Dear Editor,

We have revised the manuscript taking along the comments and suggestions provide by you and the two reviewers. In this rebuttal document, we address all editorial and review comments separately. We provide a clean version of the manuscript and a version in which all changes are indicated. Figures are updated according reviewer’s notes.

Yours sincerely,

René Verwaal

Editor comments

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

[Authors]: We have proofread the manuscript thoroughly to exclude spelling or grammar issues.

2. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. “This figure has been modified from [citation].”

[Authors]: We have requested copyright permission for Figure 2 from Wiley Global Permissions. We obtained the permission by e-mail. The correspondence and granted permission is available in a .docx file, which will be uploaded to our Editorial Manager account. We added “This Figure has been modified from Verwaal *et al*., 201819” to the legend of Figure 2.

3. Please provide an email address for each author.

[Authors]: Email addresses of all authors were added.

4. Please define all abbreviations before use.

[Authors]: The following abbreviations were explained within the text: CRISPR, Cas, PAM, crRNA, tracrRNA, GGC, NTC, G418 and bp.

5. 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. You may use the generic term followed by “(see table of materials)” to draw the readers’ attention to specific commercial names. Examples of commercial sounding language in your manuscript are: Milli-Q, NanoDrop, Puregene, QIAGEN, Nunc OmniTray, etc.

[Authors]: All commercial names were removed from the protocol and are now sufficiently referenced in the Table of Materials.

6. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

[Authors]: The protocol was checked and all personal pronouns were removed.

7. Please revise the protocol to contain only action items that direct the reader to do something (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible. Please move the discussion about the protocol to the Discussion.

[Authors]: The language used in the protocol was adapted according to your request. The previous version of the manuscript included background information of the main steps which were given as an introduction just before each section (e.g. sections 2, 2.1, 2.2, 3, 4 and 5). As recommended, only crucial background information of sections 2, 2.1 and 2.2 was kept in the protocol as “Note:” and introductions just before each section were removed. Introduction to section 3 was removed from the protocol. Instead, in the Introduction we mention genes used for the construction of the donor RNA expression cassettes (“expression cassettes encoding the carotenoid genes crtE, crtYB and crtI have been constructed”) and provide detailed design and sequences in the supplementary tables (referred in the Protocol section 3.1). The design of integration flank DNA sequences previously included in the introduction to section 4 was moved to step 4.2.

8. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step. Use sub-steps as necessary. Please move the discussion about the protocol to the Discussion.

[Authors]: As addressed in comment 7, background information belonging to Protocol sections 2, 2.1, 2.2, 3, 4 and 5 were moved to different parts of the manuscript in a shortened form. We rearranged steps that were too long in the previous version of the Protocol to describe only 2 – 3 actions in no more than 4 sentences.

9. Please add more details 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. 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. See examples below.

[Authors]: Details were added to some of the steps in the protocol. Examples are given in the responses to your comments.

10. 2.1.1-2.1.3, 3.1: Unclear what we can show here, please describe the actions. If there are no specific actions being performed, I suggest unhighlighting these.

[Authors]: Pointed steps were changed as described in our response to comments 7 and 8. These steps are not highlighted for the records in the resubmitted version of the manuscript.

11. 2.1.4, 2.2.3: Please provide the composition of buffer.

[Authors]: Composition of buffers used in the Protocol were specified where possible. For example, the composition of buffers used for yeast transformation is described, as well as the composition of the buffer used for linearization of pRN1120 (former step 2.2.3). Composition of the buffer used in a PCR reaction, *e.g.* used in former step 2.1.4, are not provided, because they are part of a commercial kit, in which the exact buffer composition is not specified. For the buffer used in a PCR reaction, we added the sentence to use “buffer required for the chosen DNA polymerase”. Further information about vendors of the materials used is given in the Table of Materials.

12. 2.1.5, 2.2.4: Please specify the loading dye and DNA ladder used.

[Authors]: In the steps describing analysis of DNA by electrophoresis we specified to use “a DNA loading dye and DNA ladder with DNA fragments in a range of 100 to 10,000 bp”. More information about the loading dye and DNA ladder is specified in the Table of Materials.

13. 2.1.6: Please describe how to purify PCR products. We need specific details for filming.

[Authors]: It is not crucial for the method to film how the PCR purification methods works according to the manufacturer. We suggest not to film the PCR purification steps but to show the kit that is used to purify PCR products.

14. 2.2.2: Please describe how to linearize plasmid pRN1120 with EcoRI-HF and XhoI.

[Authors]: The protocol for the linearization of the plasmid pRN1120 is now fully described in step 2.2.3.

15. 2.2.5: Please provide the information of the PCR purification kit in the Table of Materials.

[Authors]: The kit used (Wizard SV Gel and PCR Clean-Up System Kit) is included in the Table of Materials with a note: “Purification of PCR products and linearized pRN1120”.

16. 3.3, 3.5: Please describe how. We need specific details for filming.

[Authors]: Step 3.3. was changed to: “Transform 25 µL of purchased chemically competent *E. coli* cells”. Product name and manufacturer are given in the Table of Materials. Alike explained in comment 13, it is not crucial for the method to film steps of the plasmid purification kit. We suggest showing the plasmid purification kit mentioned in step 3.5 without recording all the steps of the purification.

17. 5.1: Please list an approximate volume of solutions to prepare.

[Authors]: Approximate volumes of stocks and solutions required in the transformation were added to step 5.1.1: “10 x TE buffer: 100mM Tris.HCl (pH 7.5), 10 mM EDTA, total volume of 50 mL. 1 M LiAc: pH 7.5, total volume of 50 mL. 50% PEG 4000, total volume of 100 mL.” and step 5.1.2: “LiAc-TE solution: 0.1 M LiAc, 10 mM Tris-HCl, 1 mM EDTA, total volume of 0.5 mL. PEG-LiAc-TE solution: 40% PEG 4000, 0.1 M LiAc, 10 mM Tris-HCl, 1 mM EDTA, total volume of 1 mL.”

18. Please combine some of the shorter Protocol steps so that individual steps contain 2-3 actions and maximum of 4 sentences per step.

[Authors]: We rearranged the Protocol steps to describe 2 – 3 actions in no more than 4 sentences in the majority of steps.

19. Please include single-line spaces between all paragraphs, headings, steps, etc.

[Authors]: Formatting was changed to single-line spacing.

20. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

[Authors]: We highlighted the most essential steps of the protocol for the video. The total length of the highlighted steps are less than 2.75 pages.

21. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.

[Authors]: Only full sentences were highlighted, all written in imperative tense.

22. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

[Authors]: We have some questions concerning this remark, which we address in a separate e-mail to the editor. We are confident, this will all be clear and agreed in the final script for filming the Protocol.

23. Please number the figures in the sequence in which you refer to them in the manuscript text. Currently Figure 5 appears in the text before Figure 2. Please reference all figures in the manuscript (currently Figure 3 is not mentioned).

[Authors]: The order of the figures was changed and an additional figure, Figure 1, was added as suggested by both reviewers. We carefully checked and confirm that figures are numbered according to the order they appear in the manuscript.

24. Figure 2: Please explain what lane 1 represents in the figure legend. Please combine all panels of this figure into a single image file.

[Authors]: Descriptions were added to all the lines in the figure. All the panels of this figure were combined into a single image.

25. Figure 3: Please label the strains with different colors in the figure, if possible.

[Authors]: Three different strains are presented in Figure 4 (former Figure 3), one per panel. The legend of Figure 3 states which transformants are present on the transformation plates: “(A) Strain 1 expressing crtE, crtYB and crtI from three strong promoters (dark orange colonies). (B) Strain 2 expressing crtE, crtYB and crtI from three medium strength promoters (orange colonies). (C) Strain 3 expressing crtE, crtYB and crtI from three low strength promoters (yellow colonies)”. Both coloured and white colonies appeared on the transformation plate. Coloured colonies were edited using the multiplex approach.

26. Figure 4: Please combine all panels of this figure into a single image file. Please describe the panels in the figure legend.

[Authors]: All the panels of this figure were combined into a single image. Descriptions of panels were added to the figure legend.

27. Discussion: As we are a methods journal, please also discuss critical steps within the protocol, any modifications and troubleshooting of the technique, and any limitations of the technique.

[Authors]: To the discussion, we have added:

* critical steps (3 items);
* modifications/troubleshooting (6 items).

We cannot think of any limitations of the technique; it has benefits as compared to other multiplexing approaches, as described in the Introduction, and results in high multiplex genome editing efficiencies.

28. Table of Materials: Please sort the items in alphabetical order according to the name of material/equipment.

[Authors]: All the items included in the Table of Material are now sorted in alphabetical order.

Reviewer 1

I miss a clear (graphical/schematic) overview of all the steps that will be taken in the introduction. I think a flowchart with the different steps would be very helpful for the readers.

[Authors]: We made and added the new Figure 1 which presents the key steps of the Protocol.

It might help the readers to keep overview by first to explain how to make a Cas12a-expressing strain, and only after that explain the cloning of the constructs required for the second transformation, and next about the second transformation/selecting transformants.

[Authors]: According to your suggestions we rewrote section 5 which describes transformation of *S. cerevisiae*. In the new version of the section 5, after describing the solutions required for transformation (step 5.1), we describe how to obtain a strain pre-expressing Cas12a (step 5.2) and then we describe that the strain pre-expressing Cas12a is transformed with all constructs needed for the multiplex genome editing experiment (step 5.3). In addition, we added a workflow of the experiment (Figure 1) which shows the two rounds of the transformation. This figure also indicates in which steps the constructs required for the second transformation are obtained.

I'm not sure why large parts of the text are highlighted in yellow?

[Authors]: Text highlighted in yellow is proposed to be recorded for the subsequent movie shoot. This is a request by the journal submission guidelines.

57: This reads like the bacteria and archaea are adapted for genome editing. Please rephrase.

[Authors]: The sentence “CRISPR/Cas genome editing systems find their origin in adaptive immune systems of bacteria and archaea which have been adapted by molecular biologists for genome editing” was rephrased by “CRISPR/Cas genome editing systems find their origin in adaptive immune systems of bacteria and archaea and these systems have been adapted by molecular biologists for genome editing.”

61: Needs explanation what Class 2 is (i.e. explain that there are different CRISPR-Cas classes and types)

[Authors]: Part of the introduction explaining the difference between class 1 and 2 was rewritten and additional information on the classification of Cas proteins was added. The original sentence: “Class 2 and specifically Cas9 is extensively studied as it is characterized by a single multidomain effector protein involved in target cleavage, in contrast to proteins with a multi-subunit categorized as class 110,11” was changed into: “Based on the recent genome analysis of CRISPR/Cas systems it was proposed to divide the CRISPR/Cas systems into two classes, five types and 16 subtypes10. The two classes are distinguished based on the organization of effector complexes involved in target cleavage. Typically, CRISPR/Cas systems with a multi-subunit organisation are categorized as class 1, whereas single subunit effector complex belong to class 210,11”.

done up to protocol

[Authors]: Part of the comment is missing, therefore this comment cannot be addressed.

108: Is there a company from which this plasmid can be acquired, or can it be deposited to Addgene? [Authors]: This information is provided in Protocol section 1 as “Note: the plasmid containing the Lachnospiraceae bacterium ND2006 Cas12a (LbCpf1, pCSN067) codon optimized for expression in S. cerevisiae, was previously constructed19 and is deposited at Addgene (ID 101748)”. The second plasmid used in this study was pRN1120 and the Addgene ID of plasmid pRN1120 is provided in the Protocol section 2.2 as “Note: The single crRNA array is expressed from the S. cerevisiae/E. coli shuttle plasmid pRN112019 which is deposited at Addgene (ID 101750)”. Furthermore, the availability of plasmids pCSN067 and pRN1120 can be found in the section Additional Information: “All plasmids are deposited at Addgene pCSN067 (ID 101748), pRN1120 (ID 101750)”.

148: Is there a company from which this plasmid can be acquired, or can it be deposited to Addgene? [Authors]: In line 148 we described construction of the donor DNA expression cassettes containing carotenogenic genes. As described in the Protocol section 3, the details of the expression cassette designs are given in Suppl. Table 3 and sequences are provided in Suppl. Table 4. The donor DNA expression cassettes were constructed in the backbones which were previously described26 and for the further information about the backbones, the reader is referred to the paper by Young *et al.* (2018)26.

194: What volume, what flasks?

[Authors]: Step 4.1.1 was changed from “Grow the strain in YEPD at 30 °C and shaking for 48 h.” to “Grow the strain in YEPD in a 500 mL shake flask filled with 100 mL of YEPD (2% glucose) medium at 30 °C and shaking at 250 rpm for 48 hours”.

196: what is physiological salt?

[Authors]: The composition of physiological salt was added to the step 4.1.3: “Resuspend the cells in physiological salt (200 µL; 0.85% NaCl solution) with RNase (10 µL, 10 mg/mL) and yeast lytic enzyme (4 µL). Incubate the cell suspension at 37 °C for 15 min”. In addition, we added the composition of physiological salt (0.85% NaCl solution) to the Table of Materials.

206: what is a small amount?

[Authors]: The step 4.1.10 “Wash the pellet with small amount of ethanol (70%)” was rewritten and renumbered to “4.1.8 Wash the pellet with 200 µL of ethanol (70%). Centrifuge at 16,000 x g at room temperature for 10 min and remove the supernatant. Evaporate the ethanol by incubating the tube at room temperature for 10 min with the lid opened. Note: If liquid in the tube is still visible, repeat step 4.1.8. Do not dry the pellet for longer than 10 min to prevent decreased solubility of the DNA”.

207: Do you remove the supernatant first?

[Authors]: Yes, first the supernatant is removed. This is included in step 4.1.8.

219: What is the protocol for amplification?

[Authors]: The amplification described in the line 219 of the old version of the manuscript now appears in step 4.4 in the new version of the manuscript. The amplification step included in the step 4.4 requires several actions that are now described in sub-steps 4.4.1 - 4.4.2.

256: can this strain be acquired commercially? Would it work for other strains too?

[Authors]: The protocol is suitable for various *S. cerevisiae* strains and this information was included in the Protocol section 5 as “Note: Perform transformation using a protocol based on the methods developed byGietz *et al*.29 and Hill *et al.*30 which can be used for various strains of *S. cerevisiae*”. Information about the collection from which strain CEN.PK.113-7D can be obtained was added to section Additional Information: “Strain *S. cerevisiae* CEN.PK113-7D can be obtained from the EUROSCARF collection (http://www.euroscarf.de)”.

283: I'm a bit puzzled here (I guess a schematic overview would be helpful here) - Do I understand correctly that the single crRNA array and the recipient plasmid for the crRNA array are transformed together? Shouldn't they be put together beforehand? Same for the donor DNA and flanking sequences?

[Authors]: This is correct, the single crRNA array and the recipient plasmid are not put together beforehand, *i.e.* before the transformation to *S. cerevisiae*. The same counts for the donor DNA and flanking sequences. The single crRNA array, recipient plasmid, donor DNA and flanking sequences are mixed (step 5.3.1) and this DNA mixture is used for transformation (step 5.3). Upon transformation, the single crRNA array and the recipient plasmid pRN1120 are assembled into a circular vector by *in vivo* recombination (described in the step 2.1: “Note: […] The single crRNA array is assembled by *in vivo* recombination into the linearized plasmid pRN1120 to generate a circular vector, thus regions homologous to plasmid pRN1120 must be present at the start and end of the single crRNA array (see the schematic structure in Figure 2A)”. *In vivo* recombination in *S. cerevisiae* is also the mechanism behind the assembly of donor DNA expression cassettes and integration flank DNA sequences, as schematically depicted in Figure 2B. To clarify our approach, we supplied the whole workflow in Figure 1.

326: This sentence is confusing. Please rephrase to something like: During PCR using these primers, the annealing temperature can be set to 62 C. (the primers don't actually anneal together, which this sentence suggests)

[Authors]: The sentence “These primers can be annealed at 62 °C in a PCR” in step 7.4.1 was changed to “When using primers KC-121 to KC-132, set the annealing temperature in the PCR program to 62 °C”.

362: should the device be cleaned afterwards in a specific way?

[Authors]: It is not required to clean the device in a specific way after using it for the described purpose. The standard maintenance procedure recommended by the manufacturer is followed. We did not include a sentence in the manuscript about a specific way to clean the device.

Reviewer 2

Major Concerns:

1. The procedure of Saccharomyces cerevisiae transformation seems to be complicated compared with other methods, such as Nature Protocols, 2007, 2(1): 1-4. So, is there any special advantages for the transformation method mentioned in this manuscript.

[Authors]: We are aware of possibly novel less complicated methods. However, we used the protocol based on the methods published by Gietz *et al.* (Yeast, 1995, 11 (4): 355-360)29 and Hill *et al.* (Nucleic acids research, 1991, 19 (20): 5791)30 as it gives high transformation efficiency in our hands. Therefore, we have not considered changing the transformation protocol. We added a note in the Discussion that other methods could be considered (“The transformation of *S. cerevisiae* might be performed using a different method than the one described in this protocol, for example the protocol described by Gietz *et al.* 200737”).

2. It is suggested that please provide an operation flow chart, such as first step: introducing Cas12a , and second step: introducing single crRNA array, linearized recipient plasmid, donor DNA and flanking sequences. It will be easy for readers to understand the whole process.

[Authors]: We made and added the new Figure 1 which presents the key step of the protocol. As suggested, we firstly presented preparation of plasmid pre-expressing LbCas12a (text box 1), single crRNA array (text box 2), recipient plasmid pRN1120 (text box 3), construction of the donor DNA expression cassettes (text box 4), generation of the integration flank DNA sequences (text box 5) and the first round of the transformation which results in the strain pre-expressing Cas12a. Secondly, we depicted that the parts listed in the previous sentence, are used in the second transformation which results in the genome editing. Finally, methods used for the quality control of the integration of the donor DNA expression cassettes and methods used to create yeast pixel art are included.

3. Line 283-286, the amount of DNA used here is the best one? It is suggested that provide further discussion about the DNA dosage used in this manuscript.

[Authors]: The amount of DNA for the transformation is now specified in step 5.3.1 (“Pipette 1 µg of the single crRNA array, 1 µg of the linearized recipient plasmid for the crRNA array, 1 µg of donor DNA and 1 µg of each flanking region in a microcentrifuge tube”). This was further described in the Discussion (“It is recommended use 1 µg of each donor DNA, linearized pRN1120 or single crRNA array expression cassette in the transformation, although the use of a lower DNA amount is also expected to result in satisfactory transformation efficiency. Perform a test transformation to determine whether lower DNA amounts can be used)”.

4. References should be checked one by one. Please provide more detailed links about reference 20; and for reference 26, it should be a paper about different strength promoters, rather than the Golden Gate Cloning assembly method.

[Authors]: Numbering of references was updated.

Minor Concerns:

1. In line 258 and line 260, the initial OD should be clearly introduced to calculate the inoculation quantity.

[Authors]: Step 5.2.2 was updated from “Measure the OD600 of the pre-culture and calculate the volume of pre-culture required for inoculation of transformation culture. The optical density of culture for transformation should be 1.0.” to “Measure the OD600 of the pre-culture (ODpc). Calculate the dilution factor (*df*) between the volume of pre-culture and the volume of fresh medium required for preparation of the cells pre-expressing Cas12a to be used in the transformation (transformation culture). In the calculations assume the optical density of the transformation culture (ODtc) to be 1.0 after the incubation step described in 5.2.3 (*ti*).

where *ti* and τ are the incubation time and doubling time, respectively. Calculate the volume of the pre-culture (*V*i) required for inoculation of the transformation culture (*V*tc) based on the dilution factor.

Terms introduced in step 5.2.2 were further used in step 5.2.3 (“Prepare the transformation culture by inoculation of 20 mL of YEPD (2% glucose) (*V*tc) with the volume of pre-culture determined in the previous step (*V*i). Incubate at 30 °C with shaking at 250 rpm”).

2. In line 264, please provide the volume of demineralized water for washing cells.

[Authors]: The volume of the demineralized water for washing the cells was specified. The sentence included in line 264 “Discard supernatant and wash cells in room temperature demineralized water” was changed to “Discard the supernatant and wash the cells in 20 mL room temperature demineralized water”.

3. In line 271:"at a low speed" , please give the exact centrifugal speed.

[Authors]: The centrifugal speed was specified. The sentence included in line 271 (“Incubate for 30 min at 30 °C while shaking at a low speed in a table top heat block”) was changed to “Incubate for 30 min at 30 °C while shaking at 450 rpm in a table top heat block”.