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.

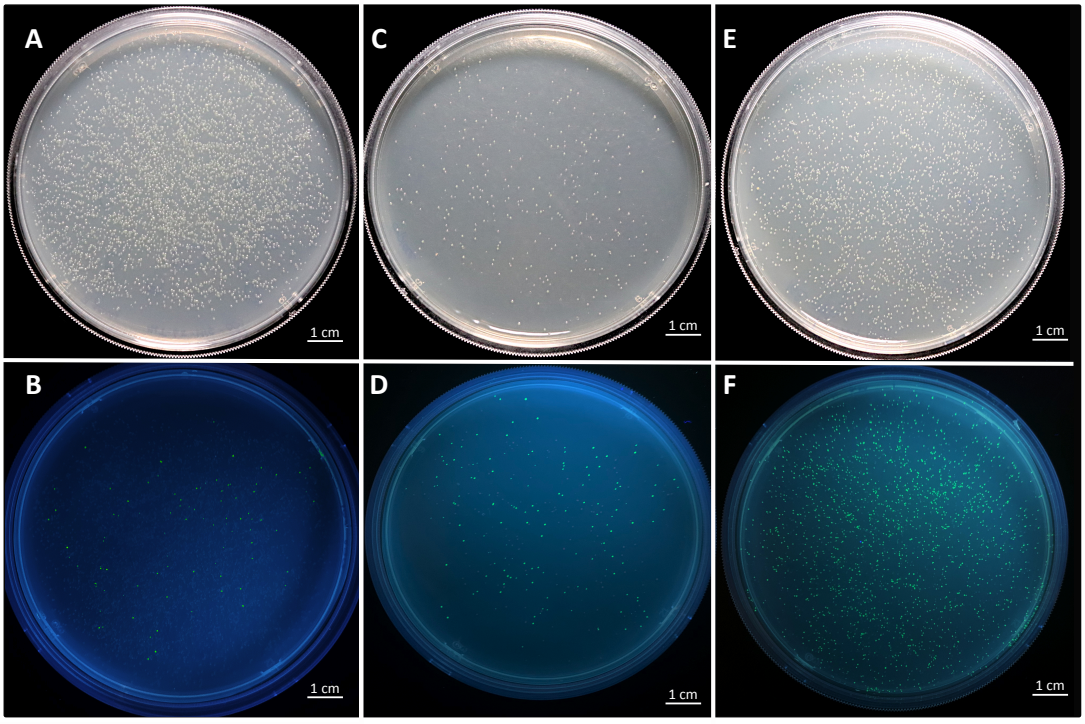


Figure 6: Representative plates of entry vector and transcription unit level cloning in E. *coli. A & B: Representative plates of the successful cloning of a gene into the entry vector pYTK001 under (A) visible light and (B) UV light. Positive colonies are white and negative colonies are green. The ratio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

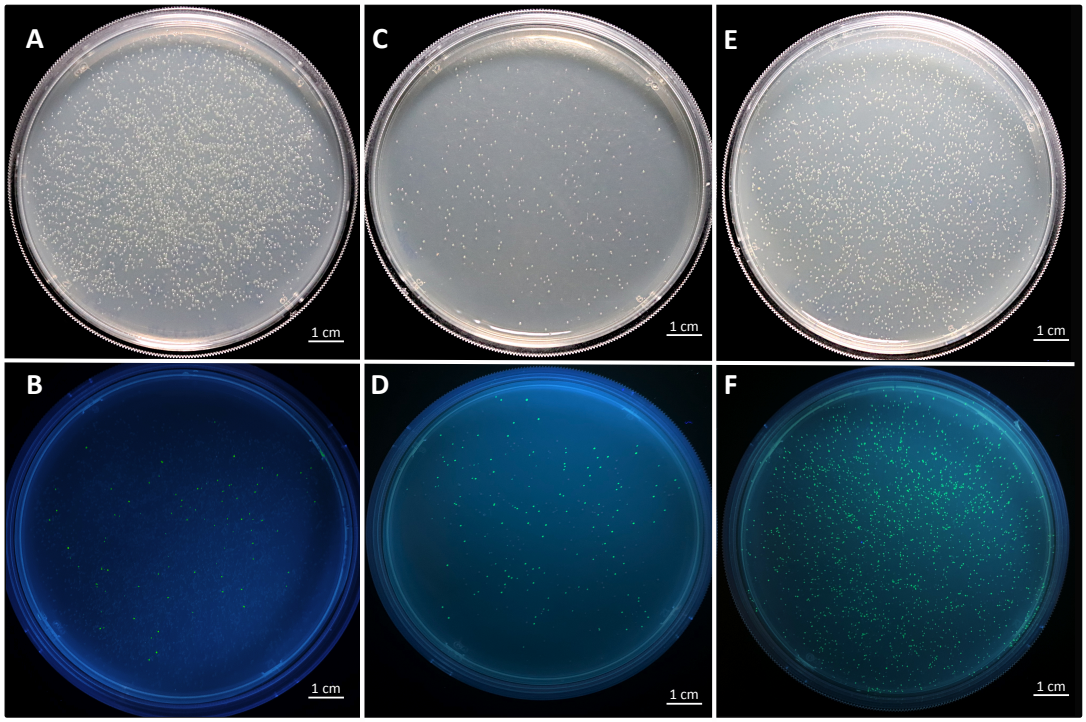
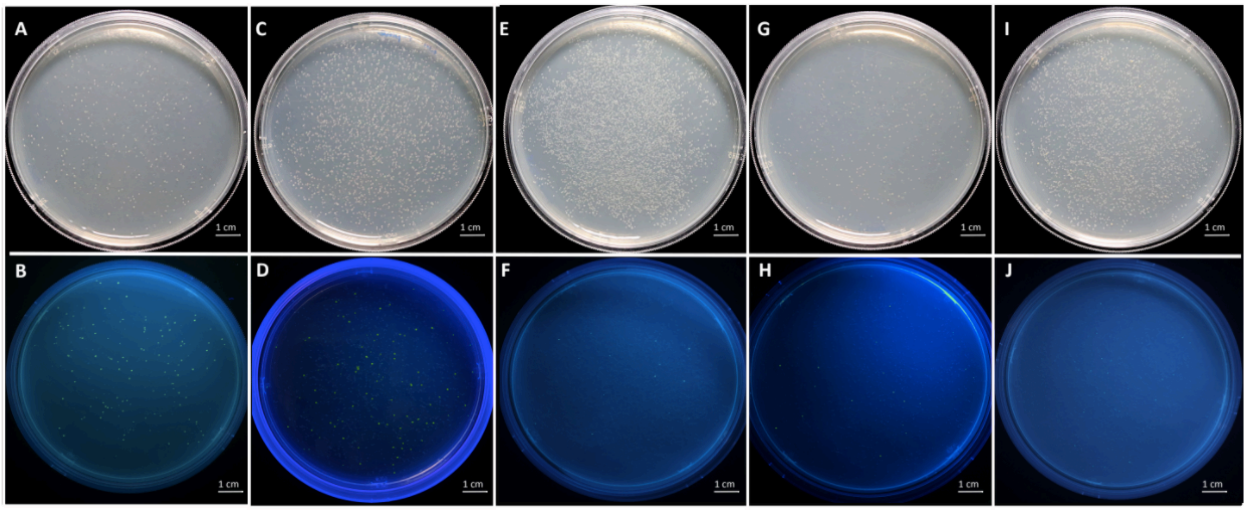


Figure 7: Representative plates of multi-gene plasmid cloning in E. coli. *Fig A & B: Assembly of the intermediate vector for multi-gene level assembly and red/green selection under (A) visible light and (B) UV light. Positive colonies are green and negative colonies are red. The ratio of positive colonies 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.

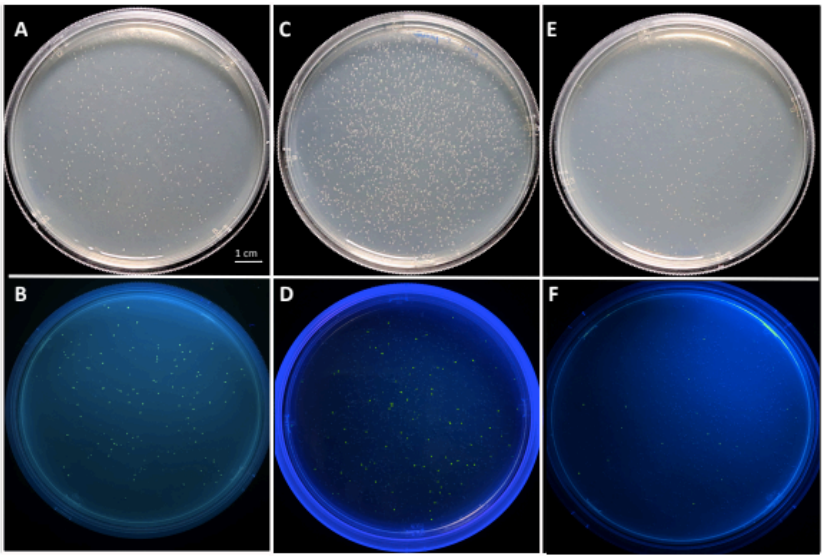
Again, we much appreciate the reviewer's insight in this matter. We have tested the final digestion temperatures at 37C vs 50C, and found that 50C significantly increased the ratio of

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
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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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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."*

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:

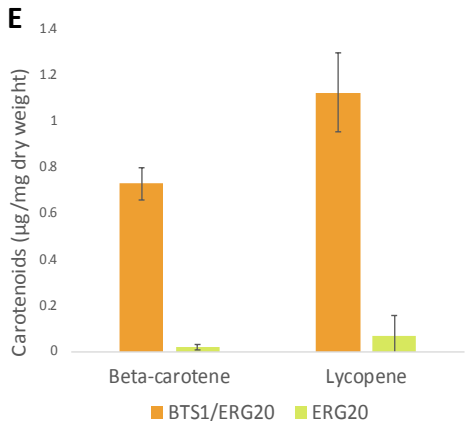


Figure 8E: *quantifying β-carotene and lycopene from yeast extract with four multigene plasmids using a UV-vis spectrometer. The maximal absorbance was recorded at 450 nm and 470 nm for β-carotene and lycopene respectively. The absolute quantification was performed using authentic standards (Figure S3). 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:

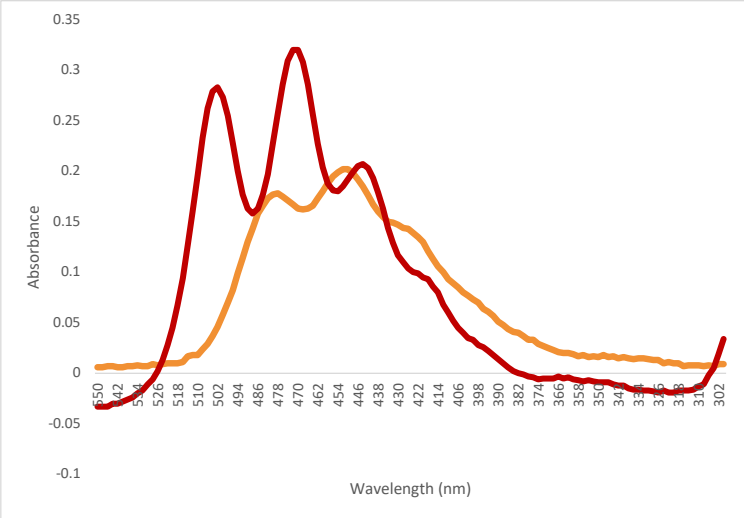


Figure S3: Absorption spectra of *β*-carotene and lycopene standards. *β*-carotene and lycopene standards were dissolved in hexane at 0.7 µg/mL and 0.65 µg/mL respectively. The absorption spectra between 300 nm and 550 nm was recorded using a UV-vis spectrometer. Red: lycopene. Orange: *β*-carotene.

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.

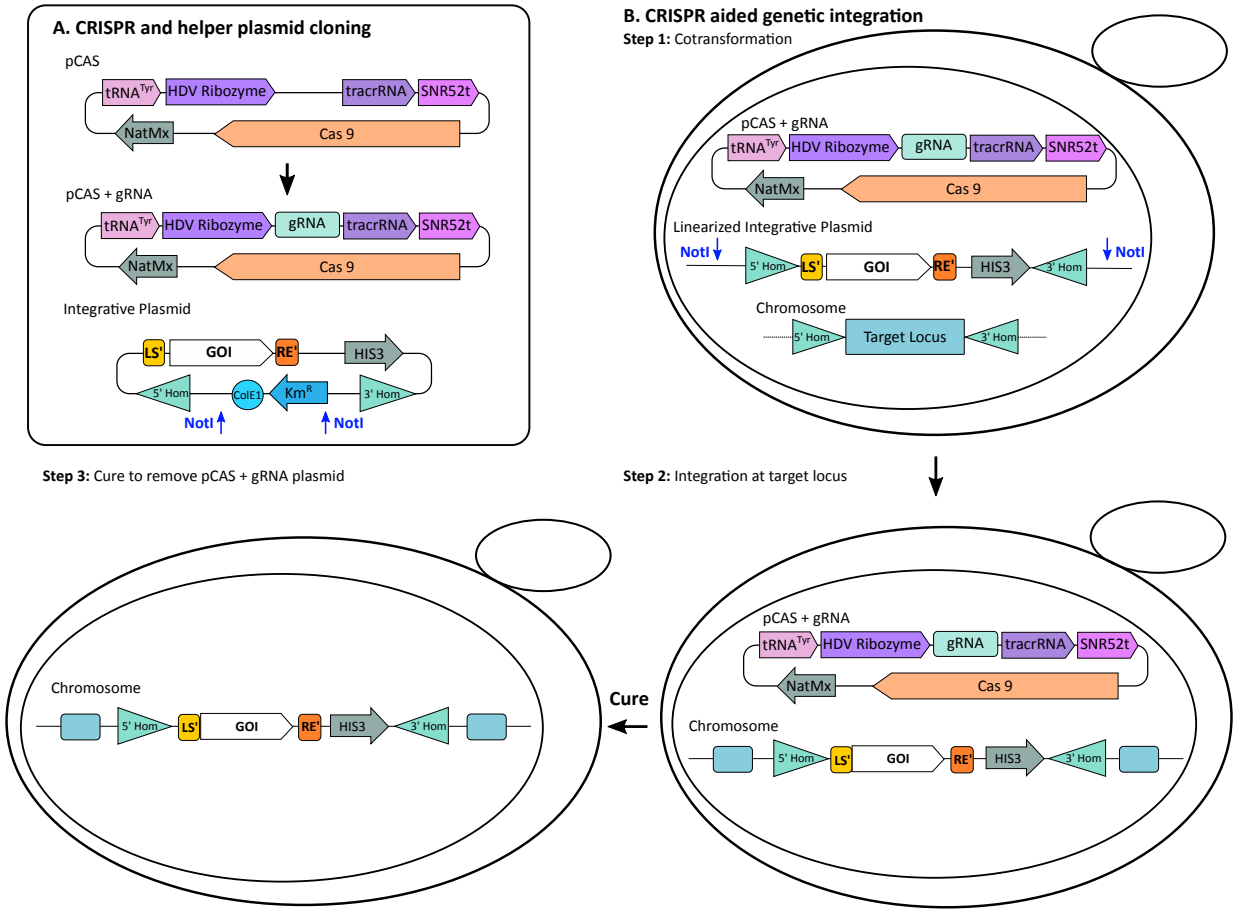
We have added more descriptions to the legends of Figure 2 and 3 as below:

"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.