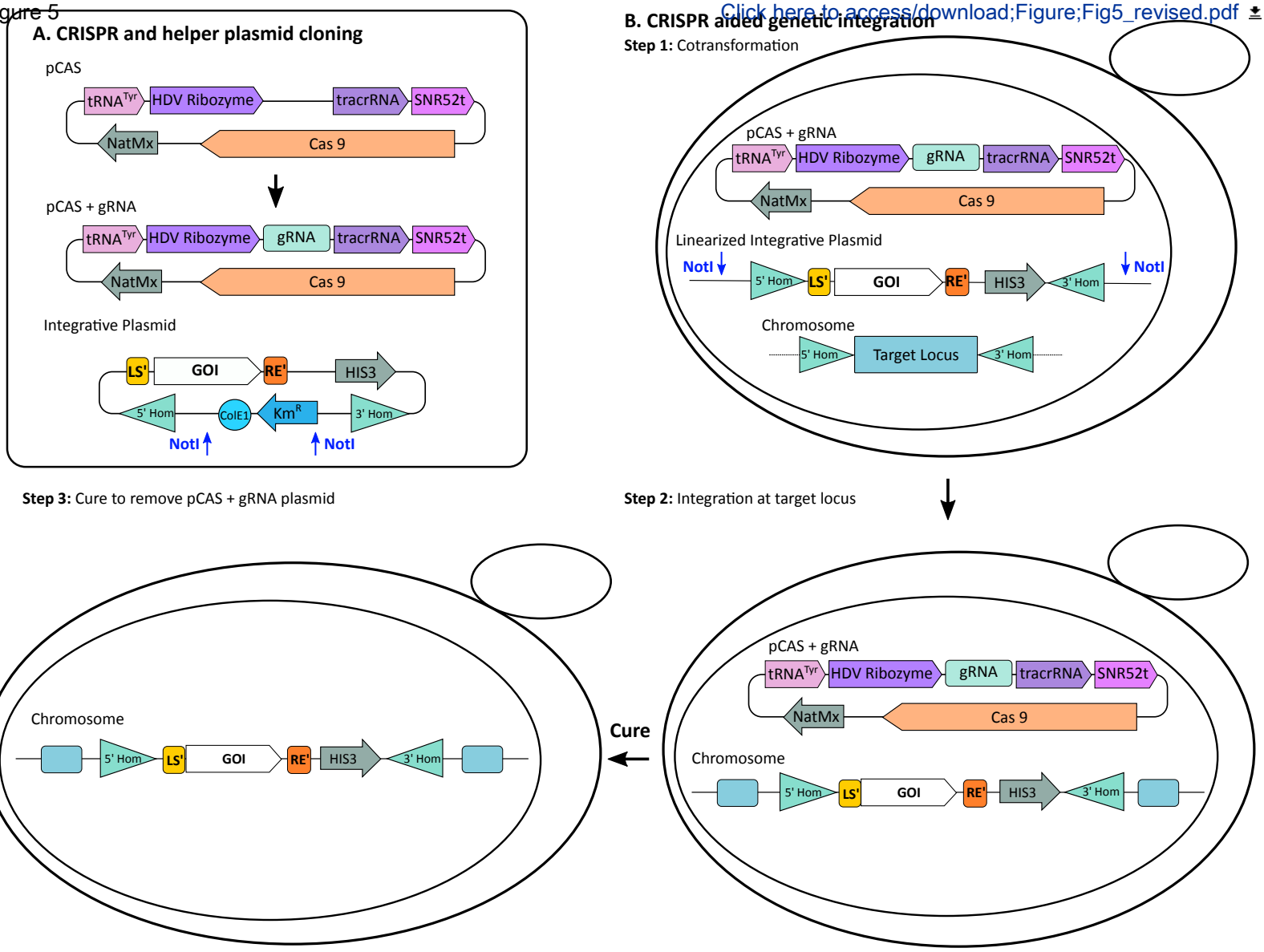

Figure 6

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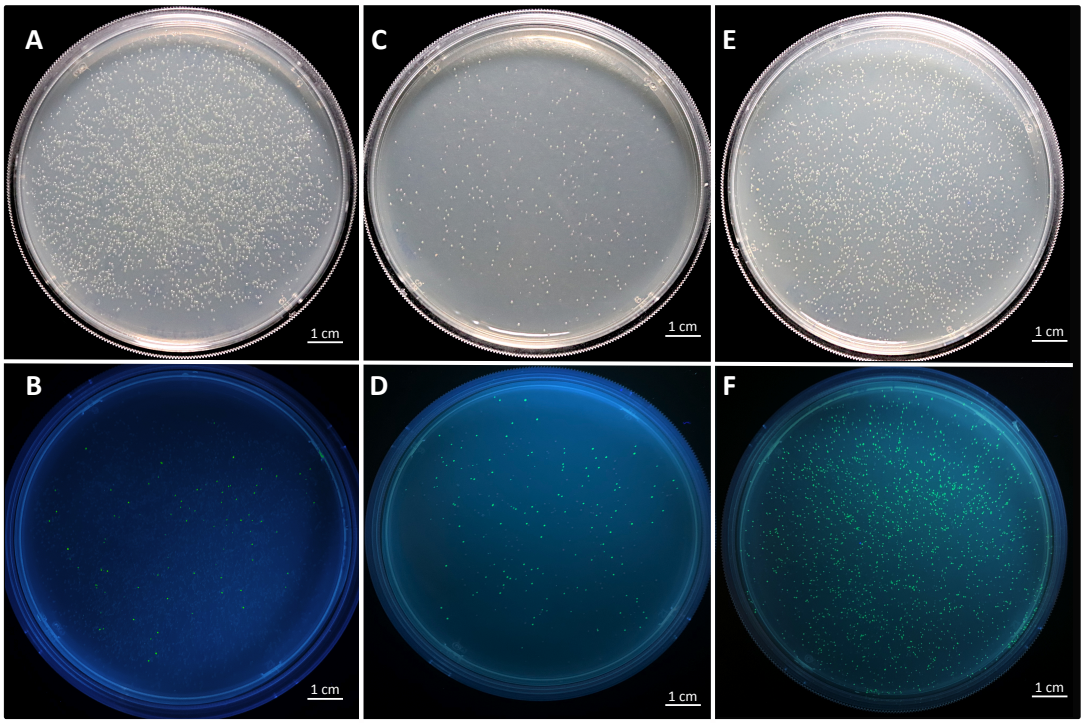

Figure 7

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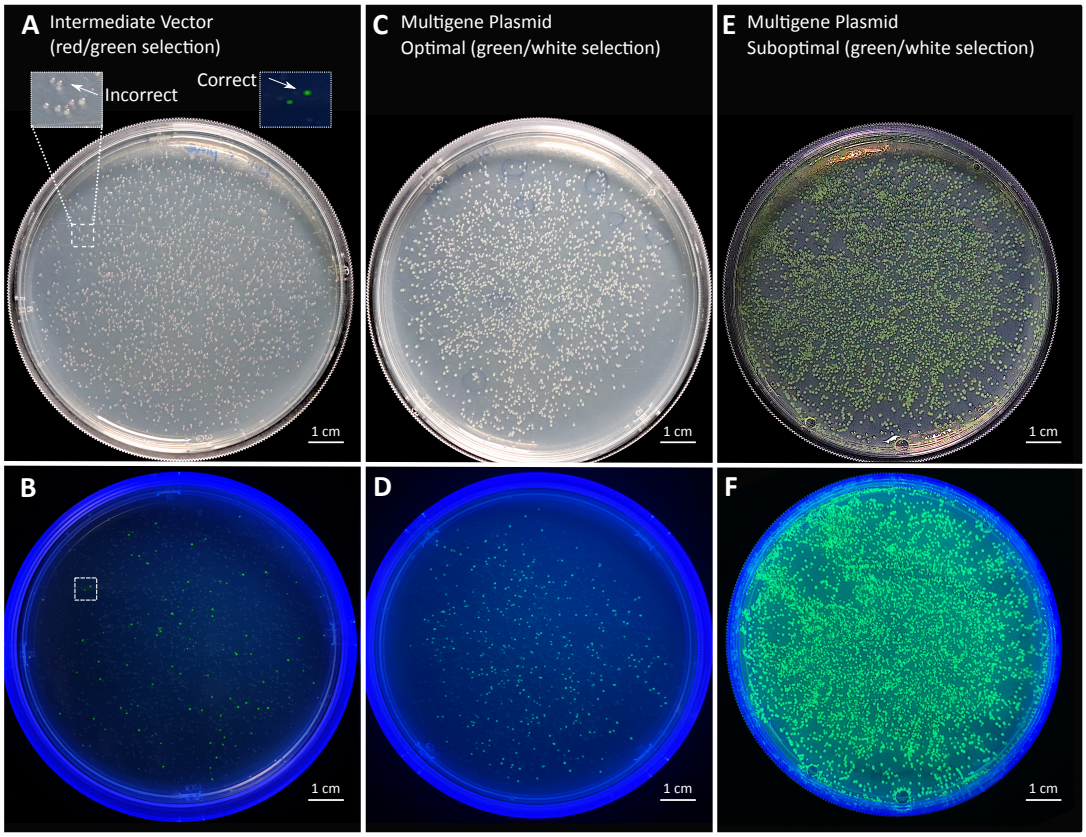
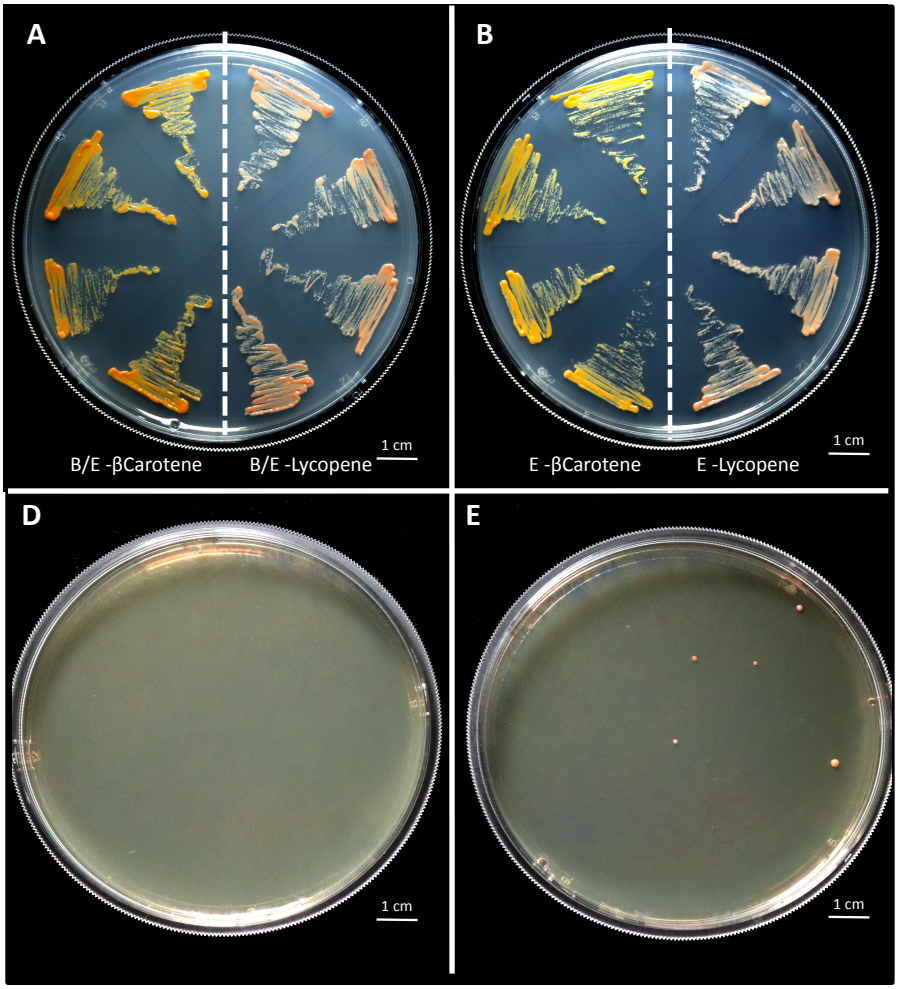


Figure 8



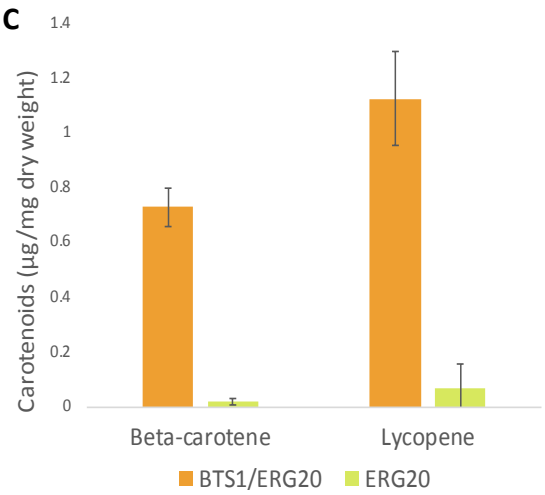


Table 1 Click here to access/download;Table;Tabel1_revised.xlsx

Table 1: Transcription units used in this study.

Name	ConLX (left connector)	Promoter	CDS	Terminator	ConRY (right connector)
BTS/ERG20 TU	LS	*pENO2*	*BTS1/ERG20*	*tTDH2*	R1
ERG20 TU	LS	*pENO2*	*ERG20*	*tTDH2*	R1
crtE TU	L1	*pTIP1*	*crtE*	*tHSP26*	R2
crtYB TU	L2	*pPDC1*	*crtYB*	*tADH2*	R3
$crtYB^{G247A}$ TU	L2	*pPDC1*	$crtYB^{G247A}$	*tADH2*	R3
crtI TU	L3	*pPYK1*	*crtI*	*tACS2*	RE

Table 2: Multi-gene plasmids used in this study.

Name	TU1	TU2	TU3	TU4	Product
B/E-*β* -carotene	*BTS1/ERG20*	*crtE*	*crtYB*	*crtI*	*β-carotene*
B/E-Lycopene	*BTS1/ERG20*	*crtE*	*crtYB*^{G247A}	*crtI*	*Lycopene*
E-*β* -carotene	*ERG20*	*crtE*	*crtYB*	*crtI*	*β-carotene*
E-Lycopene	*ERG20*	*crtE*	*crtYB*^{G247A}	*crtI*	*Lycopene*

Table of Materials

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
0.5 mm Glass beads	RPI research products	9831	For lysing yeast cells
Bacto Agar	BD & Company	214010	Component of the yeast complete synthetic medium (CSM)
Bacto Peptone	BD& Company	211677	Component of the yeast extract peptone dextrose medium (YPD)
BsaI-HFv2	New England Biolabs	R3733S	a highly efficient version of BsaI restriction enzyme
Carbenicillin	Fisher Bioreagents	4800-94-6	Antibiotic for screening at the transcription unit level
Chloramphenicol	Fisher Bioreagents	56-75-7	Antibiotic for screening at the entry vector level
CSM-His	Sunrise Sciences	1006-010	Amino acid supplement of the yeast complete synthetic medium (CSM)
Dextrose	Fisher Chemical	D16-500	Carbon source of the yeast complete synthetic medium (CSM)
Difco Yeast Nitrogen Base w/o Amino Acids	BD & Company	291940	Nitrogen source of the yeast complete synthetic medium (CSM)
dNTP mix	Promega	U1515	dNTPs for PCR
Esp3I	New England Biolabs	R0734S	a highly efficient isoschizomer of BsmBI
Frozen-EZ Yeast Trasformation II Kit	Zymo Research	T2001	For yeast transformation
Hexanes	Fisher Chemical	H302-1	For carotenoid extraction from yeast cells
Kanamycin	Fisher Bioreagents	25389-94-0	Antibiotic for screening at the multigene plasmid level
LB Agar, Miller	Fisher Bioreagents	BP1425-2	Lysogenic agar medium for *E. coli* culturing
LB Broth, Miller	Fisher Bioreagents	BP1426-2	Lysogenic liquid medium for *E. coli* culturing
Lycopene	Cayman chemicals	NC1142173	For lycopene quantification
MoClo YTK	Addgene	1000000061	Depositing Lab: John Deuber
Monarch Plasmid Miniprep Kit	New England Biolabs	T1010L	For plasmid purification from *E.coli*
Nanodrop Spectrophotometer	Thermo Scientific	ND2000c	For measuring accurate DNA concentrations
NotI-HF	New England Biolabs	R3189S	Restriction enzyme for integrative multigene plasmid linearization
Nourseothricin Sulphate	Goldbio	N-500-100	Antibiotic Selection marker for the pCAS plasmid used in this study
Phusion HF reaction Buffer (5X)	New England Biolabs	B0518S	Buffer for PCR using Phusion polymerase
Phusion High Fidelity DNA Polymerase	New England Biolabs	M0530S	High fidelity polymerase for all the PCR reactions
pLM494	Addgene	100539	Plasmid used to amplify crtI, crtYB and crtE used in this study
Quartz Cuvette	Thermo Electron	10050801	For quantifing carotenoids
T4 ligase	New England Biolabs	M0202S	Ligase for Golden Gate cloning
Thermocycler	BIO-RAD	1851148	For performing all the PCR and cloning reactions
Tissue Homogenizer	Bullet Blender	Model: BBX24	For homogenization of yeast cells
UV-Vis Spectrophotometer	Thermo Scientific	Genesys 150	For quantifing carotenoids
Yeast Extract	Fisher Bioreagents	BP1422-500	Component of the yeast extract peptone dextrose medium (YPD)
β -carotene	Alfa Aesar	AAH6010603	For *β* -carotene quantification

Dear editor and reviewers,

The authors are very grateful for your careful reading and positive feedbacks regarding the manuscript "*Raid assembly of multi-gene constructs using modular Golden Gate cloning"* by Mukherjee M, et al. submitted to Journal of Visualized Experiments. Your suggestions have strengthened the manuscript significantly. Please find the point-by-point responses to your comments as below. Please note that the line numberings have been updated in the revised manuscript.

I. Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please define all abbreviations at first use.

We have thoroughly proofread the manuscript to eliminate spelling and grammar issues. We have added the full names of all undefined abbreviations in the revised manuscript.

2. Please make the title concise: "Rapid Assembly of Multi-Gene Constructs in Yeast Using Golden Gate Cloning".

We have modified the title to be more concise: "*Rapid assembly of multi-gene constructs using modular Golden Gate cloning*".

3. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.For example: Addgene (kit # 100000061), NanoDrop, NEBioCalculator (and its link), Addgene (ID-60847), Frozen EZ Transformation II Kit, Addgene #: 100539

We have removed all commercial languages from the manuscript and referenced in the table of Materials and Reagents.

4. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

We have gone through the manuscript and added a lot more details according to the reviewer feedbacks. For example, regarding transformation, in Line 217-219, we have added the sentence:

"We recommend transforming the entire 10 µL cloned product into 35 µL chemically competent E. coli cells (2 X 10^5 cfu/mL, cfu is calculated from transforming 5 ng pYTK001 into 100 µL of the competent cells)."

Another example, in Line 440-441, we have added the sentence:

"Pellet the cells after recovery, discard the supernatant, wash with equal volume of water."

We think there are enough details in the revised manuscript to supplement the actions seen in the video.

5. 1.4: Please cite a reference for PCR of these or similar parts.

Thank you for the suggestion. We have added a reference for PCR protocol (ref 42) in step 1.4.

6. Please cite figures in order: you have cited Figure 6 after Figure 2, then Figure 3, Figure 4, Figure 7, Figure 5, Figure 8.

Thanks for the comment. We have revised the manuscript so that the figures are cited in order.

7. Please do not abbreviate journal names in the reference list.

Thank you for pointing that out. We have added the full names of the journals.

Reviewers' comments:
II. Reviewer#1:
Minor concerns:
JoVE61993
This protocol is well described.
I have just some small suggestions:
The note L127-132 could be completed "alternatively a Bsm1 free gene could be synthetized by a specific company. This could be the opportunity to order a codon optimized gene according to S. cerevisiae codon usage table.

Thank you for pointing that out. We have added it in Line 164-165: *"Alternatively, a BsmBI- and BsaI- free and codon-optimized part can be synthesized commercially".*

L358 Incubate at 37 °C for two days and at 30 °C for one day for colonies to form. (Why is it only one day at 30°C while you indicate two days at 37°C, is it one day more?

Thank you for the comment. For CRISPR/Cas9 based integration, we followed the protocol from the paper (doi: 10.7554/eLife.03703) in which gene-edited yeast was incubated 37°C for two days on a selective plate. We speculate that 37°C was used instead of 30°C (yeast optimal growth temperature) because the temperature stress increases the efficiency of homologous recombination, which facilitates the integration after CRISPR cuts. Usually colonies appear after two days at 37°C. In case no colony is observed, we normally incubate for another day or two at 30°C, which we will usually get some colonies. We have modified the sentence in Line 443-444: *"Incubate at 37 °C for two days for colonies to form. In case no colony is observed, incubate for an additional 1-2 days at 30°C".*

L527 indicate the number in number and not letter. Also is it 6 and not sis?

Thank you for identifying the error. The sentence in Line 843 is modified as: *"The kit provides 27 promoters, six terminators, seven yeast selection markers, and two yeast origin of replications."*

In table of materials the following comments could be added
For BsaI-HFv2, an improved BsaI restriction enzyme
For Esp3; a highly efficient isoschizomer of BsmBI

Thank you for the suggestion. We have added that information to the table of materials:

"BsaI-HFv2, Type II restriction enzyme for Golden Gate cloning, a highly efficient version of BsaI restriction enzyme

Esp3I, Type II restriction enzyme for Golden Gate cloning, a highly efficient isoschizomer of BsmBI"

III. Reviewer #2:
Summary:
This protocol describes Golden Gate assembly procedures compatible with different MoClo cloning kits. It describes them specifically in the context of the Yeast Toolkit.

Major Concerns:
1. Abstract, Lines 73-74, Lines 261-264: The statement that there is "no need for sequencing beyond entry vectors" should be softened. The authors mean that there is a low error rate during propagation of the part vectors and Golden Gate assembly steps. This does not mean that the insert sequences cannot mutate during cloning and that assembled plasmids should not be checked! In particular, there can be situations where an assembled operon or multigene construct leads to toxicity to *E. coli* or yeast. (We have seen these situations in our research, even toxicity to E. coli of eukaryotic sequences that do not have any bacterial gene expression parts.) It would be better to qualify this by adding something like "typically/often there is no need".

Thank you for the valuable suggestion. We agree that assembled plasmids should be sequence verified especially in cases that the construct is toxic to the host cell. We have made the following changes in the manuscript:

Abstract Line 36: "*The hierarchical workflow enables the facile cloning of a large variety of multigene constructs **typically** with no need for sequencing beyond entry vectors.*"

Line 86: "*Secondly, one needs to sequence the part plasmid but **typically** not the TU or the multigene plasmids. In most cases, screening by colony PCR or restriction digestion is sufficient for verification at the TU and multigene plasmid level."*

Line 337: "*Note: Sequencing transcription units is **typically** not necessary because the cloning involves only restriction digestion and ligation. All sequences of interest have been confirmed at the part plasmid level."*

2. Lines 200, 311, 508-515: We find that a Qubit (fluorescent-dye based assay) gives more reliable DNA concentrations than a Nanodrop, because buffer components can interfere with absorbance measurements, especially for less-concentrated DNA samples. Perhaps this, or a similar assay, should be listed as an alternative option.

Thank you for the valuable suggestion. We were not aware of the Qubit assay which is more sensitive than the Nanodrop. Unfortunately, we cannot mention either Qubit or Nanodrop because JOVE does not allow mentioning trademarks. Nevertheless, we have included this alternative in the revised manuscript:

Line 258: *"3.1.1. Purify the above six plasmids. Record their concentrations using a UV-Vis spectrophotometer or a fluorescence-based assay, and dilute each with ddH2O so that 1 µL has 20 fmol of DNA."*

Line 392: "*Record their concentrations using a UV-Vis spectrophotometer or a fluorescence-based assay."*

We added a sentence in Line 823: "*Quantifying parts using UV-vis spectrometer is usually sufficient, but more sensitive methods such as a fluorescence-based assay may give better results."*

3. Figures: In general, these are very good for showing how the restriction sites operate for each type of cloning step/aim. It would be helpful in some cases to show directionality: using arrows instead of boxes. In Figure 1 Level 3 and Fig 5 specifically: The directionality / location of 5' and 3' Hom relative to the chromosome are not obvious with how these are depicted and the direction of the text within the elements is switched as they move between different locations during cloning and integration. Someone following the protocol could misinterpret this and design a plasmid that would integrate the "Bacterial ORI and Selection Marker". Also, it would be best practices to use SBOL symbols for some of the plasmid elements. For example, a T is used instead of a lollipop for terminators.

Thanks for the valuable comments. We have included directionality and location of 5' and 3' homology arms in all figures. We have altered the direction of 5' and 3' homology arms in

Figure 5 to eliminate the impression that the bacterial ORI and selection marker are integrated to the genome. We have adapted the SBOL symbols in all the schematic figures inclduining Figure 1-5.

Minor Concerns:

4. Throughout: The proper name of the restriction enzyme is BsmB1 (with the second B capitalized). The paper uses Bsmb1, currently, and needs to be updated.

Thank you for pointing that out. We have corrected this throughout the manuscript.

5. Line 78: There is an additional broad-host-range bacterial MoClo kit (with overhangs compatible with the Yeast Toolkit and also available from Addgene) that could be used with these protocols: Leonard, S. P., Perutka, J., Powell, J. E., Geng, P., Richhart, D. D., Byrom, M., Kar, S., Davies, B. W., Ellington, A. D., Moran, N. A., Barrick, J. E. (2018) Genetic engineering of bee gut microbiome bacteria with a toolkit for modular assembly of broad-host-range plasmids. ACS Synth. Biol. 7: 1279-1290. doi:10.1021/acssynbio.7b00399

Thank you for pointing out this publication. We have added this reference in Line 93 and reference 27.

6. Line 106: The cleavage efficiency of restriction enzymes is reduced when enzymes cleave/recognize bases that are at or near the end of a DNA fragment. This detail is present in the figures, but it would be helpful to suggest here how many extra bases should be added to flank the BsmB1/Bsa1 recognition sequences or, better, one or more exact sequences that work with high efficiency (TTT is present in Fig 1).

Thank you for the suggestion. We always include TTT at the end of the primers for optimal enzyme digestion. We have added this point in Line 137: "*Design the forward and reverse primers containing: flanking nucleotides TTT at the 5' end, a BsmBI recognition site with an additional nucleotide (CGTCTCN), a 4-nucleotides (nt) overhang (TCGG) complementary to that of the entry vector, a BsaI recognition site with an additional nucleotide (GGTCTCN), and a 4-nt part-specific overhang, in addition to the template-specific sequence"*

7. Line 127: Another alternative for removing many restriction sites, which is becoming cheaper all the time and should probably be mentioned, is to *de novo* synthesize all or a portion of the part via an IDT gBlock, Twist, etc. This is briefly mentioned on Line 520, but could be made clearer.

Thank you for the suggestion. We have included a sentence in Line 164: "*Alternatively, a BsmBI- and BsaI- free and codon-optimized part can be synthesized commercially."* Unfortunately, we cannot mention commercial languages in the manuscript. We routinely use IDT gBlocks for this purpose, but Twist seems like a better alternative.

8. Line 164: What transformation method do you suggest? Take electrotransformation for example, the T4 buffer contains a certain amount of tris and dithiothreitol that can reduce

the transformation efficiency. Is there a minimum or recommended volume-ratio between the competent cells and the assembly reaction that is being transformed?

We typically transform using heat shock with chemically competent *E. coli* cells. We usually transform 10 µL of the cloning product into 35 µL $2*10^5$ cfu/mL chemically competent *E. coli* cells. The colony formation unit was assessed by transforming 5 ng pYTK001 into 100 µL of the competent cell. However, slightly more or less competent cells may be used. We have included this in the manuscript in Line 217-219:

"We recommend transforming the entire 10 µL cloned product into 35 µL chemically competent E. coli cells (2 X 10^5 cfu/mL, cfu is calculated from transforming 5 ng pYTK001 into 100 µL of the competent cells)."

9. Line 172: A blue light transilluminator can also be used (and is less hazardous).

Thank you for the suggestion. We have included the "blue light transilluminator" along with the UV light in Line 225, Line 283, Line 330, Line 815.

10. Line 175: Could you specify an expected positive/negative colony ratio or a typical range of ratios for then assembly has been successful?

We have included a sentence in Line 229-230: "*The cloning is usually successful if there are ~30% to close to 100% white colonies."*

11. Line 348: What is your recommended volume of integrative plasmid and pCAS-gRNA? Since the Frozen EZ Transformation II Kit recommends that the volume of DNA should be less than 5 µL, your instructions will help readers of this article determine how they should dilute or concentrate their plasmids.

As per the editor's comment, we have removed the Frozen EZ Transformation II Kit and therefore the specific instructions from the protocol. Usually, we transform 2-3 ul of pCAS-gRNA and ~5-10 ul of integrative plasmid. We try to keep the total volume minimal but we have seen the Frozen EZ Transformation II Kit works well enough even if the total volume is higher than 5 ul.

12. Lines 58-59: One alternative DNA assembly method that is not mentioned in this list is "Yeast Assembly": Gibson, D. G. (2009) Synthesis of DNA fragments in yeast by one-step assembly of overlapping oligonucleotides. Nucleic Acids Res. 37: 6984-6990. doi:10.1093/nar/gkp687; and several follow-up papers. It seems like this should be mentioned/cited, as it can be a very flexible way to construct yeast plasmids without a need for removing restriction sites, even though Golden Gate cloning is likely to be more efficient and to have lower error rates.

Thank you for the suggestion. We have included it in Line 72 (reference 13).

IV. Reviewer #3:

Wang and Co-workers show the method and technology of golden gate cloning for the assembling of multigene plasmids. This is based on a previous publication and they wish to demonstrate the details of each step and procedure. The presented methodologies seem sound and valid and the comments are helpful.

Only minor revisions are due:

*** LB medium is not Luria-Bertani but lysogenic broth. Please change this throughout the document**

Thanks for the suggestion. We have made the correction throughout the document.

*** Line 78-79: please also add the recent BioRxiv Paper as Saccharomyces/Pichia kit doi: https://doi.org/10.1101/2020.07.22.216432**

Thanks for the suggestion. We have included it in Line 93 (reference 22).

*** Section 1 Primer design: there are two primer design tools for Golden Gate. Golden Braid (https://doi.org/10.1007/978-1-4939-2444-8_20) and Golden Mutagenesis (https://doi.org/10.1038/s41598-019-47376-1). These should be cited here for Domestication and for Golden Mutagenesis also for later primer design.**

Thank you for the suggestions. We have included them in Lines 141 (reference 37 and 38).

*** General: minutes is min not mins. And for hours please write h.**

Thanks. We have changed these throughout the manuscript.

*** Capitalise liter as in µL instead of µl.**

Thanks. We have made the correction throughout the manuscript.

V. Reviewer #4:

Manuscript Summary:

The manuscript presents detailed protocols for assembling metabolic pathways over several rounds using the MoClo standard for Golden Gate assembly and covers their application in a Yeast toolkit including two ways of expression in yeast. Additionally, and probably the key feature of interest for Jove, they present a very clear screening system taking advantage of fluorescent proteins to allow rapid identification of colonies likely to contain the desired insert. Colonies with empty vector can be quickly discarded based on this visual screen, though preliminary identified positive colonies may need verification to ensure they contain the full, correctly ordered insert.

Major Concerns:

While the subject matter is excellent for the journal and clear protocols for modular GGA are quite useful, I have significant concern with the extremely low correct colony rate evident in several assembly rounds. This method additionally changes the MoClo cycling protocol in ways that differ significantly from the literature standard without explanation, and these changes are likely resulting in the extremely poor positive colony rate/high empty vector the authors report. Further the protocol recommendation to do a partial assembly round that leaves in two internal BsaI creates an extremely trickly assembly step, and the design choice is poorly justified in the text. I feel these extremely low positive rates will make this protocol unnecessarily difficult to use and highly sensitive to minor changes in fragment concentration or quality, leading to a protocol likely to fail and create frustration for the user. An easy to read screen does little good if the assembly fails.

Thank you for the valuable comments. We agree assembly of an intermediate vector that retains the BsaI sites seems tricky. However, in practice, we have routinely observed that omitting the final digestion and heat inactivation steps can successfully assemble intermediate vectors retaining two BsaI sites. We have included Figure 6 C&D to show a typical result for TU level intermediate assembly and Figure 7A&B for multi-plasmid level intermediate assembly. The positive rates are 17.56% and 1.83% respectively. The screening of the positives was made easy by the green fluorescence.

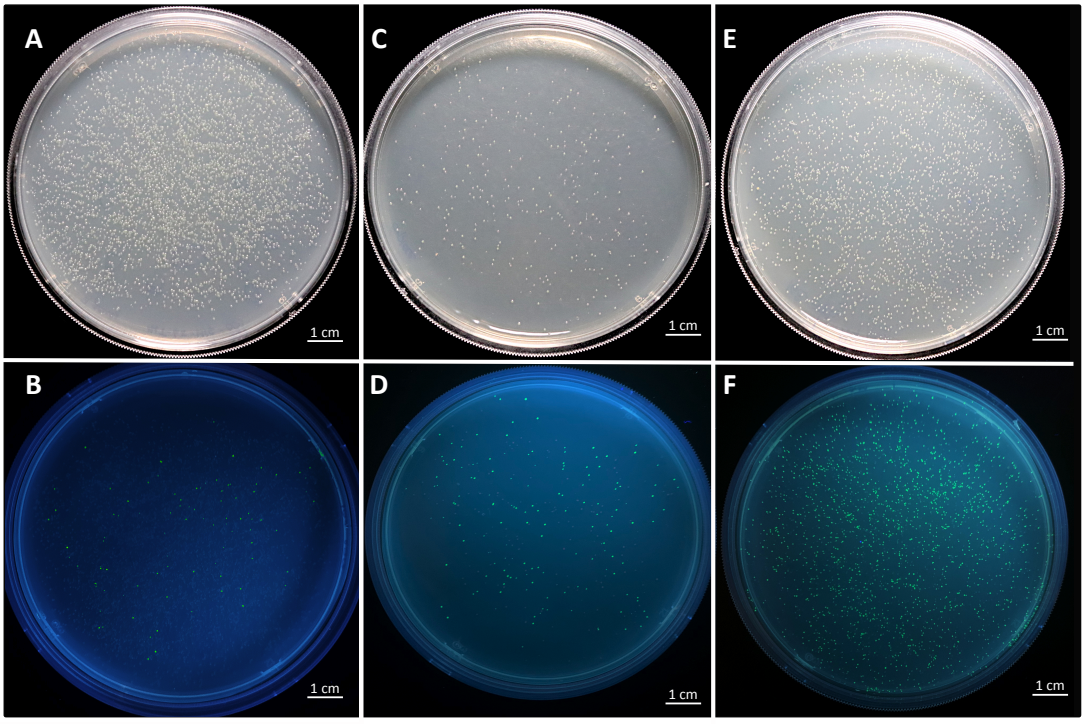


Figure 6: Representative plates of entry vector and transcription unit level cloning in E. *coli. A & B: Representative plates of the successful cloning of a gene into the entry vector pYTK001 under (A) visible light and (B) UV light. Positive colonies are white and negative colonies are green. The ratio of positive colonies overall colonies is: 90.35 ± 4.22 %. C & D: Successful assembly of the intermediate vector for transcription unit level assembly and green/red selection under (C) visible light and (D) UV light. Positive colonies are green. The ratio of positive colonies overall colonies is: 17.56 ± 3.32 %. E & F: Successful*

assembly of transcription unit from the intermediate vector and green/white screening under (E) visible light and (F) UV light. Positive colonies are white and negative colonies are green. The ratio of positive colonies overall colonies is: 65.02 ± 3.32 %. Data are from three biological replicates.

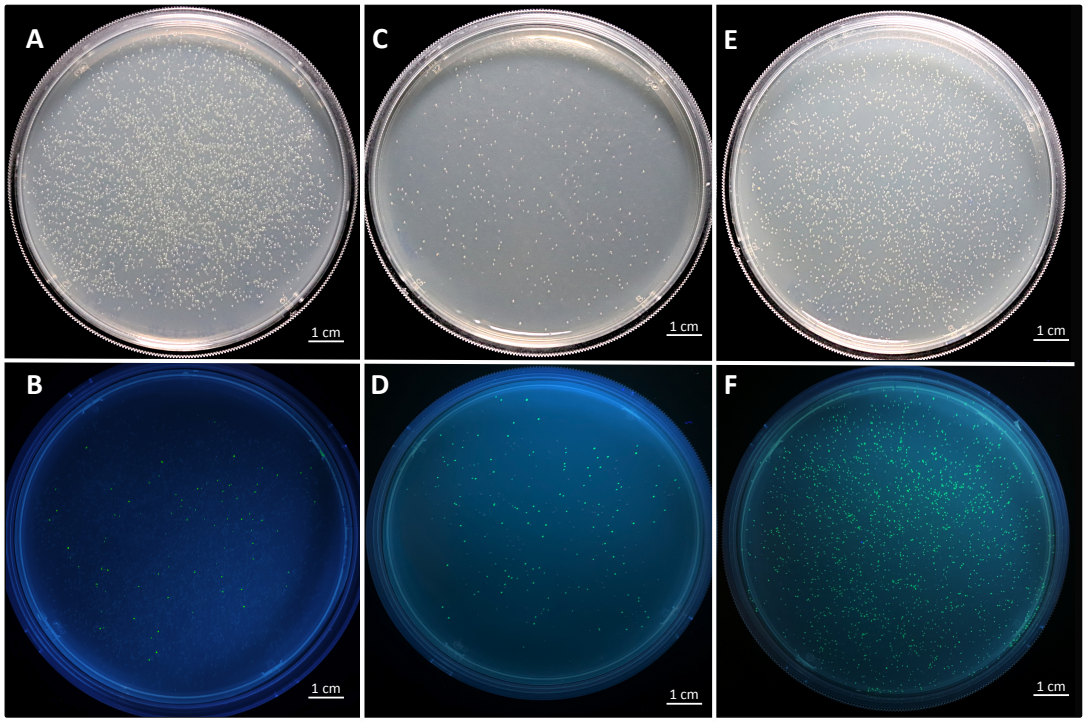
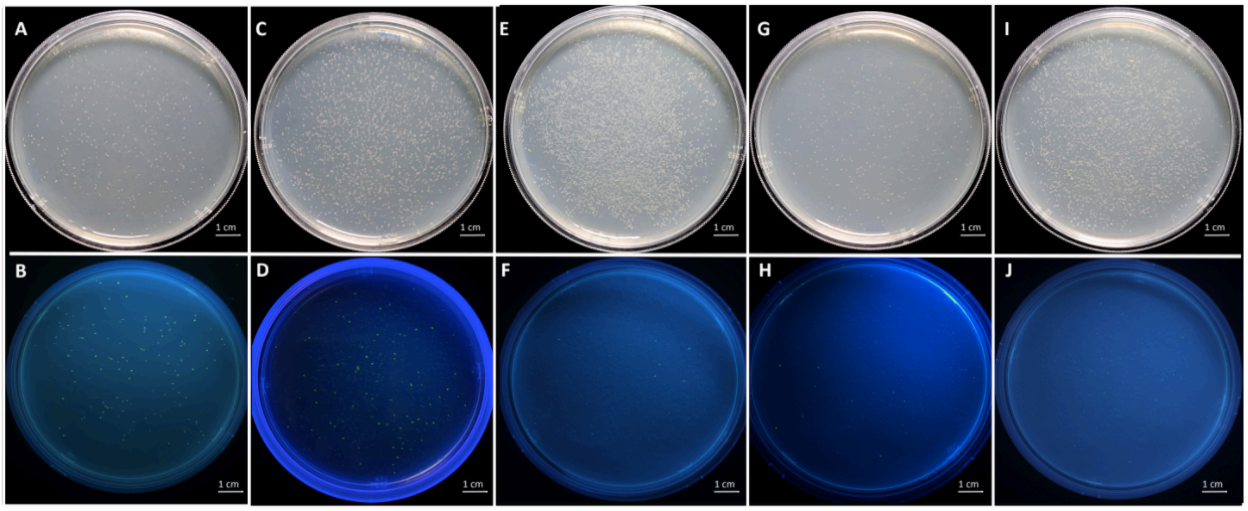


Figure 7: Representative plates of multi-gene plasmid cloning in E. coli. *Fig A & B: Assembly of the intermediate vector for multi-gene level assembly and red/green selection under (A) visible light and (B) UV light. Positive colonies are green and negative colonies are red. The ratio of positive colonies overall colonies is 1.83 ± 0.15%. C-F: Successful assembly of multiple TUs from the intermediate vector and green/white selection (C) under visible light and (D) UV light. Positive colonies are white. The ratio of positive colonies overall colonies is 93.77 ± 1.65%. Suboptimal assembly of multi-gene plasmid under (E) visible light and (F) UV light. The number of positive colonies is negligible. Data are from three biological replicates.*

Regarding the value of assembling intermediate vectors, if assemble TU directly from part plasmids, it relies on screening for red colonies. However, the red colonies are often too pale to visualize (Figure 7A, insert). Cloning an intermediate vector allows screening for green colonies, which is much easier. Another advantage is that an intermediate vector greatly facilitates combinatorial assembly with varied promoters, CDSs and terminators. For example, for another project (unpublished), we assembled 33 different combinations of TU level plasmids with different promoter, gene and terminator combinations very easily because we had the intermediate vector cloned. We could get away with doing 33 4-piece assembly instead of 33 8-piece ones. An 8-piece GG assembly is significantly less efficient than a 4-piece assembly (Figure S2, will discuss further). The value of intermediate vectors is also mentioned in the supplementary material of the paper by Lee et al (2015) https://doi.org/10.1021/sb500366v.

In the 2009 moclo paper, and in many other peer reviewed and manufacturer protocols for GGA, typical cycling condition are alternating 37C/16C for equal times. The low ligation temperature is needed for highly efficient ligation, and consequently, good production of full-length assemblies during the reaction, while the higher T is used to get efficient cutting. At higher ligation temperatures, reduced annealing of the short overhangs can result in reduced efficiency in the ligation step and thus fewer colony forming units created per cycle. Why do the authors use the surprisingly high ligation cycle T of 25C, which likely contributes to the very poor "correct" rate the authors observe in figures 6EF and 7ABCD. The authors at minimum should provide a reference justifying this temperature change, but preferably show data to at least the reviewers that this change improves the outcome over the literature standard protocols. Likewise I would like to see justification for the different hold times at each temperature. Is this an arbitrary change, one based on a literature report, or do the authors have data that shows this to be an improvement over the usual MoClo parameters? Please cite the source of this protocol change and preferably provide data for review that shows this is an improvement over standard MoClo cycling protocols.

Thanks for the valuable comments. We have tested the effect of different ligation temperatures (16C vs 37C), different hold times (2 min vs 5 min) per reviewer's suggestions. The data shows that the reviewer's comment was correct that 16C is better for ligation (condition 1 vs condition 3) and 5 min may be better for holding (condition 4 vs condition 5). For a 4-piece assembly, our initial condition only gave 3.6% positive colonies, while the condition recommended by the reviewer (Condition 5: 16C ligation, 5 min digestion, and 50C final digestion) in combination achieved 99.5%. positive colonies. We have included these data in Figure S1 and Table S1. We have also updated Figure 6 & 7 using the optimized condition.



Condition 1	Condition 2	Condition 3	Condition 4	Condition 5
$\begin{matrix}37\ °C\ -2\ min\\25\ °C\ -5\ min\end{matrix}\Big]30$ X	$\begin{matrix}37\ °C\ -2\ min\\25\ °C\ -5\ min\end{matrix}\Big]30$ X	$\begin{matrix}37\ °C\ -2\ min\\16\ °C\ -5\ min\end{matrix}\Big]30$ X	$\begin{matrix}37\ °C\ -2\ min\\16\ °C\ -5\ min\end{matrix}\Big]30$ X	$\begin{matrix}37\ °C\ -5\ min\\16\ °C\ -5\ min\end{matrix}\Big]30$ X

37 °C – 10 min	50 °C – 10 min	37 °C – 10 min	50 °C – 10 min	50 °C – 10 min
80 °C – 10 min	80 °C – 10 min	80 °C – 10 min	80 °C – 10 min	80 °C – 10 min
4 °C – ∞	4 °C – ∞	4 °C – ∞	4 °C – ∞	4 °C – ∞

Figure S1: Optimizing temperature and time conditions for Golden Gate cloning for a 4-piece assembly: Different temperatures were tested for ligation by T4 ligase (25 °C and 16 °C) and final digestion by BsaI-v2 (37 °C and 50 °C) and different time durations (2 min vs 5 min) were tested for the initial digestion step. One representative plate from each cycle shown in the figure. White colonies contain potentially correct constructs. The first panel was taken under visible light and the second panel was taken under the UV light.

Table S1: Results obtained for different cycle conditions:

Condition used	Approximate percentage of white (potentially correct) colonies*
Condition 1	$3.56 \pm 5.2\%$
Condition 2	$6.28 \pm 3.7\%$
Condition 3	$4.12 \pm 3.9\%$
Condition 4	$97.6 \pm 2.4\%$
Condition 5	$99.5 \pm 1.3\%$

* $Percentage\ of\ white\ colonies = \frac{Number\ of\ white\ colonies}{Total\ number\ of\ colonies} \times 100$ (Calculated from 3 plates per condition)

Next, it should be noted that while melting of the overhangs greatly reduces ligase activity at 37C, this temperature does not inactivate T4 DNA ligase and it will be still active when the reaction is cooled prior to transformation. This is why the MoClo paper uses a final hold of 50 C (with many other protocols using a hold in the 45 - 65C range)- this temperature inactivates the ligase but not the restriction enzyme. This step is critical for low background, as it linearizes any remaining empty vector, preventing replication in bacteria. The end hold temperature of 37C is insufficient to inactivate the ligase, with the expected result an extremely high percentage of colonies with missing inserts (as the authors observe in several experiments). An explanation for this change, with again a minimum provided literature reference and preferably data to demonstrate this change improves over the literature standard 50C end hold is needed.

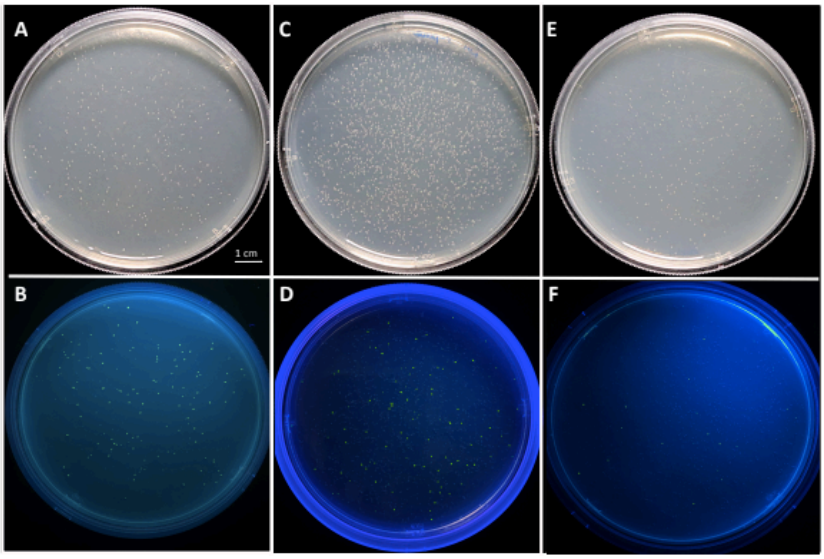
Again, we much appreciate the reviewer's insight in this matter. We have tested the final digestion temperatures at 37C vs 50C, and found that 50C significantly increased the ratio of

positive colonies (condition 1 vs 2 in the table above). More importantly, ligation at 16C and final digestion at 50C have a synergistic effect, resulting in dramatically increased ratio of positive colonies (condition 4: 97.6%). The reviewer's insights on this greatly strengthened the manuscript and helped future cloning in our lab.

Finally, their middle step seems to create a wholly unnecessary challenge to the user. By asking the user to assemble an insert containing multiple internal sites for the restriction enzyme being used, they are guaranteed to have a very poor rate of full-length construct formed as the restriction enzymes will leave many of these internal sites cut. Coupled with the total lack of an end hold ensuring the percentage of religated empty vector will also be high, I am surprised they see any insert-bearing colonies at all. This design is flawed and risks frustrating users with a high chance complete failure at this step. The justification that the authors use - assembly of 7 fragments is much less efficient than 4 - is not supported by the literature, with multiple labs reporting assembly of 12 fragments in one round routine, and the need to make the screening easier to see will be much less necessary if a protocol providing a much lower empty vector background is used.

We have discussed the advantage of creating the intermediate vector in above sections. In addition to that, we would like to emphasize that the middle step of creating an intermediate vector at the TU level is optional but we highly recommend as this one step made our workflow creating a large combinatorial library much easier. The ratio of positive colonies of TU level intermediate assembly is 17.56% (Figure 6 C&D)). The ratio of positive colonies of multi-gene level intermediate assembly is 1. 83% (Figure 7 A&B). Although the success rate is relatively low, the screening of the positives was made easy by screening for the green fluorescence.

Regarding our claim that efficiency of cloning decreases with larger number of inserts, we have observed decrease in number of green colonies when we assembled 6-pieces vs 7-pieces vs 8- pieces and therefore believe it to be true (Please see below). It is certainly possible to assemble 8 pieces and do a red-white screening, but in our experience the assembly of intermediate vector makes the workflow much simpler. This figure is included as Figure S2 in the manuscript.



Number of parts assembled	No. of green colonies	No. of total colonies	Approximate percentage of green (potentially correct) colonies
6	80	300	26.6%
7	45	2658	1.7%
8	15	2275	0.65%

Figure S2: **Decreasing efficiency of Golden Gate Assembly with increasing number of parts:** Golden Gate assembly of 6 parts (A and B), 7 parts (C and D), and 8 parts (E and F) show a progressive decrease in number of potentially correct green colonies. The first panel shows plates under visible light and the second panel shows plats under the UV light.

The protocol is likely to be substantially improved and shortened if this step is removed, especially with restoration of the 50 C end hold to remove background.
In short, the authors need to provide experimental evidence and/or cite appropriate literature to demonstrate that their new protocols are better than the literature they cite. Given the extremely poor positive rates seen, it seems unlikely this will be the case, and thus I would recommend the authors redo the work using more robust assembly protocols that result in the a much higher positive colony rate at each step. If they can provide those results and report the modified conditions, the protocols coupled with the GFP screen will make an excellent addition to the journal.

The reviewer's insights here are extremely helpful. We have tested the suggested condition (condition 5 in Figure S1 and Table S1) and found it dramatically increased the positive rate

from 3.5% to 99.5% for a 4-piece assembly. We have added the following paragraph in Line 697-703:

"We tested five different conditions for the digestion/ligation cycles of Golden Gate cloning with T4 DNA ligase. We found that 30 cycles of digestion at 37 °C for 5 min and ligation at 16 °C for 5 min, followed by a final digestion step at 50 °C for 10 min and protein inactivation step at 80 °C for 10 min resulted in 99.5% colonies been potentially correct in a 4-piece assembly (Figure S1 and Table S1). Ligation at 16 °C ensures optimal activities of ligase and annealing of overhangs. Final digestion at 50 °C prevents digested products from re-ligation. We have followed this cycle for all the assembly reactions unless otherwise specified."

Minor Concerns:
In addition to my key objection, I have some additional suggestions that the authors should consider in revision:
-The manuscript could use a pass for grammar editing. For example, lines 59-61 is not a complete sentence.

Thanks for the comment. We have completed grammar editing throughout the revised manuscript. We have edited Line 70-73 as: "*Many multipart cloning methods such as the Gibson cloning[4,5], the ligation-independent cloning (SLIC)[6], the uracil excision-based cloning (USER)[7], the ligase cycling reaction (LCR)[8], and the in vivo recombination (DNA Assembler)[9] have been developed so far."*

The term "efficiency" is used several times with unclear (and I think, multiple) meanings. In some cases the authors seem to be referring to the percentage of correct colonies, other times to the total number or the speed at which full length constructs are formed. Please clarify meaning when this term is used. (line 67 vs line 153)

Thanks for the valuable comment. We have limited the use of word "efficient" only when describing enzymes, Exp. Line 267-268: *BsaI-HFv2 (a highly efficient version of BsaI)*

Line 77-80: *"Applying this cloning strategy to the modular cloning (MoClo) system has enabled the assemble of up to 10 DNA fragments with over 90% transformants screened containing the correctly assembled construct".*

Introduction could use some additional information on the method. Many of these points are discussed throughout the protocol, but it would benefit the reader to have them in the introduction. Abstract: not all type IIs enzymes create 4 base overhangs.

Thank you for the helpful comments. We have added the following sentences in the introduction in Line 116-122:

"During our extensive use of this kit, we found that accurate measurement of DNA concentrations is the key to ensure equimolar distribution of each part in the Golden Gate reaction. We also recommend the T4 DNA ligase over the T7 DNA ligase because the former worked better with larger numbers of overhangs. Lastly, any internal recognition sites of BsmBI

and BsaI must be removed or domesticated prior to assembly. Alternatively, one may consider synthesizing parts to remove multiple internal sites and achieve codon optimization simultaneously."

We have changed the sentence in Abstract Line 31 to*: "It utilizes type IIS restriction enzymes that cut outside of their recognition sites and create a short overhang."*

Line 58 - despite using the word "recently" only the original 2009 paper is cited here, would suggest adding more (and more recent) references.

Thank you for the suggestion. We changed "*recently*" into "*in the past decade*" and cited 4 additional papers about recent developments in Golden Gate Cloning in Line 69 (refs 4-7).

Line 62 - if you are restricted to specific junctions/fusion sites by your assembly standard, the assembly is arguably not "scarless" as it is unlikely the native sequence contains only the moclo standard junctions at the appropriate places, especially if you are assembling within a gene.

We agree with the reviewer that 4 nt overhang sometime can result in scars, especially using the standard junctions. However, because the junctions flank the functional parts, the overhangs usually won't affect function. In addition, arbitrary 4nt can be chosen within the gene or any other parts, allowing true scar-less assembly. Such examples in this manuscript include the domestication and creating fusion proteins. While Golden Gate cloning allows true scar-less cloning, MoClo does not. We have softened the sentences accordingly:

Line 34-35 in Abstract: "*The Golden Gate cloning strategy is of tremendous advantage because it* ***allows*** *scar-less, directional, and modular assembly in a one-pot reaction."*

Line 833-834: *"The MolClo strategy is advantageous over alternative multi-part assembly methods4,6-9 because it allows modular and highly versatile cloning."*

Lines 64-67 some clarification of the one pot/cycled nature of the reaction is needed, it is confusing to refer to GGA as "one step" then talk about cycling.

We agree with the review and have eliminated the use of "one-pot" in the text.

Line 73-76 what about the possibility of insertions/deletions of fragments? Worth a comment that it may be needed to at least verify the size of your insert via colony PCR.

Thank you for the suggestion. We have inserted a sentence in Line 87-89*: "In most cases, screening by colony PCR or restriction digestion is sufficient for verification at the TU and multigene plasmid level."*

Also worth mentioning domestication and the need to avoid internal sites in the introduction, not mentioned until the protocol section.

We have added a sentence regarding domestication in the introduction in Line 120-121: "*Lastly, any internal recognition sites of BsmBI and BsaI must be removed or domesticated prior to assembly.*"

Figures: Figure2 B in the final assembled entry vector, the "NNNN" should be moved to the box of GOI. Figure 2, B Clone into Entry Vector, 2nd of the three diagrams, the BsmBI recognition sequence downstream of the green GFP Dropout is reversed (says 5' to 3', ctctgc instead of cgtctc). Figure3 The Kmr maker in the intermediate vector is an error. It should be Ampr. Figures 6 & 7 - please indicate the percentage of positive colonies observed in the figure legend and in the text. For some it is very hard to pick out the few white or few green colonies on the plate by eye.

Thank you so much for finding these errors. We have modified Figure 2B and Figure 3 accordingly. We have updated Figure 6 and 7 with new pictures and included the percentage of positive colonies observed:

Figure 6:

Level	Figure 6	% of positive colonies
Entry vector	A&B	90.35 ± 4.22
Intermediate vector for TU	C&D	17.56 ± 3.32
TU assembly	E&F	65.02 ± 4.99

Figure 7

Level	Figure 7	% of positive colonies
Intermediate vector for multigene plasmid	A&B	1.83 ± 0.15
Multigene plasmid assembly-optimal	C&D	93.77 ± 1.65
Multigene plasmid assembly-suboptimal	E&F	0.15

These stats are included in the figure legends:

"Figure 6: *Representative plates of entry vector and transcription unit level cloning in E. coli. A & B: Representative plates of successful cloning of a gene into the entry vector pYTK001 under (A) visible light and (B) UV light. Positive colonies are white and negative colonies are green. The*

ratio of positive colonies over all colonies is: 90.35 ± 4.22 %. C & D: Successful assembly of the intermediate vector for transcription unit level assembly and green/red selection under (C) visible light and (D) UV light. Positive colonies are green. The ratio of positive colonies over all colonies is: 17.56 ± 3.32 %. E & F: Successful assembly of transcription unit from the intermediate vector and green/white screening under (E) visible light and (F) UV light. Positive colonies are white and negative colonies are green. The ratio of positive colonies over all colonies is: 65.02 ± 3.32 %. Data are from three biological replicates."

***"Figure 7:** Representative plates of multigene plasmid cloning in E. coli. Fig A & B: Assembly of the intermediate vector for multigene level assembly and red/green selection under (A) visible light and (B) UV light. Positive colonies are green and negative colonies are red. The ratio of positive colonies over all colonies is 1.83 ± 0.15%. C-F: Successful assembly of multiple TUs from the intermediate vector and green/white selection (C) under visible light and (D) UV light. Positive colonies are white. The ratio of positive colonies over all colonies is 93.77 ± 1.65%. Suboptimal assembly of multigene plasmid under (E) visible light and (F) UV light. The number of positive colonies are negligible. Data are from three biological replicates."*

The stats are also included in the text:

In text Line 487-488, we've added: *"Figure 6A&B shows a representative plate of successful cloning of a part plasmid and provides an example of the green/white screening with 90.35 ± 4.22% total colonies being positive (white)."*

In Line 495-497, we've added: "*Figure 6C&D shows a representative plate of a successful assembly of the intermediate vector and provides an example of the red/green screening with 17.56 ± 3.32% total colonies being positive (green). Although this ratio is relatively low, screening is greatly facilitated by the green fluorescence."*

In Line 501-502, we've added: "*Figure 6E&F shows a representative plate of a successful assembly of the TUs and provides an example of a green/white screening method with 65.02 ± 4.99% total colonies being positive (white).*"

In Line 509-514, we've added: "*The ratio of potentially correct colonies (green) for the intermediate vector cloning was 1.83 ± 0.15% (Figure 7 A/B). Although this number seems low, the screening was made easy by detecting the green fluorescence (Figure 7 B). Once the intermediate plasmid was cloned, the success rate of assembling multigene plasmid (white) from the intermediate was 93.77± 1.65% (Figure 7 C/D). Figure 7E&F shows a suboptimal assembly of multigene plasmid as the numbers of white positive colonies were negligible."*

Some minor protocol suggestions: Lines 146-148 Gel purification is fine if laborious, perhaps the authors can suggest purification by spin columns or other methods (though I strongly agree with the advice the authors give that fragments must be clean and accurately quantified).

We have added a sentence in Line 186-187: "*Using purified DNA is strongly recommended, if gel purification is laborious, use at least a spin column to purify the PCR product."*

Throughout the protocol, refers to adding 1 ul T4 DNA ligase buffer; this should be 1 ul 10X T4 DNA ligase buffer.

We apologize for the mistake. We have corrected it throughout the protocol.

Lines 224-227; suggestion about using a transilluminator to identify the green colonies. Would it not be better to also recommend, if possible, using a UV source with a wavelength known to be less damaging to DNA/cells?

We have included the "blue light transilluminator" along with the UV light in Line 225, Line 283, Line 330, Line 815 as suggested by reviewer #2 and #4.

Lines 257-259; colony PCR is best performed on colonies spotted/streaked from the transformation plate onto a new selection plate. The surface of the original transformation plate can contain extraneous assembly incompletes that complicate the colony PCR result analysis.

Thank you for the suggestion. We have modified the sentence in Line 333 as: *"Streak out and grow 8-10 white colonies and perform a colony PCR"*

Line 510 - I do not think Esp3I is anywhere reported to be a high-fidelity version of BsmBI, but please reference if it has been.

We apologize for the mistake. We agree the word "high-fidelity" is not accurate. We have removed it from the manuscript and instead mentioning that Esp3I is "highly-efficient" for its faster digestion.

Line 512 - a recent reference (ref 44) discusses the high bias of T7 ligase as a good reason to not use it.

Thanks. We have included this reference in Line 826 as: "*Regarding the ligase, we found that the T4 ligase works well for both the overhangs in the kit and customized overhangs necessary to fuse of two parts, which is consistent with literature*[36]*."*

VI. Reviewer #5:

Manuscript Summary:

This manuscript describes some of the hands-on techniques required to recapitulate the methods of Lee et al 2015 (ref 15) using an already described Golden Gate cloning technique. The authors provide a further description of the cloning system and have constructed some new parts for testing. They attempt to show the efficacy of the cloning system along with some representative results. Overall I found the manuscript to come up short in terms of providing a thorough resource for use of the Lee et al yeast toolkit.

Major Concerns:

Title- "assembly of multi-gene constructs in yeast". I find the title misleading as it suggests that yeast are being used to select for properly assembled constructs. The constructs are first assembled in bacteria and then transformed into yeast.

Thanks for the valuable comments. We agree with the reviewer and have changed the title to: *"Rapid assembly of multi-gene constructs using modular Golden Gate cloning"*

Figure 6 and 7. What is being shown? Bacteria or yeast? You should indicate. No scale bars. I think these figures could be supplemental material. If they remain figures there should be quantitative data associated along with statistical tests.

We have updated Figure 6 and 7 with new pictures showing representative plates at the TU level and the multi-gene level cloning respectively. These are bacterial colonies for cloning purposes. The legends have been modified to reflect this change:

"Figure 6: *Representative plates of entry vector and transcription unit level cloning in E. coli."*

"Figure 7: *Representative plates of multigene plasmid cloning in E. coli."*

We think these are essential figures for the main text allowing readers to replicate the protocol and assessing their success. We have added the scale bars and ratios of potential successfully colonies over total number of colonies in the figure legend and text Line. The ratios are below:

Figure 6:

Level	Figure 6	% of positive colonies
Entry vector	A&B	90.35 ± 4.22
Intermediate vector for TU	C&D	17.56 ± 3.32
TU assembly	E&F	65.02 ± 4.99

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These stats are included in the figure legends:

"Figure 6: *Representative plates of entry vector and transcription unit level cloning in E. coli. A & B: Representative plates of successful cloning of a gene into the entry vector pYTK001 under (A) visible light and (B) UV light. Positive colonies are white and negative colonies are green. The ratio of positive colonies over all colonies is: 90.35 ± 4.22 %. C & D: Successful assembly of the intermediate vector for transcription unit level assembly and green/red selection under (C) visible light and (D) UV light. Positive colonies are green. The ratio of positive colonies over all colonies is: 17.56 ± 3.32 %. E & F: Successful assembly of transcription unit from the intermediate vector and green/white screening under (E) visible light and (F) UV light. Positive colonies are white and negative colonies are green. The ratio of positive colonies over all colonies is: 65.02 ± 3.32 %. Data are from three biological replicates."*

"Figure 7: *Representative plates of multigene plasmid cloning in E. coli. Fig A & B: Assembly of the intermediate vector for multigene level assembly and red/green selection under (A) visible light and (B) UV light. Positive colonies are green and negative colonies are red. The ratio of positive colonies over all colonies is 1.83 ± 0.15%. C-F: Successful assembly of multiple TUs from the intermediate vector and green/white selection (C) under visible light and (D) UV light. Positive colonies are white. The ratio of positive colonies over all colonies is 93.77 ± 1.65%. Suboptimal assembly of multigene plasmid under (E) visible light and (F) UV light. The number of positive colonies are negligible. Data are from three biological replicates."*

The stats are also included in the text:

In text Line 487-488, we've added: *"Figure 6A&B shows a representative plate of successful cloning of a part plasmid and provides an example of the green/white screening with 90.35 ± 4.22% total colonies being positive (white)."*

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Figure 8. I fail to see the breakthrough, both A and B appear to be inefficient. The paper of Lee et showed a much higher success rate when applying Cas9 to aid integration. C and D should be followed up with a quantitative measurement to confirm the product. I wouldn't only use colour as an indication. No quantitative data, no statistics.

Thanks for the comments. We agree that the integration efficiency was lower. However, our integration scenario was very different from the one in Lee et al. Some of the differences listed below explain the lower efficiency we observed:

1. Lee et al used different genomic loci. They used *LEU2, HIS3, MET15*, and *TRP1.* We used *ADE2*.
2. Different loci used results in different sgRNA designed. The efficiencies of different sgRNA vary dramatically.
3. While Lee et al used synthetic medium for auxotrophic selection, we used YPD plus antibiotic for selection. Using antibiotics as selection markers leads to far fewer colonies than using auxotrophic markers in our experience.
4. Lee et al used PCR products directly as the helper DNA, we used the linearized multigene plasmid. The PCR products could have higher concentrations than the digested plasmid.
5. The distance between the 5' and 3' homology regions on the genome was very different. While in Lee et al, this distance was only 40 bp, in our case it was 500 bp. This could also lead to decreased efficiency.
6. While in Lee et al, background colonies (without helper DNA) were always present, our background was zero.

For these reasons, we think that their system was completely different than ours and therefore cannot be directly compared.

We agree with the reviewer that quantitative measurements are necessary for beta-caratenoids and lycopenes in Figure 8. Thus we have added absolute quantification of these two compounds with authentic standards using UV-vis spectrophotometer. The data is included in Figure 8:

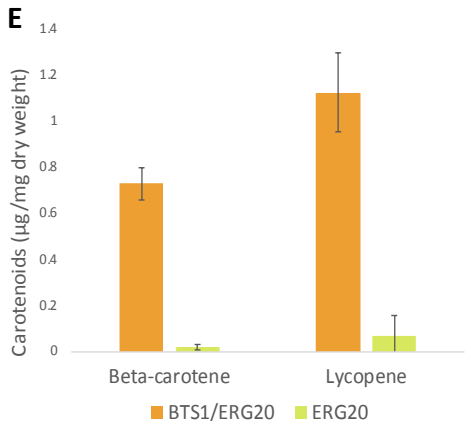


Figure 8E: *quantifying β-carotene and lycopene from yeast extract with four multigene plasmids using a UV-vis spectrometer. The maximal absorbance was recorded at 450 nm and 470 nm for β-carotene and lycopene respectively. The absolute quantification was performed using authentic standards (Figure S3). Fusion of BTS1-ERG20 leads to the production of ~35 fold higher β-carotene and ~16.5 fold higher lycopene compared to ERG20 alone.*

We have added the following descriptions in Line 521-526:

"Upon extraction[48] and quantification of the carotenoids by UV-Vis spectrophotometry, it is seen that fusion of BTS1-ERG20 leads to the production of 0.729 µg/mg β-carotene which is ~35 fold higher than 0.021 µg/mg β-carotene produced by the strain with ERG20 alone. Likewise, the production of lycopene is ~16.5 fold higher in the strain with BTS1-ERG20 (1.126 µg/mg) compared to ERG20 (0.068 µg/mg) alone"

We have also included the absorption spectra of authentic standards in Supplemental Figure S3:

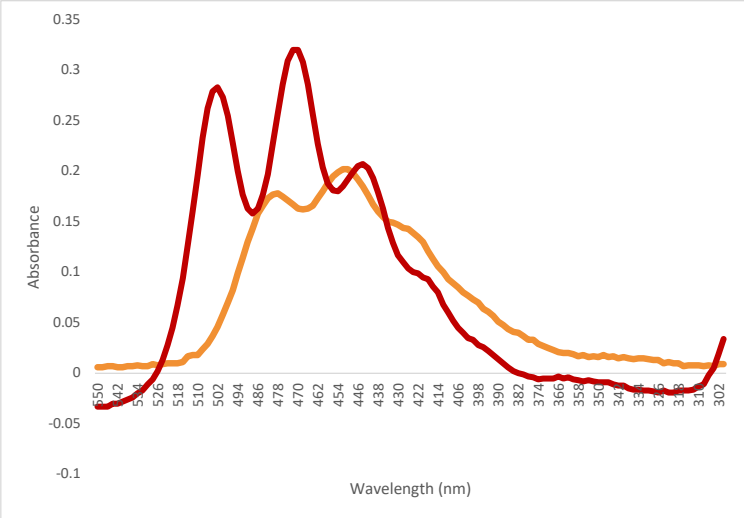


Figure S3: Absorption spectra of *β*-carotene and lycopene standards. *β*-carotene and lycopene standards were dissolved in hexane at 0.7 µg/mL and 0.65 µg/mL respectively. The absorption spectra between 300 nm and 550 nm was recorded using a UV-vis spectrometer. Red: lycopene. Orange: *β*-carotene.

Citations: The authors did not cite any of their own work. This manuscript seems to describe the methods of the Lee et al paper (Ref 15) but none of the authors were part of that manuscript. I'm a bit confused by this situation.

Although we are not the developers of this kit, we are the experienced users of this toolkit. We have been using this toolkit for the past three years to create combinatorial libraries with hundreds of constructs and developed expertise on how to use the toolkit more efficiently. Although our unpublished work will only be submitted in 2021, we feel that our experience in using this kit would be invisible to the community without this JOVE article. The original paper provided a sufficient but not user-friendly protocol in the supplemental information. JOVE

being a video-based journal, provides an excellent platform to explore this protocol in detail for seasoned users and make this protocol more accessible to beginners.

Minor Concerns:
Line 31- define MoClo

Thanks. We have modified the sentence as: *"This modular cloning (MoClo) system uses a hierarchical workflow where different DNA parts…"*

Line 35- Golden Gate is normally not "scar-less" as there are 4bp scars with typical Type IIS enzymes

We agree with the reviewer and reviewer #4 that 4 nt overhang sometime can result in scars, especially using the standard junctions. However, because the junctions flank the functional parts, the overhangs usually won't affect function. In addition, arbitrary 4nt can be chosen within the gene or any other parts, allowing true scar-less assembly. Such examples in this manuscript include the domestication and creating fusion proteins. While Golden Gate cloning allows true scar-less cloning, MoClo does not. We have softened the sentences accordingly:

Line 34-35 in Abstract: "*The Golden Gate cloning strategy is of tremendous advantage because it **allows** scar-less, directional, and modular assembly in a one-pot reaction."*

Line 833-834: *"The MolClo strategy is advantageous over alternative multi-part assembly methods4,6-9 because it allows modular and highly versatile cloning."*

Line 58: "recently"? from 2009

We have changed "*recently*" into "*in the past decade*", and included more citations for Golden Gate Cloning.

Line 58: " the Golden Gate cloning".. perhaps add "technique"

We have made the suggested change.

Line 131: "If multiple undesirable sites are present, site-directed mutagenesis". Perhaps mention that one can easily have the fragment synthesized with the desired mutations.

Thanks. We have made the suggested addition. This has been mentioned by multiple reviewers. We have added it in Line 164: *"Alternatively, a BsmBI- and BsaI- free and codon-optimized part can be synthesized commercially".*

Line 170: jumped from Figure 2B previously to now Figure 6A. Should be in order.

Thanks for the comment. We have revised the manuscript so that the figures are cited in order.

Line 348: 15-17 µg- is that much plasmid necessary?

15-17 µg is indeed a lot of plasmids. The DNA amount depends on the size of the helper DNA and the selective media used. For example, if the helper DNA is a PCR product, then usually 1-5 µg is enough. If plasmids are used, then higher amounts are required because multigene plasmids usually exceed 10 kb. Using antibiotics as selection markers leads to far fewer colonies than using auxotrophic markers in our experience. And the ADE2 selection was based on antibiotic selection. That is why here we have to use at least 15 µg plasmid. If the selection is based on auxotrophic markers, 5~10 µg plasmid is enough.

We have modified the sentence in Line 435: "*5.1.3. Linearize 5-15 µg integrative multigene plasmid with 1 µL NotI-HF enzyme overnight.*"

Line 354: Overnight? This seems long and would lead to colonies that are not independent transformants.

For marker-less integration and growth on an antibiotic containing plate, we have seen overnight recovery results in a greater number of colonies than a standard 3-4 hours recovery, hence the suggestion.

Line 356: If you are plating on CSM dropout plate do you directly add cells that were previously resuspended in YPD? Normally we resuspend cells in water for this situation.

We do that too, we have added the sentence in Line 440: *"Pellet the cells after recovery, discard the supernatant, wash with equal volume of water."* Thank you for pointing that out.

Line 360: Which protocol? There are 3 options for colony PCR

We suggest the NaOH lysis method. However, we had to delete the link per journal requirements. We have cited the paper for the protocol: http://cshprotocols.cshlp.org/content/early/2020/08/06/pdb.prot098152

Line 369: 100 ng is usually sufficient to produce lots of colonies with a single plasmid.

We have made the suggested change in Line 454: "*5.2.1. Transform 100 ng-1 µg pure multigene plasmid into S. cerevisiae competent cells."*

Lines 382-385: The part sequences should be added to the supplementary files.

We have added a supplementary file as Supplementary Table 2.

Table 1 and Table 2: Perhaps give each plasmid a unique name for identification.

Thanks for the comment. We have included unique names in Table 2 for multigene plasmids.

Table 2: Multi-gene Plasmids used in this study.

Name	TU1	TU2	TU3	TU4	Product
B/E-β-carotene	*BTS1/ERG20*	*crtE*	*crtYB*	*crtI*	*β-carotene*
B/E-lycopene	*BTS1/ERG20*	*crtE*	*crtYB^{G247A}*	*crtI*	*Lycopene*
E-β-carotene	*ERG20*	*crtE*	*crtYB*	*crtI*	*β-carotene*
E-lycopene	*ERG20*	*crtE*	*crtYB^{G247A}*	*crtI*	*Lycopene*

Figure 2 and 3. Figure legends much too brief to describe what is being shown.

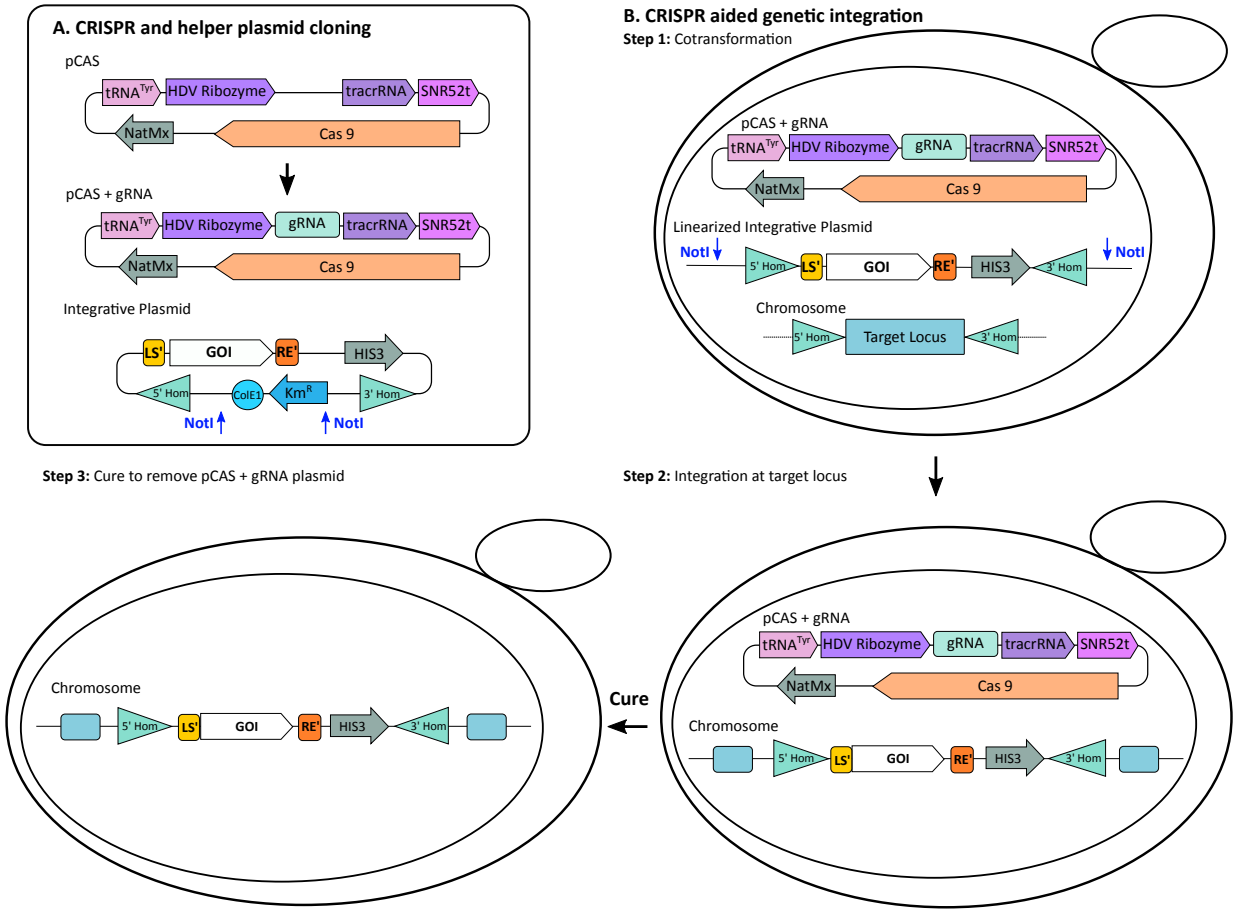
We have added more descriptions to the legends of Figure 2 and 3 as below:

"Figure 2: Primer design and cloning of part plasmids. (A) Primer design for amplifying individual parts, domesticating genes or creating point mutations, and assembling fusion proteins. Primers include BsmBI and BsaI recognition and cut sites, and the MoClo overhang for proper assembly. MoClo overhangs are represented as 1,2,3,4, and 5,6,7,8. Internal primers for domestication or creating fusion protein contains the BsmBI but not the BsaI sites. The overhangs for these are customized internal sequences (NNNN and N'N'N'N' in purple). Terminal "ttt" are included for optimal enzyme digestion. GOI: gene of interest. (B) Cloning amplified parts into the pYTK001 entry vector using BsmBI (or Esp3I). Complementary overhangs lead to the integration of the part and the removal of the BsmBI recognition site. ColE1: E. coli origin of replication; CmR: Chloramphenicol resistant gene."

"Figure 3: Transcription unit assembly. To assemble the TU plasmids, we recommend assembly of an intermediate vector to facilitate combinatorial TU assembly. To assemble the intermediate vector, clone the Con LX (X: one of the five left connectors), the sfGFP dropout, the Con RY (Y: one of the five right connectors), a yeast ORI (origin of replication), and a yeast marker parts into the mRFP1 dropout vector using the BsaI enzyme. The intermediate plasmid is resistant to ampicillin. The BsaI recognition sites are retained for TU plasmid cloning. To clone the TU plasmid, a promoter, a CDS, and a terminator are assembled into the intermediate vector using BsaI. The cloned TU will have BsmBI sites at the ConLX and ConRY regions for the next step multigene assembly. The cloned TU is also resistant to ampicillin."

Figure 5. Label each diagram separately and described in more detail. It is hard to follow what you are trying to show. Do you actually make a construct with a GFP dropout and transform into yeast? If not, why show this?

We have labeled each diagram separately and described in more detail in Figure 5:



"Figure 5: CRISPR integration. (A) CRISPR and helper plasmid cloning. The pCAS plasmid contains the Cas9 endonuclease and components (tRNA promoter, SNR52 terminator, HDV ribozyme and tracrRNA) for optimal expression of a gRNA. Clone the pCAS+gRNA plasmid by assembling the synthetic gRNA with the linearized pCAS using Gibson cloning. (B) CRISPR aided genetic integration. Step 1: Cotransformation: pCAS +gRNA was co-transformed into yeast with the integrative plasmid containing gene of interest (GOI), a yeast selective marker, and 5' and 3' homology region targeting the genomic locus. For optimal integration, linearize the integrative plasmid with NotI. Step 2: Integration at target locus; Growing the transformed yeast on a plate selective for the yeast marker, either antibiotic or auxotrophic. Perform genotyping to confirm the integration. Step 3: cure the pCAS + gRNA plasmid by streaking yeast on a non-selective plate."

Yes, we did make the integrative construct with the GFP dropout and transformed into yeast. But the GFP dropout gene only expressed in *E. coli,* not in yeast because it is under an bacterial promoter. The reason we included the non-functional GFP dropout is to comply with the MoClo overhangs. The GFP can certainly be omitted or substituted by gene(s) of interest for integration. Since our goal here was just to disrupt *ADE2*, we chose to use the non-functional GFP here. We have changed "*GFP dropout*" into "*GOI*" (gene of interest) in the Figure 5 above.

Supplementary information for

Rapid Assembly of Multi-gene Constructs Using Molular Golden Gate Cloning

Minakshi Mukherjee[1,*], Emily Caroll[1,*], Zhen Q. Wang[1, †]

1. Department of Biological Sciences, University at Buffalo, State University of New York, Buffalo, NY14260, USA

Table of Contents:

Table S1: List of primers

Primers	Sequence (5'-3')	Description
BTS1 F	tttcgtctcgtcggggtctcgtatggaggccaagatagatgag	Forward primer to amplify *BTS1* from the yeast genome
BTS1-GGGS R	tttcgtctctccatagaaccaccacccaattcggataagtggtc	Reverse primer with a GGGS linker sequence to amplify *BTS1* from the yeast genome
ERG20 F	tttcgtctcgatggcttcagaaaaagaaattaggag	Forward primer to amplify *ERG20* from the yeast genome
ERG20 R	tttcgtctctggtcggtctccggatctatttgcttctcttgtaaactttgttc	Reverse primer to amplify *ERG20* from the yeast genome
crtE F	tttcgtctcgtcggggtctcgtatggattacgcgaacatcctc	Forward primer to amplify *crtE* from pLM494 plasmid
crtE R	tttcgtctctggtcggtctccggattcacagagggatatcggctag	Reverse primer to amplify *crtE* from pLM494 plasmid
crtI F	tttcgtctcgtcggggtctcgtatgggaaaagaacaagatcagg	Forward primer to amplify *crtI* from pLM494 plasmid
crtI R	tttcgtctctggtcggtctccggattcagaaagcaagaacaccaac	Reverse primer to amplify *crtI* from pLM494 plasmid
crtYB F	tttcgtctcgtcggggtctcgtatgacggctctcgcatattac	Forward primer to amplify *crtYB* from pLM494 plasmid
crtYB R	tttcgtctctggtcggtctccggattcactgcccttcccatc	Reverse primer to amplify *crtYB* from pLM494 plasmid
crtYB mut F	tttcgtctctacgctttctttgtcattcaaac	Forward primer to amplify second half of *crtYB* from pLM494 in order to introduce G247A mutation
crtYB mut R	tttcgtctcagcgtacttttcatatggaacatc	Reverse primer to amplify the first half of *crtYB* from pLM494 to introduce the G247A mutation
pENO2 F	tttcgtctcgtcggggtctcgaacggtgtcgacgctgcg	Forward primer to amplify the *ENO2* promoter sequence from the yeast genome
pENO2 R	tttcgtctcgggtcggtctcccatatattattgtatgttatagtattagttgcttgg	Reverse primer to amplify the *ENO2* promoter sequence from the yeast genome
pPDC1 F	tttcgtctcgtcggggtctcgaacgcatgcgactgggtgag	Forward primer to amplify the *PDC1* promoter sequence from the yeast genome
pPDC1 R	tttcgtctcgggtcggtctcccatatttgattgatttgactgtgttatttgc	Reverse primer to amplify the *PDC1* promoter sequence from the yeast genome
pPYK1 F	tttcgtctcgtcggggtctcgaacggaaagtttttccggcaagc	Forward primer to amplify the *PYK1* promoter sequence from the yeast genome
pPYK1 R	tttcgtctcgggtcggtctcccatatgtgatgatgtttatttgttttgattg	Reverse primer to amplify the *PYK1* promoter sequence from the yeast genome
pTIP1 F	tttcgtctcgtcggggtctcgaacgataagcttgatatcgaattcctgc	Forward primer to amplify the *TIP1* promoter sequence from the yeast genome

pTIP1 R	tttcgtctcgggtcggtctcccatattttattttatttag cagagggtatagttatg	Reverse primer to amplify the *TIP1* promoter sequence from the yeast genome
tTDH2 F	tttcgtctcgtcggggtctcgatcctaaatttaactcct taagttactttaatg	Forward primer to amplify the *TDH2* terminator sequence from the yeast genome
tTDH2 R	tttcgtctcgggtcggtctcccagcgcgaaaagccaa ttagtg	Reverse primer to amplify the *TDH2* terminator sequence from the yeast genome
tHSP26 F	tttcgtctcgtcggggtctcgatccagtgacctggctct atagtg	Forward primer to amplify the *HSP26* terminator sequence from the yeast genome
tHSP26 R	tttcgtctcgggtcggtctcccagcacgaggttagatt ccttcgttaaaac	Reverse primer to amplify the *HSP26* terminator sequence from the yeast genome
tADH2 F	tttcgtctcgtcggggtctcgatccgcggatctcttatg tctttacg	Forward primer to amplify the *ADH2* terminator sequence from the yeast genome
tADH2 R	tttcgtctcgggtcggtctcccagcatgagaaatatcg agggatacgattc	Reverse primer to amplify the *ADH2* terminator sequence from the yeast genome
tACS2 F	tttcgtctcgtcggggtctcgatccaatgagataaaat ttcgc	Forward primer to amplify the *ACS2* terminator sequence from the yeast genome
tACS2 R	tttcgtctcgggtcggtctcccagccactaagtgcata aagtctttg	Reverse primer to amplify the *ACS2* terminator sequence from the yeast genome
ADE2 5' hom F	tttcgtctcgtcggggtctcacaatgaaacttcatgct cgaaaaagatc	Forward primer to amplify a 500 bp sequence that serves as the 5' homology arm during integration at the *ADE2* locus
ADE2 5' hom R	tttcgtctcgggtcggtctcaagggtcattactatcatt actaaatataccaactgttctagaatccatac	Reverse primer to amplify a 500 bp sequence plus 6 stop codons that would serve as the 5' homology arm during integration at the *ADE2* locus and stop *ADE2* expression
ADE2 3'hom F	tttcgtctcgtcggggtctcagagtgctttggaagtact gaaggatc	Forward primer to amplify a 500 bp sequence that would serve as the 3' homology arm during integration at the *ADE2* locus
ADE2 3' hom R	tttcgtctcgggtcggtctcatcggctttcgcaagtttc tagctc	Forward primer to amplify 500 bp sequence that would serve as the 3' homology arm during integration at the *ADE2* locus

All the primers are designed for cloning the parts into the entry vector (pYTK001)

Table S2: List of sequences for part plasmids

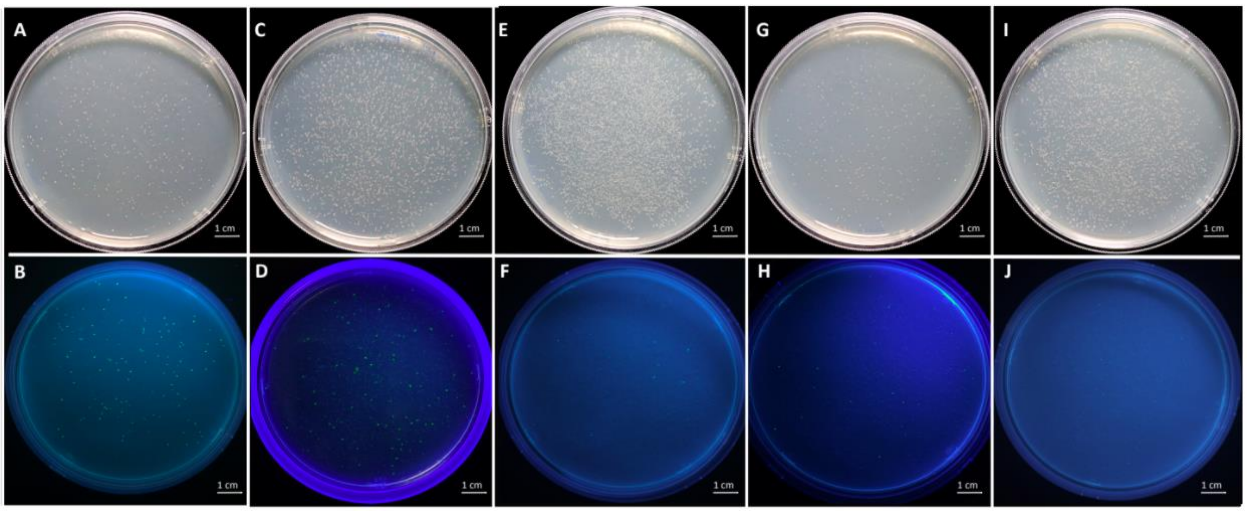
Name	Sequence
pENO2 (promoter)	GTGTCGACGCTGCGGGTATAGAAAGGGTTCTTTACTCTATAGTACCTCCTCGCTCAG CATCTGCTTCTTCCCAAAGATGAACGCGGCGTTATGTCACTAACGACGTGCACCAACT TGCGGAAAGTGGAATCCCGTTCCAAAACTGGCATCCACTAATTGATACATCTACACA CCGCACGCCTTTTTTCTGAAGCCCACTTTCGTGGACTTTGCCATATGCAAAATTCATG AAGTGTGATACCAAGTCAGCATACACCTCACTAGGGTAGTTTCTTTGGTTGTATTGAT CATTTGGTTCATCGTGGTTCATTAATTTTTTTTCTCCATTGCTTTCTGGCTTTGATCTTA CTATCATTTGGATTTTTGTCGAAGGTTGTAGAATTGTATGTGACAAGTGGCACCAAG CATATATAAAAAAAAAAAGCATTATCTTCCTACCAGAGTTGATTGTTAAAAACGTATT TATAGCAAACGCAATTGTAATTAATTCTTATTTTGTATCTTTTCTTCCCTTGTCTCAATC TTTTATTTTTATTTTATTTTTCTTTTCTTAGTTTCTTTCATAACACCAAGCAACTAATACT ATAACATACAATAATA
pTIP1 (promoter)	ATAAGCTTGATATCGAATTCCTGCAGCCCCCAGTATAACTTCGTATAATGTACATTAT ACGAAGTTATTATTAGCGGCGCCGGGAAATCCAGCATATTCTCGCGGCCCTGAGCAG TAGGTGTCTCGGGCAGTCAATATTCCCAAAAAGAGCATCAGACGATCTGGTTATGGT TTTTCTTGACTATAACCTTAATTATGAGACTAATGTCTTCGGGAGGTCCCTTTTCCGAT TTTCCGACTCTTTTCCGTTGAAGAATGTACTTGTGGTTTTGAATCCTACGGCAGTTATT GCGGCGGTTTGGCCCTTTCTTTCAAAGATTGTGATGGAAATAATTGATTGTTCCGGG AAATGTGTCTTATTTTCTAAAAGCATCTTTTTTTCTCTCCAATTCTTCGAGCTATTTCCA GTAAAGGAAAAAAAAGGTTTGCTGTAAGGGTGAATATGTCTCCAACCTCTTTGAGGT ACTGCGTTGCTTCATTCACCATTTAATATAAATAGTACATTGGCAGCCCTCTTTCAAAC GTCAATTATTCTCGCTTGCCTAACTTTGTTCGGACCGAAATTATAAAGGCATTCAATC AGTAACAATAATTGCTATTGCATAACTATACCCTCTGCTAAATAAAATAAAA
pPDC1 (promoter)	CATGCGACTGGGTGAGCATATGTTCCGCTGATGTGATGTGCAAGATAAACAAGCAA GGCAGAAACTAACTTCTTCTTCATGTAATAAACACACCCCGCGTTTATTTACCTATCTC TAAACTTCAACACCTTATATCATAACTAATATTTCTTGAGATAAGCACACTGCACCCAT ACCTTCCTTAAAAACGTAGCTTCCAGTTTTTGGTGGTTCCGGCTTCCTTCCCGATTCCG CCCGCTAAACGCATATTTTTGTTGCCTGGTGGCATTTGCAAAATGCATAACCTATGCA TTTAAAAGATTATGTATGCTCTTCTGACTTTTCGTGTGATGAGGCTCGTGGAAAAAAT GAATAATTTATGAATTTGAGAACAATTTTGTGTTGTTACGGTATTTTACTATGGAATA ATCAATCAATTGAGGATTTTATGCAAATATCGTTTGAATATTTTTCCGACCCTTTGAGT ACTTTTCTTCATAATTGCATAATATTGTCCGCTGCCCCTTTTTCTGTTAGACGGTGTCT TGATCTACTTGCTATCGTTCAACACCACCTTATTTTCTAACTATTTTTTTTTTAGCTCAT TTGAATCAGCTTATGGTGATGGCACATTTTTGCATAAACCTAGCTGTCCTCGTTGAAC ATAGGAAAAAAAAATATATAAACAAGGCTCTTTCACTCTCCTTGCAATCAGATTTGG GTTTGTTCCCTTTATTTTCATATTTCTTGTCATATTCCTTTCTCAATTATTATTTTCTACT CATAACCTCACGCAAAATAACACAGTCAAATCAATCAAA
pPYK1 (promoter)	GAAAGTTTTTCCGGCAAGCTAAATGGAAAAAGGAAAGATTATTGAAAGAGAAAGAA AGAAAAAAAAAAAATGTACACCCAGACATCGGGCTTCCACAATTTCGGCTCTATTGT TTTCCATCTCTCGCAACGGCGGGATTCCTCTATGGCGTGTGATGTCTGTATCTGTTAC TTAATCCAGAAACTGGCACTTGACCCAACTCTGCCACGTGGGTCGTTTTGCCATCGAC AGATTGGGAGATTTTCATAGTAGAATTCAGCATGATAGCTACGTAAATGTGTTCCGC

	ACCGTCACAAAGTGTTTTCTACTGTTCTTTCTTCTTTCGTTCATTCAGTTGAGTTGAGT GAGTGCTTTGTTCAATGGATCTTAGCTAAAATGCATATTTTTTCTCTTGGTAAATGAA TGCTTGTGATGTCTTCCAAGTGATTTCCTTTCCTTCCCATATGATGCTAGGTACCTTTA GTGTCTTCCTAAAAAAAAAAAAAGGCTCGCCATCAAAACGATATTCGTTGGCTTTTTT TTCTGAATTATAAATACTCTTTGGTAACTTTTCATTTCCAAGAACCTCTTTTTTCCAGTT ATATCATGGTCCCCTTTCAAAGTTATTCTCTACTCTTTTTCATATTCATTCTTTTTCATCC TTTGGTTTTTTATTCTTAACTTGTTTATTATTCTCTCTTGTTTCTATTTACAAGACACCA ATCAAAACAAATAAAACATCATCACA
ERG20 (CDS)	ATGGCTTCAGAAAAAGAAATTAGGAGAGAGAGATTCTTGAACGTTTTCCCTAAATTA GTAGAGGAATTGAACGCATCGCTTTTGGCTTACGGTATGCCTAAGGAAGCATGTGAC TGGTATGCCCACTCATTGAACTACAACACTCCAGGCGGTAAGCTAAATAGAGGTTTG TCCGTTGTGGACACGTATGCTATTCTCTCCAACAAGACCGTTGAACAATTGGGGCAA GAAGAATACGAAAAGGTTGCCATTCTAGGTTGGTGCATTGAGTTGTTGCAGGCTTAC TTCTTGGTCGCCGATGATATGATGGACAAGTCCATTACCAGAAGAGGCCAACCATGT TGGTACAAGGTTCCTGAAGTTGGGGAAATTGCCATCAATGACGCATTCATGTTAGAG GCTGCTATCTACAAGCTTTTGAAATCTCACTTCAGAAACGAAAAATACTACATAGATA TCACCGAATTGTTCCATGAGGTCACCTTCCAAACCGAATTGGGCCAATTGATGGACTT AATCACTGCACCTGAAGACAAAGTCGACTTGAGTAAGTTCTCCCTAAAGAAGCACTC CTTCATAGTTACTTTCAAGACTGCTTACTATTCTTTCTACTTGCCTGTCGCATTGGCCA TGTACGTTGCCGGTATCACGGATGAAAAGGATTTGAAACAAGCCAGAGATGTCTTG ATTCCATTGGGTGAATACTTCCAAATTCAAGATGACTACTTAGACTGCTTCGGTACCC CAGAACAGATCGGTAAGATCGGTACAGATATCCAAGATAACAAATGTTCTTGGGTAA TCAACAAGGCATTGGAACTTGCTTCCGCAGAACAAAGAAAGACTTTAGACGAAAATT ACGGTAAGAAGGACTCAGTCGCAGAAGCCAAATGCAAAAAGATTTTCAATGACTTG AAAATTGAACAGCTATACCACGAATATGAAGAGTCTATTGCCAAGGATTTGAAGGCC AAAATTTCTCAGGTCGATGAGTCTCGTGGCTTCAAAGCTGATGTCTTAACTGCGTTCT TGAACAAAGTTTACAAGAGAAGCAAATAG
BTS1- ERG20 (CDS)	ATGGAGGCCAAGATAGATGAGCTGATCAATAATGATCCTGTTTGGTCCAGCCAAAAT GAAAGCTTGATTTCAAAACCTTATAATCACATCCTTTTGAAACCTGGCAAGAACTTTA GACTAAATTTAATAGTTCAAATTAACAGAGTTATGAATTTGCCCAAAGACCAGCTGG CCATAGTTTCGCAAATTGTTGAGCTCTTGCATAATTCCAGCCTTTTAATCGACGATAT AGAAGATAATGCTCCCTTGAGAAGGGGACAGACCACTTCTCACTTAATCTTCGGTGT ACCCTCCACTATAAACACCGCAAATTATATGTATTTCAGAGCCATGCAACTTGTATCG CAGCTAACCACAAAAGAGCCTTTGTATCATAATTTGATTACGATTTTCAACGAAGAAT TGATCAATCTACATAGGGGACAAGGCTTGGATATATACTGGAGAGACTTTCTGCCTG AAATCATACCTACTCAGGAGATGTATTTGAATATGGTTATGAATAAAACAGGCGGCC TTTTCAGATTAACGTTGAGACTCATGGAAGCGCTGTCTCCTTCCTCACACCACGGCCA TTCGTTGGTTCCTTTCATAAATCTTCTGGGTATTATTTATCAGATTAGAGATGATTACT TGAATTTGAAAGATTTCCAAATGTCCAGCGAAAAAGGCTTTGCTGAGGACATTACAG AGGGGAAGTTATCTTTTCCCATCGTCCACGCCCTTAACTTCACTAAAACGAAAGGTCA AACTGAGCAACACAATGAAATTCTAAGAATTCTCCTGTTGAGGACAAGTGATAAAGA TATAAAACTAAAGCTGATTCAAATACTGGAATTCGACACCAATTCATTGGCCTACACC AAAAATTTTATTAATCAATTAGTGAATATGATAAAAAATGATAATGAAAATAAGTATT TACCTGATTTGGCTTCGCATTCCGACACCGCCACCAATTTACATGACGAATTGTTATA

	TATAATAGACCACTTATCCGAATTGggtggtggttctATGGCTTCAGAAAAAGAAATTAG GAGAGAGAGATTCTTGAACGTTTTCCCTAAATTAGTAGAGGAATTGAACGCATCGCT TTTGGCTTACGGTATGCCTAAGGAAGCATGTGACTGGTATGCCCACTCATTGAACTA CAACACTCCAGGCGGTAAGCTAAATAGAGGTTTGTCCGTTGTGGACACGTATGCTAT TCTCTCCAACAAGACCGTTGAACAATTGGGGCAAGAAGAATACGAAAAGGTTGCCA TTCTAGGTTGGTGCATTGAGTTGTTGCAGGCTTACTTCTTGGTCGCCGATGATATGAT GGACAAGTCCATTACCAGAAGAGGCCAACCATGTTGGTACAAGGTTCCTGAAGTTG GGGAAATTGCCATCAATGACGCATTCATGTTAGAGGCTGCTATCTACAAGCTTTTGA AATCTCACTTCAGAAACGAAAAATACTACATAGATATCACCGAATTGTTCCATGAGGT CACCTTCCAAACCGAATTGGGCCAATTGATGGACTTAATCACTGCACCTGAAGACAA AGTCGACTTGAGTAAGTTCTCCCTAAAGAAGCACTCCTTCATAGTTACTTTCAAGACT GCTTACTATTCTTTCTACTTGCCTGTCGCATTGGCCATGTACGTTGCCGGTATCACGG ATGAAAAGGATTTGAAACAAGCCAGAGATGTCTTGATTCCATTGGGTGAATACTTCC AAATTCAAGATGACTACTTAGACTGCTTCGGTACCCCAGAACAGATCGGTAAGATCG GTACAGATATCCAAGATAACAAATGTTCTTGGGTAATCAACAAGGCATTGGAACTTG CTTCCGCAGAACAAAGAAAGACTTTAGACGAAAATTACGGTAAGAAGGACTCAGTC GCAGAAGCCAAATGCAAAAAGATTTTCAATGACTTGAAAATTGAACAGCTATACCAC GAATATGAAGAGTCTATTGCCAAGGATTTGAAGGCCAAAATTTCTCAGGTCGATGAG TCTCGTGGCTTCAAAGCTGATGTCTTAACTGCGTTCTTGAACAAAGTTTACAAGAGAA GCAAATAG
crtE (CDS)	ATGGATTACGCGAACATCCTCACAGCAATTCCACTCGAGTTTACTCCTCAGGATGATA TCGTGCTCCTTGAACCGTATCACTACCTAGGAAAGAACCCTGGAAAAGAAATTCGAT CACAACTCATCGAGGCTTTCAACTATTGGTTGGATGTCAAGAAGGAGGATCTCGAGG TCATCCAGAACGTTGTTGGCATGCTACATACCGCTAGCTTATTAATGGACGATGTGG AGGATTCATCGGTCCTCAGGCGTGGGTCGCCTGTGGCCCATCTAATTTACGGGATTC CGCAGACAATAAACACTGCAAACTACGTCTACTTTCTGGCTTATCAAGAGATCTTCAA GCTTCGCCCAACACCGATACCCATGCCTGTAATTCCTCCTTCATCTGCTTCGCTTCAAT CATCAGTCTCCTCTGCATCCTCCTCCTCCTCGGCCTCGTCTGAAAACGGGGGCACGTC AACTCCTAATTCGCAGATTCCGTTCTCGAAAGATACGTATCTTGATAAAGTGATCACA GACGAGATACTTTCCCTCCATAGAGGGCAGGGCCTGGAGCTATTCTGGAGAGATAG TCTGACGTGTCCTAGCGAAGAGGAATATGTGAAAATGGTTCTTGGAAAGACGGGAG GTTTGTTCCGTATAGCGGTCAGATTGATGATGGCAAAGTCAGAATGTGACATAGACT TTGTCCAGCTTGTCAACTTGATCTCAATATACTTCCAGATCAGGGATGACTATATGAA CCTTCAGTCTTCTGAGTATGCCCATAATAAGAATTTTGCAGAGGACCTCACAGAAGG GAAATTCAGTTTTCCCACTATCCACTCGATTCATGCCAACCCCTCATCGAGACTCGTC ATCAATACGTTGCAGAAGAAATCGACCTCTCCTGAGATCCTTCACCACTGTGTAAACT ACATGCGCACAGAAACCCACTCATTCGAATATACTCAGGAAGTCCTCAACACCTTGTC AGGTGCACTCGAGAGAGAACTAGGAAGGCTTCAAGGAGAGTTCGCAGAAGCTAACT CAAGGATGGATCTTGGTGACGTAGATTCGGAAGGAAGAACGGGGAAGAACGTCAA ATTGGAAGCGATCCTGAAAAAGCTAGCCGATATCCCTCTGTGA
crtI (CDS)	ATGGGAAAAGAACAAGATCAGGATAAACCCACAGCTATCATCGTGGGATGTGGTAT CGGTGGAATCGCCACTGCCGCTCGTCTTGCTAAAGAAGGTTTCCAGGTCACGGTGTT CGAGAAGAACGACTACTCCGGAGGTCGATGCTCTTTAATCGAGCGAGATGGTTATC GATTCGATCAGGGGCCCAGTTTGCTGCTCTTGCCAGATCTCTTCAAGCAGACATTCG

	AAGATTTGGGGGAGAAGATGGAAGATTGGGTCGATCTCATCAAGTGTGAACCCAAC TATGTTTGCCACTTCCACGATGAAGAGACTTTCACTTTTTCAACCGACATGGCGTTGC TCAAGCGGGAAGTCGAGCGTTTTGAAGGCAAAGATGGATTTGATCGGTTCTTGTCGT TTATCCAAGAAGCCCACAGACATTACGAGCTTGCTGTCGTTCACGTCCTGCAGAAGA ACTTCCCTGGCTTCGCAGCATTCTTACGGCTACAGTTCATTGGCCAAATCCTGGCTCT TCACCCCTTCGAGTCTATCTGGACAAGAGTTTGTCGATATTTCAAGACCGACAGATTA CGAAGAGTCTTCTCGTTTGCAGTGATGTACATGGGTCAAAGCCCATACAGTGCGCCC GGAACATATTCCTTGCTCCAATACACCGAATTGACCGAGGGCATCTGGTATCCGAGA GGAGGCTTTTGGCAGGTTCCTAATACTCTTCTTCAGATCGTCAAGCGCAACAATCCCT CAGCCAAGTTCAATTTCAACGCTCCAGTTTCCCAGGTTCTTCTCTCTCCTGCCAAGGA CCGAGCGACTGGTGTTCGACTTGAATCCGGCGAGGAACATCACGCCGATGTTGTGA TTGTCAATGCTGACCTCGTTTACGCCTCCGAGCACTTGATTCCTGACGATGCCAGAAA CAAGATTGGCCAACTGGGTGAAGTCAAGAGAAGTTGGTGGGCTGACTTAGTTGGTG GAAAGAAGCTCAAGGGAAGTTGCAGTAGTTTGAGCTTCTACTGGAGCATGGACCGA ATCGTGGACGGTCTGGGCGGACACAATATCTTCTTGGCCGAGGACTTCAAGGGATC ATTCGACACAATCTTCGAGGAGTTGGGACTCCCAGCCGATCCTTCCTTTTACGTGAAC GTTCCCTCGCGAATCGATCCTTCTGCCGCTCCCGAAGGCAAAGATGCTATCGTCATTC TTGTGCCGTGTGGCCATATCGACGCTTCGAACCCTCAAGATTACAACAAGCTTGTTGC TCGGGCAAGGAAGTTTGTGATCCAAACGCTTTCCGCCAAGCTTGGACTTCCCGACTT TGAAAAAATGATTGTGGCAGAGAAGGTTCACGATGCTCCCTCTTGGGAGAAAGAAT TTAACCTCAAGGACGGAAGCATCTTGGGACTGGCTCACAACTTTATGCAAGTTCTTG GTTTCAGGCCGAGCACCAGACATCCCAAGTATGACAAGTTGTTCTTTGTCGGGGCTT CGACTCATCCCGGAACTGGGGTTCCCATCGTCTTGGCTGGAGCCAAGTTAACTGCCA ACCAAGTTCTCGAATCCTTTGACCGATCCCCAGCTCCAGATCCCAATATGTCACTCTC CGTACCATATGGAAAACCTCTCAAATCAAATGGAACGGGTATCGATTCTCAGGTCCA GCTGAAGTTCATGGATTTGGAGAGATGGGTATACCTTTTGGTGTTGTTGATTGGGGC CGTGATCGCTCGATCCGTTGGTGTTCTTGCTTTCTGA
crtYB (CDS)	ATGACGGCTCTCGCATATTACCAGATCCATCTGATCTATACTCTCCCAATTCTTGGTCT TCTCGGCCTGCTCACTTCCCCGATTTTGACAAAATTTGACATCTACAAAATATCGATCC TCGTATTTATTGCGTTTAGTGCAACCACACCATGGGACTCATGGATCATCAGAAATG GCGCATGGACATATCCATCAGCGGAGAGTGGCCAAGGCGTGTTTGGAACGTTTCTA GATGTTCCATATGAAGAGTACGCTTTCTTTGTCATTCAAACCGTAATCACCGGCTTGG TCTACGTCTTGGCAACTAGGCACCTTCTCCCATCTCTCGCGCTTCCCAAGACTAGATC GTCCGCCCTTTCTCTCGCGCTCAAGGCGCTCATCCCTCTGCCCATTATCTACCTATTTA CCGCTCACCCCAGCCCATCGCCCGACCCGCTCGTGACAGATCACTACTTCTACATGCG GGCACTCTCCTTACTCATCACCCCACCTACCATGCTCTTGGCAGCATTATCAGGCGAA TATGCTTTCGATTGGAAAAGTGGCCGAGCAAAGTCAACTATTGCAACAATCATGATC CCGACGGTGTATCTGATTTGGGTAGATTATGTTGCTGTCGGTCAAGACTCTTGGTCG ATCAACGATGAGAAGATTGTAGGGTGGAGGCTTGGAGGTGTACTACCCATTGAGGA AGCTATGTTCTTCTTACTGACGAATCTAATGATTGTTCTGGGTCTGTCTGCCTGCGAT CATACTCAGGCCCTATACCTGCTACACGGTCGAACTATTTATGGCAACAAAAAGATG CCATCTTCATTTCCCCTCATTACACCGCCTGTGCTCTCCCTGTTTTTTAGCAGCCGACC ATACTCTTCTCAGCCAAAACGTGACTTGGAACTGGCAGTCAAGTTGTTGGAGAAAAA GAGCCGGAGCTTTTTTGTTGCCTCGGCTGGATTTCCTAGCGAAGTTAGGGAGAGGCT

	GGTTGGACTATACGCATTCTGCCGGGTGACTGATGATCTTATCGACTCTCCTGAAGT ATCTTCCAACCCGCATGCCACAATTGACATGGTATCCGATTTTCTTACCCTACTATTTG GGCCCCCGCTACACCCTTCGCAACCTGACAAGATCCTTTCTTCGCCTTTACTTCCTCCT TCGCACCCTTCCCGACCCACGGGAATGTATCCCCTCCCGCCTCCTCCTTCGCTCTCGCC TGCCGAGCTCGTTCAATTCCTTACCGAAAGGGTTCCCGTTCAATACCATTTCGCCTTC AGGTTGCTCGCTAAGTTGCAAGGGCTGATCCCTCGATACCCACTCGACGAACTCCTT AGAGGATACACCACTGATCTTATCTTTCCCTTATCGACAGAGGCAGTCCAGGCTCGG AAGACGCCTATCGAGACAACAGCTGACTTGCTGGACTATGGTCTATGTGTAGCAGGC TCAGTCGCCGAGCTATTGGTCTATGTCTCTTGGGCAAGTGCACCAAGTCAGGTCCCT GCCACCATAGAAGAAAGAGAAGCTGTGTTAGTGGCAAGCCGAGAGATGGGAACTG CCCTTCAGTTGGTGAACATTGCTAGGGACATTAAAGGGGACGCAACAGAAGGGAGA TTTTACCTACCACTCTCATTCTTTGGTCTTCGGGATGAATCAAAGCTTGCGATCCCGAC TGATTGGACGGAACCTCGGCCTCAAGATTTCGACAAACTCCTCAGTCTATCTCCTTCG TCCACATTACCATCTTCAAACGCCTCAGAAAGCTTCCGGTTCGAATGGAAGACGTACT CGCTTCCATTAGTCGCCTACGCAGAGGATCTTGCCAAACATTCTTATAAGGGAATTG ACCGACTTCCTACCGAGGTTCAAGCGGGAATGCGAGCGGCTTGCGCGAGCTACCTA CTGATCGGCCGAGAGATCAAAGTCGTTTGGAAAGGAGATGTCGGAGAGAGAAGGA CAGTTGCCGGATGGAGGAGAGTACGGAAAGTCTTGAGTGTGGTCATGAGCGGATG GGAAGGGCAGTGA
tTDH2 (terminator)	TAAATTTAACTCCTTAAGTTACTTTAATGATTTAGTTTTTATTATTAATAATTCATGCTC ATGACATCTCATATACACGTTTATAAAACTTAAATAGATTGAAAATGTATTAAAGATT CCTCAGGGATTCGATTTTTTTGGAAGTTTTTGTTTTTTTTTCCTTGAGATGCTGTAGTA TTTGGGAACAATTATACAATCGAAAGATATATGCTTACATTCGACCGTTTTAGCCGTG ATCATTATCCTATAGTAACATAACCTGAAGCATAACTGACACTACTATCATCAATACTT GTCACATGAGAACTCTGTGAATAATTAGGCCACTGAAATTTGATGCCTGAAGGACCG GCATCACGGATTTTCGATAAAGCACTTAGTATCACACTAATTGGCTTTTCGC
tHSP26 (terminator)	AGTGACCTGGCTCTATAGTGTTGTCCCTCTCGCGAGGACCATTGTTGCTTGCATATGG GCTTGAAACATATGGTCATCACATCTGAGCGATTTTACCTCTTAGAATTAGTTTAGAT ATATATGAGTTGATGAATAAATAGTTATAAAAACTTGCTTTGGCTTCGATATATGACC GTTATTTTTGACTAAGTTTTAACGAAGGAATCTAACCTCGT
tADH2 (terminator)	GCGGATCTCTTATGTCTTTACGATTTATAGTTTTCATTATCAAGTATGCCTATATTAGT ATATAGCATCTTTAGATGACAGTGTTCGAAGTTTCACGAATAAAAGATAATATTCTAC TTTTTGCTCCCACCGCGTTTGCTAGCACGAGTGAACACCATCCCTCGCCTGTGAGTTG TACCCATTCCTCTAAACTGTAGACATGGTAGCTTCAGCAGTGTTCGTTATGTACGGCA TCCTCCAACAAACAGTCGGTTATAGTTTGTCCTGCTCCTCTGAATCGTATCCCTCGATA TTTCTCAT
tACS2 (terminator)	AATGAGATAAAATTTCGCTCCTTTTCTGTTTTCTATTTTCTATTTTCCCAACTTTTGCTC TATTCAGTTATAAATTACTATTTATCCATCAGTTAAAAAACAAGATCTTTTACTGGTCA GCTAGGAAAGCGAAAATACAAAGACTTTATGCACTTAGTG



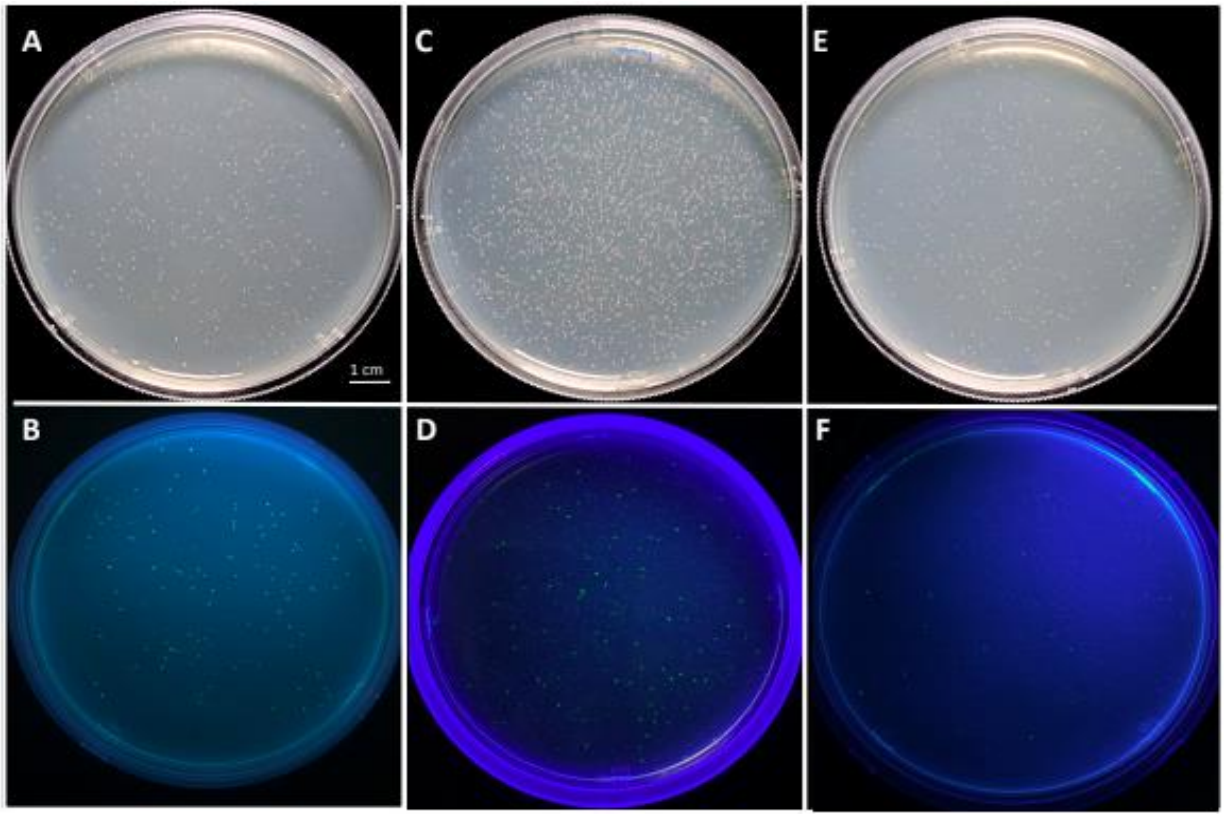
Condition 1	Condition 2	Condition 3	Condition 4	Condition 5
$\left.\begin{matrix}37\ °C\ -2\ min\\25\ °C\ -5\ min\end{matrix}\right]$ 30X	$\left.\begin{matrix}37\ °C\ -2\ min\\25\ °C\ -5\ min\end{matrix}\right]$ 30X	$\left.\begin{matrix}37\ °C\ -2\ min\\16\ °C\ -5\ min\end{matrix}\right]$ 30X	$\left.\begin{matrix}37\ °C\ -2\ min\\16\ °C\ -5\ min\end{matrix}\right]$ 30X	$\left.\begin{matrix}37\ °C\ -5\ min\\16\ °C\ -5\ min\end{matrix}\right]$ 30X
37 °C – 10 min	50 °C – 10 min	37 °C – 10 min	50 °C – 10 min	50 °C – 10 min
80 °C – 10 min	80 °C – 10 min	80 °C – 10 min	80 °C – 10 min	80 °C – 10 min
4 °C – ∞	4 °C – ∞	4 °C – ∞	4 °C – ∞	4 °C – ∞

Figure S1: Optimizing conditions of Golden Gate cloning for a 4-piece assembly: Different temperatures were tested for ligation by T4 ligase (25 °C and 16 °C) and final digestion by BsaI-v2 (37 °C and 50 °C). Different time durations (2 min vs 5 min) were tested for the initial digestion step. One representative plate from each cycle was shown in the figure. White colonies contained potentially correct constructs. The first panel was taken under visible light and the second panel was taken under the UV light.

Table S3: Results obtained from different cycle conditions:

Condition used	**Approximate percentage of white (potentially correct) colonies***
Condition 1	$3.56 \pm 5.2\%$
Condition 2	$6.28 \pm 3.7\%$
Condition 3	$4.12 \pm 3.9\%$
Condition 4	$97.6 \pm 2.4\%$
Condition 5	$99.5 \pm 1.3\%$

* $Percentage\ of\ white\ colonies = \frac{Number\ of\ white\ colonies}{Total\ number\ of\ colonies} \times 100$ (Calculated from 3 biological replicates)



Number of parts assembled	No. of green colonies	No. of total colonies	percentage of potentially correct (green) colonies
6	80	300	26.6%
7	45	2658	1.7%
8	15	2275	0.65%

Figure S2: Decreasing efficiency of Golden Gate Assembly with increasing number of parts: Golden Gate assembly of 6 parts (A and B), 7 parts (C and D), and 8 parts (E and F) showed a progressive decrease in the ratio of potentially correct green colonies. The first panel shows plates under visible light and the second panel shows plats under the UV light.

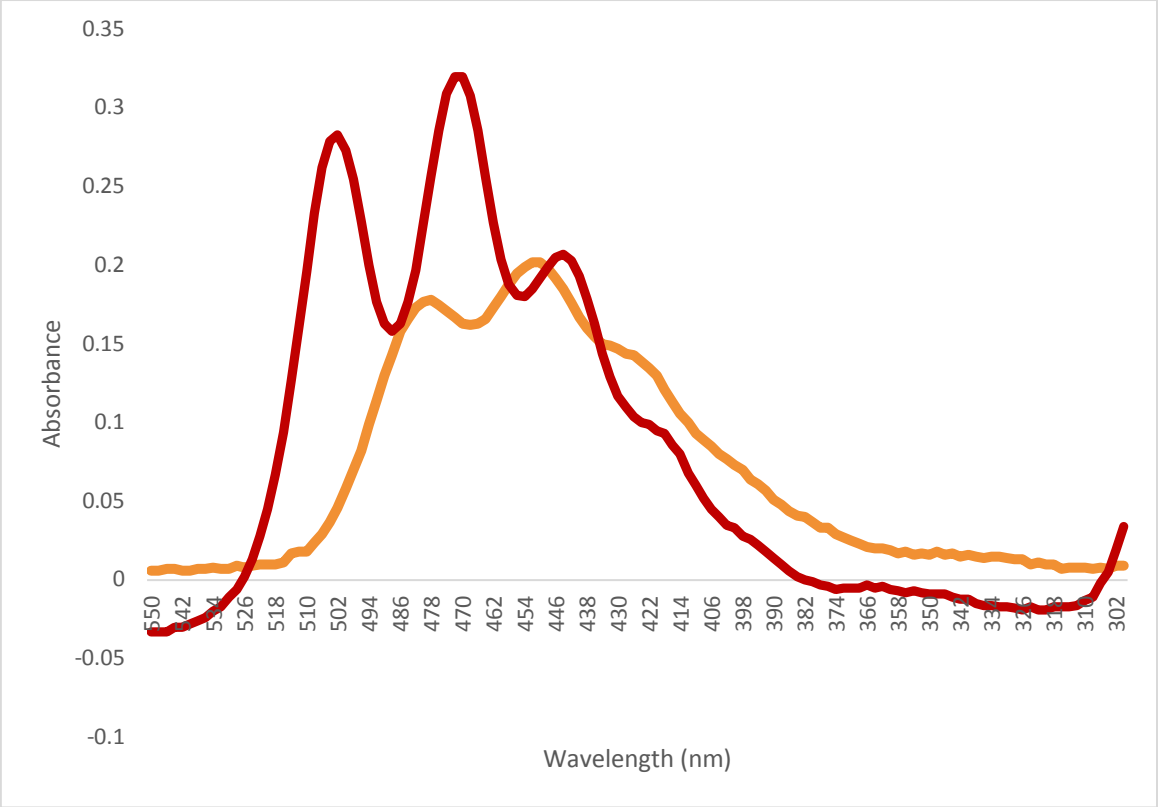


Figure S3: Absorption spectra of *β*-carotene and lycopene standards. *β*-carotene and lycopene standards were dissolved in hexane at 0.7 µg/mL and 0.65 µg/mL respectively. The absorption spectra between 300 nm and 550 nm was recorded using a UV-vis spectrometer. Red: lycopene. Orange: *β*-carotene.