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

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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[4,5,6,7], 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[6,18,19,20,21,22], bacteria[7,23,24,25,26,27], 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. 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.

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. 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.

    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.

    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].

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. 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.
    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.
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)

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. 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.
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.
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.
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.
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).
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**).

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**).
    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.
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. 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**).
4. Transform the entire reaction mix into the DH5α 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.
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.

   Delete: since they are mentioned later
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.

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.

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

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

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

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

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.

5. After 16-18 h, take the plate out from the incubator. Delete: since they are mentioned later 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.

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.

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

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).

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

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.

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

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

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.
    1. Clone the 5' and 3' homology arms from yeast genomic DNA into the entry vector-pYTK001. Follow steps 1 and 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.
    3. For assembly and screening, follow step 4.1.1.

2. Assembly of the multi-gene plasmid
    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.
    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.
    3. To set up the cloning reaction, follow step 2.2.
    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.
    5. Perform the green/white screening as in step 3.2.5.
    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

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

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.

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

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.

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

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

   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

To demonstrate, we have assembled

~~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

Add: also

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]

Add: were cloned

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.

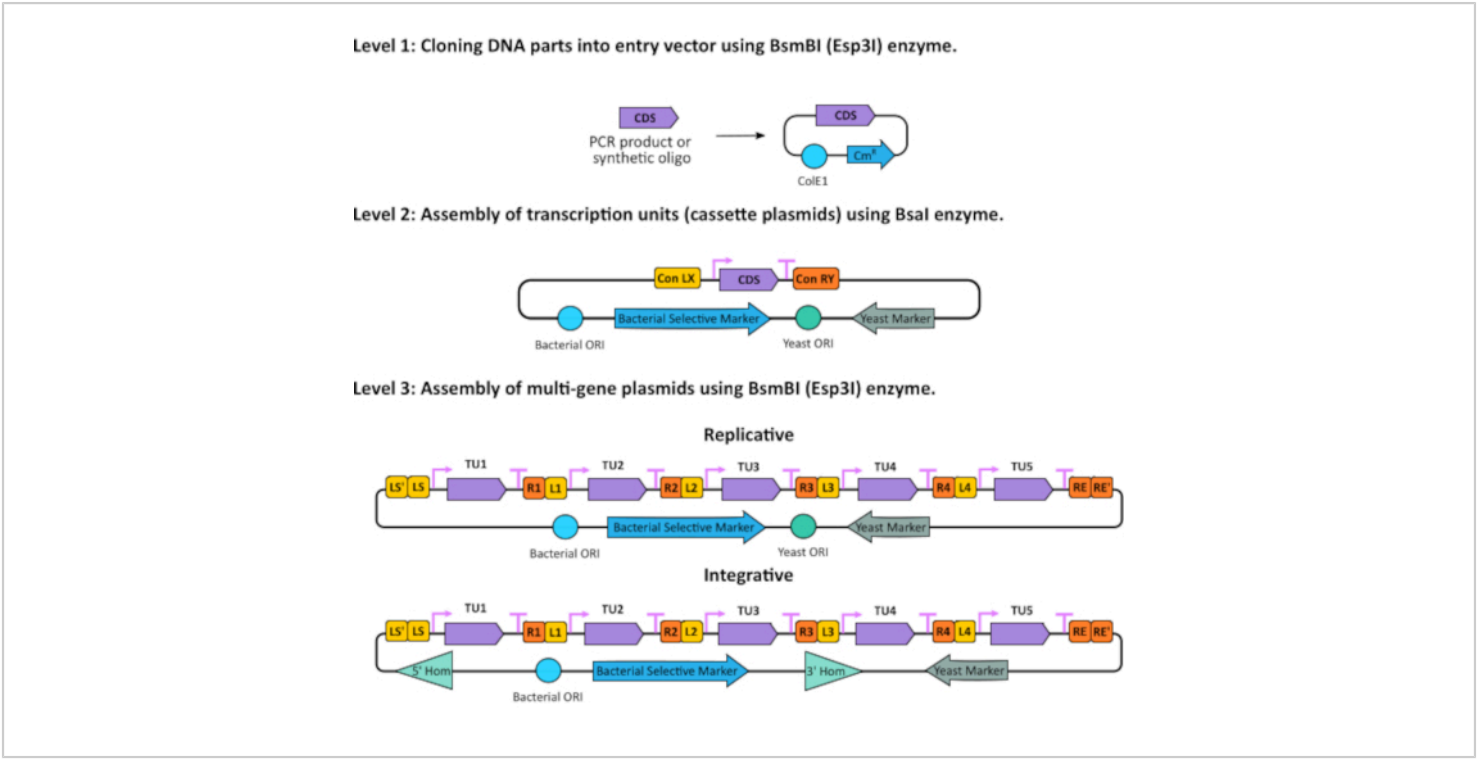
Next, the TUs for *BTS1-ERG20, ERG20*, *crtE, crtYB, crtYB(G247A)*, and *crtI* were assembled following step 3.2 (**Table 1**). **Figures 6E and 6F** show a representative plate of a successful assembly of the TUs and provide an example of a green/white screening method, with 65.02 ± 4.99% total colonies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



Change to: In level 1

Figure 1: Overview of multi-gene assembly. Assembly takes place in three levels. In0 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. Please click here to view a larger version of this figure.

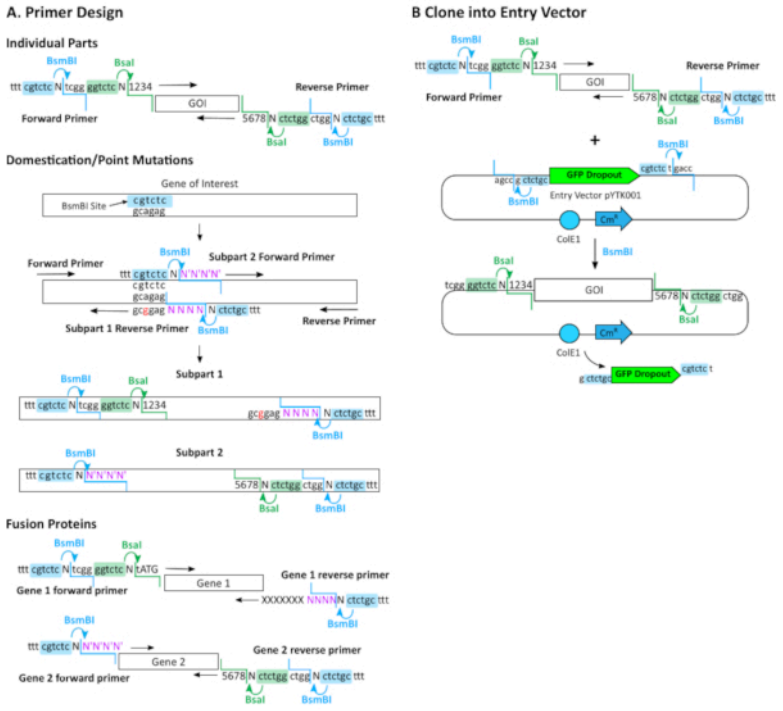


Figure 2: Primer design and cloning of part plasmids. (**A**) Primer design for amplifying individual parts, domesticating genes or creating point mutations, and assembling fusion proteins. Primers include BsmBI and BsaI recognition and cut sites and the MoClo overhang for proper assembly. MoClo overhangs 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. Please click here to view a larger version of this figure.

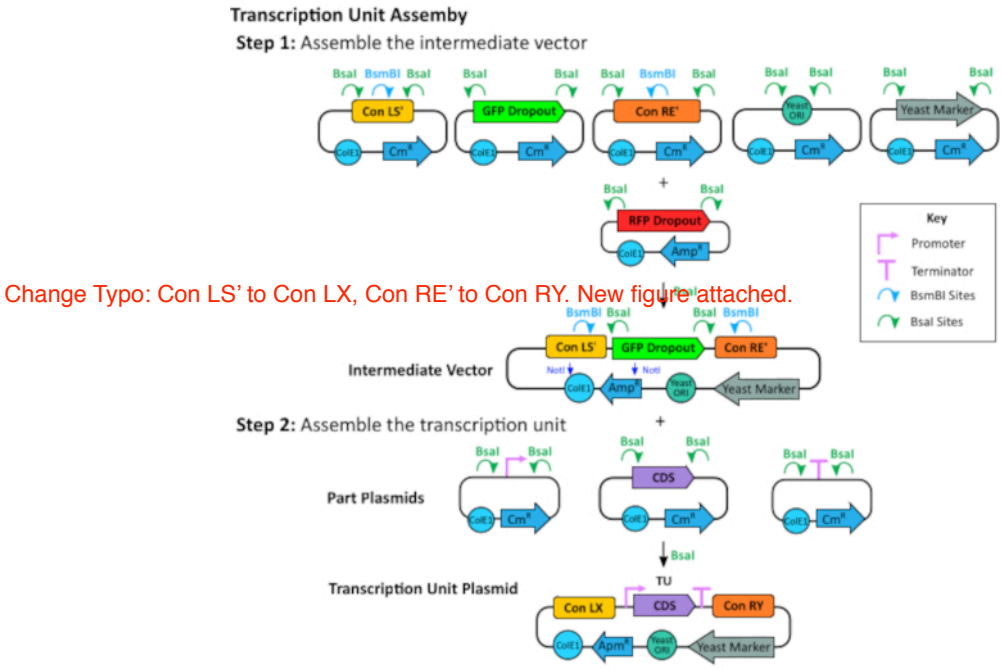
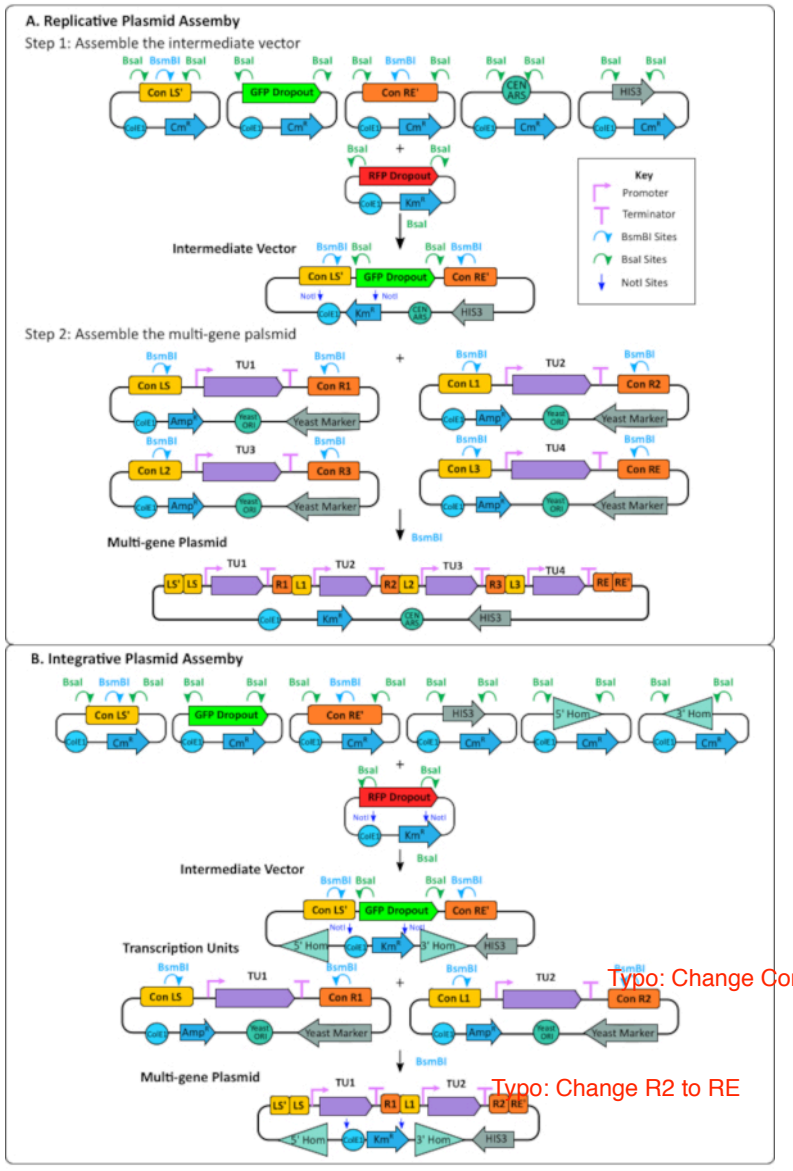


Figure 3: Transcription unit assembly. To assemble the TU plasmids, the assembly of an intermediate vector is recommended to facilitate combinatorial TU assembly. To assemble the intermediate vector, clone the Con LX (X: one of the five left connectors), the sfGFP dropout, the Con RY (Y: one of the five 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. Please click here to view a larger version of this figure.

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 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*.



New figure attached.

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. Please click here to view a larger version of this figure.

Capitalize carotene, New Table 2 attached

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 2: Multi-gene Plasmids used in this study.

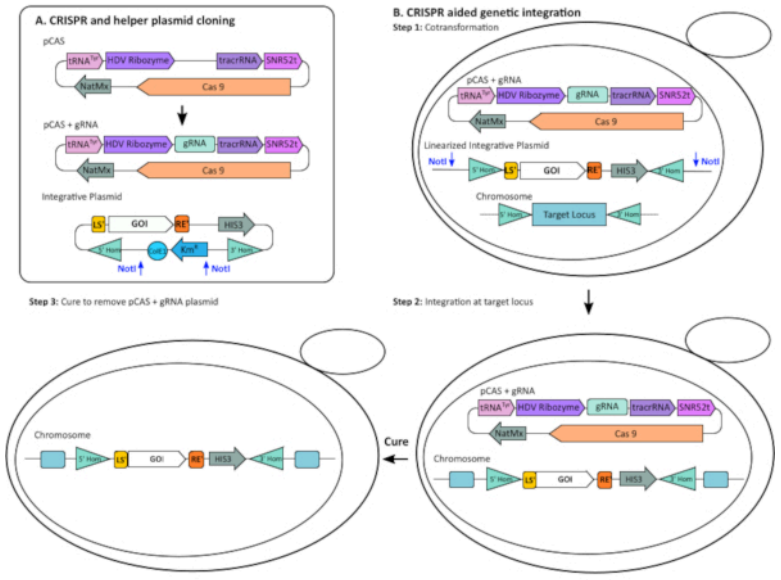


Figure 5: CRISPR integration. (**A**) CRISPR and helper plasmid cloning. The pCAS plasmid contains the Cas9 endonuclease and components (tRNA promoter, SNR52 terminator, HDV ribozyme, and tracrRNA) for optimal expression of a gRNA. Clone the pCAS+gRNA plasmid by assembling the synthetic gRNA with the 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.

Please click here to view a larger version of this figure.

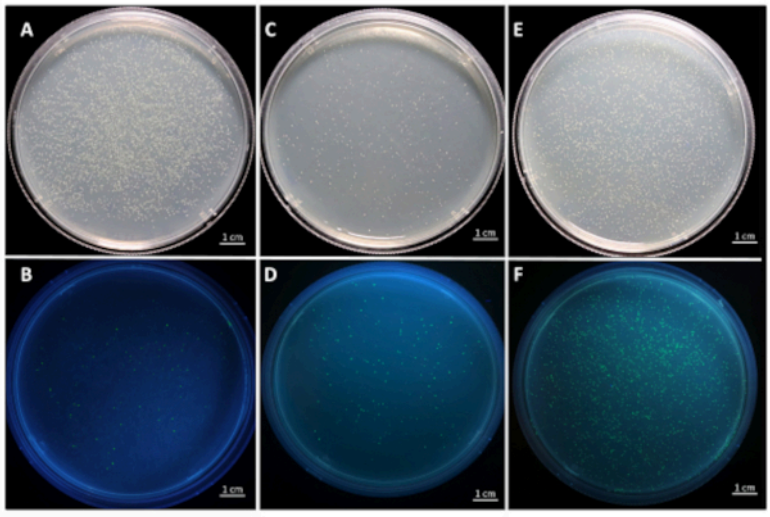


Figure 6: Representative plates of entry vector and transcription unit level cloning in *E. coli.* Representative plates of the successful cloning of a gene into the entry vector pYTK001 under (**A**) visible light and (**B**) UV light. Positive colonies are white and negative colonies are green. The 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. Please click here to view a larger version of this figure.

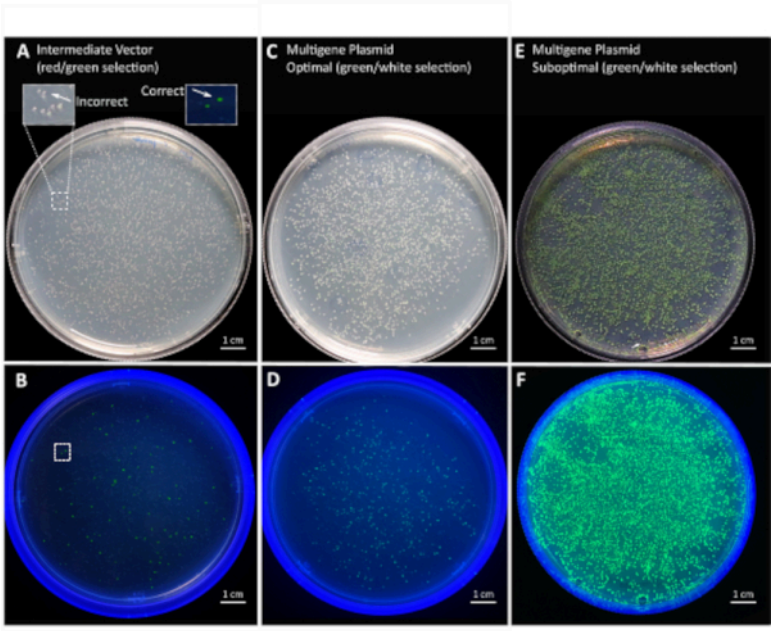


Figure 7: Representative plates of multi-gene plasmid cloning in *E. coli*. Assembly of the intermediate vector for multi-gene level assembly and red/green selection under (**A**) visible light and (**B**) UV light. Positive colonies are green and negative colonies are red. The ratio of positive colonies 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. Please click here to view a larger version of this figure.

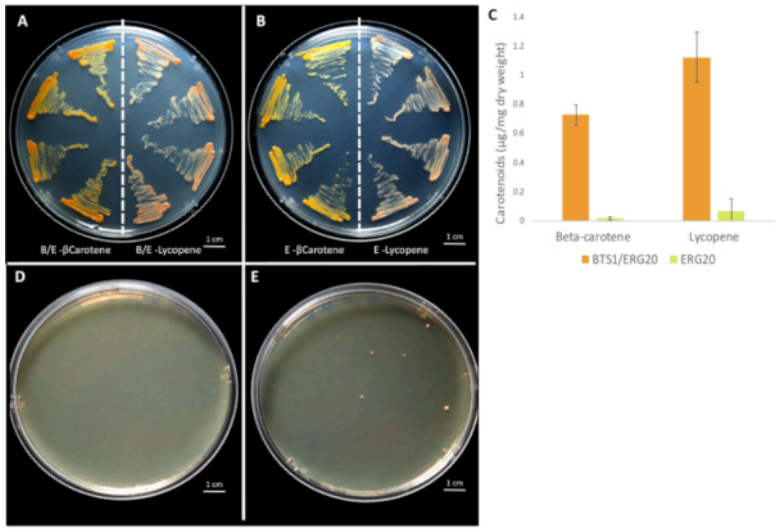


Figure 8: Representative plates of integrative and replicative plasmids in yeast. Replicative multi-gene plasmids transformed into yeast with (**A**) *BTS1-ERG20* fusion TU and (**B**) *ERG20* TU. On each plate, yeast on the left side has plasmids containing crtE TU, crtYB TU and crtI TU for the production 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**). Please click here to view a larger version of this figure.

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 MoClo strategy is advantageous over alternative multi-part assembly methods[8,9,10,11,12] 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.

Disclosures

The authors have nothing to disclose.

Acknowledgments

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