Journal of Visualized Experiments

CRISPR/Cas12a multiplex genome editing of Saccharomyces cerevisiae and the creation of yeast pixel art

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE59350R1
Full Title:	CRISPR/Cas12a multiplex genome editing of Saccharomyces cerevisiae and the creation of yeast pixel art
Keywords:	CRISPR/Cas12a, CRISPR/Cpf1, CRISPR/Cas9, multiplex genome editing, Saccharomyces cerevisiae, yeast pixel art
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Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Open Access (US$4,200)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Delft, Zuid-Holland, the Netherlands

TITLE:

CRISPR/Cas12a Multiplex Genome Editing of *Saccharomyces Cerevisiae* and the Creation of Yeast Pixel Art

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KEYWORDS:

CRISPR/Cas12a, CRISPR/Cpf1, CRISPR/Cas9, multiplex genome editing, *Saccharomyces cerevisiae*, yeast pixel art

SUMMARY:

The CRISPR/Cas12a system in combination with a single crRNA array enables efficient multiplex editing of the *S. cerevisiae* genome at multiple loci simultaneously. This is demonstrated by constructing carotenoid producing yeast strains which are subsequently used to create yeast pixel art.

ABSTRACT:

The high efficiency, ease of use and versatility of the clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9 (CRISPR)/Cas9 system has facilitated genetic modification of *S. cerevisiae*, a workhorse organism in biotechnology, extending its capability as a cell factory. CRISPR-associated protein 12a (Cas12a) is an RNA-guided endonuclease with features distinguishable from Cas9, further extending the molecular toolbox for genome editing purposes. A benefit of the CRISPR/Cas12a system is that it can be used in multiplex genome editing with multiple guide RNAs expressed from a single transcriptional unit (single CRISPR RNA (crRNA) array). We present a protocol for multiplex integration of multiple heterologous genes into independent loci of the *S. cerevisiae* genome using the CRISPR/Cas12a system with multiple crRNAs expressed from a single crRNA array construct. The proposed method exploits the ability

of *S. cerevisiae* to perform in vivo recombination of DNA fragments to assemble the single crRNA array into a plasmid that can be used for transformant selection, as well as the assembly of donor DNA sequences that integrate into the genome at intended positions. Cas12a is pre-expressed constitutively, facilitating cleavage of the *S. cerevisiae* genome at the intended positions upon expression of the single crRNA array. The protocol includes the design and construction of a single crRNA array and donor DNA expression cassettes, and exploits an integration approach making use of unique 50-bp DNA connectors sequences and separate integration flank DNA sequences, which simplifies experimental design through standardization and modularization and extends the range of applications. Finally, we demonstrate a straightforward technique for creating yeast pixel art with an acoustic liquid handler using differently colored carotenoid producing yeast strains that were constructed.

INTRODUCTION:

CRISPR/Cas enzymes have unquestionably revolutionized molecular biology and been widely adopted as tools for engineering genomes at a speed that was previously unfeasible[1]. The first modification of a *Saccharomyces cerevisiae* genome by the CRISPR/Cas9 genome editing system was reported by DiCarlo et al.[2], demonstrating successful gene knock-out and making point mutations using externally introduced oligonucleotides. Further yeast CRISPR toolbox developments included: transcriptional regulation by fusion of catalytically inactive dead Cas9 (dCas9) with transcriptional effector domains to enable activation and silencing of transcription[3], application for both genome editing and regulatory functions for metabolic pathway engineering by simultaneous activation, repression and deletion[4], deletion of large fragments from the *S. cerevisiae* genome[5], and multiple-chromosome fusions[6].

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. Their functionality is based on the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) DNA regions encoding RNA responsible for the recognition of the foreign DNA or RNA and the CRISPR associated genes (Cas) which encodes RNA-guided endonucleases[1,7,8,9]. 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 subtypes[10]. 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 complexes belong to class 2[10,11]. In this paper, we explore the class 2 type V Cas12a, formerly called Cpf1[10,12], which is an alternative to the class 2 type II Cas9. Although Cas9 is well-characterized and widely used in research, Cas12a offers additional features[12]. Firstly, Cas12a forms a complex with crRNA of 42 to 44 nucleotides without requiring an additional trans-activating CRISPR RNA (tracrRNA). Therefore, a shorter guide RNA can be utilized in genome editing with CRISPR/Cas12a systems compared to CRISPR/Cas9. Secondly, the unique endonuclease and endoribonuclease activity of Cas12a enables maturation of its pre-crRNA[13]. This RNase activity allows for the encoding of multiple crRNA on a single CRISPR crRNA array, whereas Cas9 requires the separate expression of each so-called single-guide RNAs (sgRNAs) or alternatively for example expression of an additional endonuclease (e.g., Csy4) in combination with recognition motifs for Csy4 surrounding each sgRNA[14,15]. Thirdly, Cas12a target site

recognition requires a protospacer adjacent motif (PAM) at the 5' end from the target and cleaves after the +18/+23 position from its PAM resulting in cleaved DNA with sticky ends, whereas Cas9 requires a PAM located on the 3' end from the target and cleaves after the -3 position creating blunt end cuts in the DNA[12]. Fourthly, the consensus nucleotide sequence of the PAM differs between Cas12a ((T)TTV) and Cas9 (NGG), which makes Cas12a a promising candidate for targeting T-rich promoter and terminator sequences[16]. Finally, a recent study reported greater target specificity for Cas12a than for the native Cas9[17].

We present a protocol for using the CRISPR/Cas12a system for genome editing of *Saccharomyces cerevisiae* with a particular focus on the introduction of multiple DNA expression cassettes into independent genomic loci simultaneously (multiplex genome editing) using a single crRNA array. The key steps of the protocol are depicted in **Figure 1**. As a proof of concept, the CRISPR/Cas12a system was applied for introduction of three expression cassettes into the genome of *S. cerevisiae* which enable the production of β-carotene[18] as schematically shown in **Figure 2**. Production of β-carotene affects the phenotype of *S. cerevisiae*: i.e., upon successful introduction of all three heterologous genes required for carotenoids biosynthesis, the white *S. cerevisiae* cells turn yellow or orange, depending on the expression strength of each gene's promoter. Due to the simple visual read-out of this pathway, it has been introduced to develop advanced CRISPR-based systems and methods for genome editing[19,20]. In this work, expression cassettes encoding the carotenoid genes *crtE*, *crtYB* and *crtI* have been constructed using a Golden Gate cloning (GGC) approach[21] with heterologous promoters and homologous terminators used to drive expression of the genes. The expression cassettes are surrounded by unique 50-base pairs (bp) sequences, called connectors, that allow for in vivo assembly with integration flank DNA sequences (flanking regions) with the same 50-bp sequences, and subsequent integration into the genomic DNA of yeast at the position determined by the flanking regions. By using different promoter strengths, strains with different levels of carotenoids production were obtained resulting in variation in color of the cells. These strains—inspired by the "Yeast Art Project"[22]—were used in a spotting setup with an acoustic liquid handler to create a 4-color high-resolution "yeast photograph" of Rosalind Franklin. Franklin (1920–1958) was an English chemist and X-ray crystallographer well known for her contribution to the discovery of the DNA structure by Photo 51[23,24,25].

PROTOCOL:

1. Preparation of the Cas12a plasmids

NOTE: The plasmid containing the *Lachnospiraceae* bacterium ND2006 Cas12a (LbCpf1, pCSN067) codon optimized for expression in *S. cerevisiae*, was previously constructed[19], deposited at a plasmid repository (see the **Table of Materials**). This is a single-copy episomal *S. cerevisiae*/*E. coli* shuttle plasmid containing a KanMX resistance marker gene to allow for selection of *S. cerevisiae* transformants on geneticin (G418).

1.1. Obtain the pCSN067 plasmid (see the **Table of Materials**).

1.2. Amplify the pCSN067 plasmid to obtain a high amount.

1.2.1. Transform 25 µL of purchased chemically competent *E. coli* cells with the plasmid pCSN067 according to the manufacturer's protocol. Dilute the transformation mix 10 and 50 times in 2x peptone-yeast (PY). Plate out 10x and 50x dilutions on 2x PY agar plates containing ampicillin (0.1 g/L) and incubate overnight at 37 °C.

1.2.2. Pick 2 to 3 colonies and inoculate each colony in 3 mL of 2x PY and grow overnight at 37 °C in a shaking incubator at 180 rpm.

1.2.3. Purify the plasmid using a plasmid purification kit according to manufacturer's instructions.

2. Preparation of the single crRNA array expression cassette

2.1. Prepare the single crRNA array.

NOTE: The single crRNA array comprises an *SNR52* RNA polymerase III promoter from *S. cerevisiae*[2], a direct repeat specific for LbCas12a and a spacer (genomic target sequence), together repeated for each target[19] and ends with a *SUP4* terminator from *S. cerevisiae*[2]. The single crRNA array is assembled by in vivo recombination into the linearized plasmid pRN1120 to generate a circular plasmid, thus regions homologous to plasmid pRN1120 must be present at the start and end of the single crRNA array (see **Figure 2A**). It is recommended to in advance evaluate the functionality of a number of designed crRNAs separately[19]. This information is subsequently used to select most functional crRNAs to combine these into the direct repeat and spacer sequences to create a single crRNA array for the multiplexing purpose.

2.1.1. Order the single crRNA array for multiplex genome editing experiments as synthetic DNA (see the DNA sequence of the single crRNA array in **Supplementary Table 1**).

2.1.2. Amplify the ordered single crRNA array (e.g., using primers KC-101 and KC-102 (**Supplemental Table 2**)). Prepare the PCR amplification mix containing: 0.5 µL of DNA polymerase, 10 µL of 5x buffer required for the DNA polymerase, 1 µL of 10 mM dNTPs, 2.5 µL of 10 µM forward primer, 2.5 µL of 10 µM reverse primer, 2 µL of DNA template at a concentration of 5 ng/µL and ultrapure H_2O up to a total volume of 50 µL.

2.1.2.1. Perform the reaction in a thermocycler using the following program: (i) 98 °C for 3 min, (ii) 98 °C for 10 s, (iii) 60 °C for 20 s, (iv) 72 °C for 15 s – repeat steps (ii) to (iv) 30 times, (v) 72 °C for 5 min (vi) hold at 12 °C until further analysis.

2.1.3. Analyze the PCR products by electrophoresis by running the samples on a 0.8% agarose gel at 5 V/cm for 40 min using a DNA loading dye and DNA ladder with DNA fragments in a range of 100 to 10,000 bp.

2.1.4. Purify the PCR products using a PCR purification kit according to the instructions of the manufacturer.

2.2. Prepare the single crRNA array recipient plasmid.

NOTE: The single crRNA array is expressed from the *S. cerevisiae*/*E. coli* shuttle plasmid pRN1120[19] (see the **Table of Materials**). This multi-copy plasmid contains a NatMX resistance marker gene to allow selection of *S. cerevisiae* transformants on nourseothricin (NTC).

2.2.1. Obtain the pRN1120 plasmid.

2.2.2. Amplify the pRN1120 plasmid to obtain a high amount.

2.2.2.1. Transform 25 µL of purchased chemically competent *E. coli* cells with plasmid pRN1120 according to the manufacturer's protocol. Dilute the transformation mix 10 and 50 times in 2x PY. Plate out 10x and 50x dilutions on 2x PY agar plates containing ampicillin (0.1 g/L) and incubate overnight at 37 °C.

2.2.2.2. Pick 2 to 3 colonies and inoculate each colony in 3 mL of 2x PY and grow overnight at 37 °C in a shaking incubator at 180 rpm.

2.2.2.3. Purify the plasmid using a plasmid purification kit according to the manufacturer's instructions.

2.2.3. Linearize plasmid pRN1120 with *Eco*RI-HF and *Xho*I. For this, prepare a digestion mix composed of 1 µg of pRN1120, 5 µL of 10x buffer (1x buffer contains 50 mM potassium acetate, 20 mM Tris-acetate, 10 mM magnesium acetate, 100 µg/mL bovine serum albumin [BSA]; pH 7.9), 1 µL of *Eco*RI-HF (20 U), 1 µL of *Xho*I (20 U) and ultrapure H_2O up to a total volume of 50 µL. Incubate the digestion mix at 37 °C for 2 h and inactivate at 65 °C for 20 min.

2.2.4. Analyze the linearized plasmid by electrophoresis on an agarose gel (0.8%, 40 min, 5 V/cm) using a DNA loading dye and DNA ladder with DNA fragments in a range of 100 to 10,000 bp. As a control include a circular plasmid in the analysis.

2.2.5. Purify the linearized plasmid using a PCR purification kit according to the instructions of the manufacturer.

3. Preparation of Promoter-ORF-Terminator (POT) donor DNA constructs

3.1. Order a set of promoter (P) of different strength, open reading frame (O) and terminator (T) sequences as synthetic DNA such that each element contains standardized 4-bp recognition sequences that are flanked by *Bsa*I sites to enable Golden Gate Cloning (GGC) assembly[26] (see the detailed designs in **Supplementary Table 3** and sequences in Supplementary **Table 4**).

3.2. Assemble POT expression cassettes composed of a promoter, open reading frame, terminator and connectors sequences via a 4-part assembly using a GGC reaction[21], into a destination vector that already contains pre-specified 50-bp connectors sequences (see **Supplementary Table 4** and references[26,27]).

3.2.1. Measure the concentration of DNA parts using a spectrophotometer. Dilute each DNA part in ultrapure H_2O to a final concentration of 15 fmol/µL.

3.2.2. Prepare a reaction mix composed of DNA fragments: 2 µL of promoter, 2 µL of open reading frame, 2 µL of terminator and 2 µL backbone (Level 1 destination vectors as described in [26]), 4 µL of 5x T4 DNA ligase buffer, 2.5 µL of 1 U/µL T4 DNA Ligase, 1.5 µL of 20 U/µL *Bsa*I-HF and ultrapure H_2O up to a total volume of 20 µL.

3.2.3. Perform the GGC reaction in a thermocycler using the following program: (i) 37 °C for 2 min, (ii) 16 °C for 5 min – repeat steps (i) and (ii) 50 times, (iii) 50 °C for 60 min, (iv) 80 °C for 45 min, (v) hold at 12 °C until further analysis.

3.3. Transform 25 µL of purchased chemically competent *E. coli*[28] cells with 3 µL of the GGC reaction mix according to manufacturer's protocol. Dilute the transformation mix 10 and 50 times in 2x PY. Plate out 10x and 50x dilutions on 2x PY agar plates containing ampicillin (0.1 g/L) and incubate overnight at 37 °C.

3.4. Pick 2 to 3 colonies and inoculate each colony in 3 mL of 2x PY and grow overnight at 37 °C in a shaking incubator at 180 rpm.

3.5. Purify the plasmids using a plasmid purification kit according to manufacturer's instructions.

3.6. Check if POT expression cassettes were assembled correctly in the GGC reaction by PCR.

3.6.1. Design primers complementary to the connector sequence present at the start and the end of each expression cassette (see **Figure 2B**). For connectors chosen in this protocol use primers KC-103 to KC-108 (see **Supplementary Table 2**).

3.6.2. Prepare PCR amplification mixes for each plasmid containing: 0.5 µL of proofreading DNA polymerase, 10 µL of 5x buffer required for the DNA polymerase, 1 µL of 10 mM dNTPs, 2.5 µL of 10 µM forward primer, 2.5 µL of 10 µM reverse primer, 2 µL of DNA template with a concentration of 5 ng/µL, and ultrapure H_2O up to a total volume of 50 µL.

3.6.3. Perform the PCR reaction in a thermocycler using the following program: (i) 98 °C 3 min, (ii) 98 °C for 10 s, (iii) 60 °C for 20 s, (iv) 72 °C for 2 min 30 s – repeat steps (ii) to (iv) 30 times, (v) 72 °C for 5 min, (vi) hold at 12 °C until further analysis.

NOTE: Resulting PCR products consist of 50-bp of the 5' connector, promoter, open reading frame, terminator and 50-bp of the 3' connector.

3.7. Analyze the PCR products by electrophoresis by running samples on a 0.8% agarose gel at 5 V/cm for 40 min using a DNA loading dye and DNA ladder with DNA fragments in a range of 100 to 10,000 bp.

4. Preparation of integration flank DNA sequences containing connectors sequences

4.1. Purify genomic DNA from wild type *S. cerevisiae* CEN.PK113-7D[29].

4.1.1. Grow the strain in a 500 mL shake flask filled with 100 mL of yeast extract peptone dextrose (YEPD, 2% glucose) medium at 30 °C and shaking at 250 rpm for 48 hours.

4.1.2. Harvest the cells by centrifugation of 2 mL of broth at 16,000 x *g* for 1 min and discard the supernatant.

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.

4.1.4. Add 300 µL of cell lysis solution (see **Table of Materials**) and vortex shortly.

4.1.5. Add 168 µL of protein precipitation solution (see **Table of Materials**) and vortex vigorously for 20 s.

4.1.6. Separate the protein fraction by centrifugation at 16,000 x *g* and 4 °C for 10 min. Collect 600 µL of supernatant in a new tube and mix with 600 µL of isopropanol and vortex shortly.

4.1.7. Recover DNA by spinning down at 16,000 x *g* at room temperature for 10 min. Discard the supernatant and keep the pellet.

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 the step 4.1.8. Do not dry the pellet for longer than 10 min to prevent decreased solubility of the DNA.

4.1.9. Dissolve DNA in 50 µL of TE buffer. Store purified DNA at –4 °C.

4.2. For each integration site, design integration flank DNA sequences (approx. 500 bp) such that approximately 1000 bp of genomic DNA will be removed upon introduction of donor DNA (see the schematic design in **Figure 2B** and sequences in **Supplementary Table 4**).

4.3. Design primers to generate the flanking regions by PCR.

4.3.1. For the **left flanking region**, design forward and reverse primers to amplify approximately 500 bp of the genomic DNA region positioned 5' (left) of the integration site of interest.

NOTE: The forward primer includes 20 bp of homology with the intended flanking region. The reverse primer includes 20 bp with homology with the intended flanking region and contains the desired 50-bp connector sequence to enable in vivo assembly in the Cas12a editing on the genome later on.

4.3.2. For the **right flanking region**, design forward and reverse primers to amplify approximately 500 bp of the genomic DNA region positioned 3' (right) of the integration site of interest.

NOTE: The forward primer includes 20 bp with homology with the intended flanking region and contains the desired 50-bp connector sequence to enable in vivo assembly in the Cas12a editing on the genome later on. The reverse primer includes 20 bp of homology with the intended flanking region.

4.4. Amplify the flanking regions with the designed primers (e.g., primers KC-109 to KC-120 enclosed in **Supplementary Table 2**).

4.4.1. Measure the concentration of purified genomic DNA that will serve as the template in the PCR. Adjust the DNA concentration to 50 ng/µL.

4.4.2. Prepare PCR amplification mixes composed of genomic DNA (1 – 4 µL of 50 ng/µL genomic DNA dilution) purified in step 4.1, forward and reverse primer (10 µM each), 1 µL of 10 mM dNTPs, 10 µL of 5x buffer required for the DNA polymerase, 0.5 µL of DNA polymerase (1.0 U), and ultrapure H_2O up to total volume of 50 µL.

4.4.3. Perform PCRs in a thermocycler using the following program: (i) 98 °C for 3 min, (ii) 98 °C for 20 s, (iii) 60 °C for 20 s, (iv) 72 °C for 15 s, repeat steps (ii) to (iv) 30 times, (v) 72 °C for 5 min, (vi) hold at 12 °C until further analysis.

4.5. Analyze the PCR products by electrophoresis on a 0.8% agarose gel at 5 V/cm for 40 min using a DNA loading dye and DNA ladder with DNA fragments in a range of 100 to 10,000 bp.

4.6. Purify the correct PCR products using a PCR purification kit according the instructions of the manufacturer.

5. Transformation to *S. cerevisiae*

NOTE: Perform transformation using a protocol based on the methods developed by Gietz et al. (1995)[30] and Hill et al.[31] which can be used for various strains of *S. cerevisiae*. The protocol described below is sufficient for 1 transformation.

5.1. Prepare solutions required for transformation.

5.1.1. Prepare the following stock solutions and filter-sterilize:10x TE buffer containing 100 mM Tris-HCl (pH 7.5), 10 mM EDTA, total volume of 50 mL; 1 M LiAc at pH 7.5, total volume of 50 mL. 50% PEG 4000, total volume of 100 mL.

NOTE: Always check that PEG 4000 stock is at pH 5. This stock should not be stored longer than one month.

5.1.2. Prepare the following solutions using stocks: Prepare LiAc-TE solution containing 0.1 M LiAc, 10 mM Tris-HCl, 1 mM EDTA, total volume of 0.5 mL. Prepare PEG-LiAc-TE solution containing 40% PEG 4000, 0.1 M LiAc, 10 mM Tris-HCl, 1 mM EDTA, total volume of 1 mL.

NOTE: It is crucial for successful transformation that PEG-LiAc-TE and LiAc-TE solutions are freshly prepared.

5.2. First transformation round (prepare the strain pre-expressing Cas12a).

NOTE: In all the transformation steps, use water with a pH higher than 5. It is recommended to use demineralized water in all the steps of the transformation.

5.2.1. Prepare a pre-culture by growing strain CEN.PK113-7D in a 100 mL shake flask containing 20 mL of YEPD (2% glucose) medium and incubate overnight at 30 °C with shaking at 250 rpm.

5.2.2. Measure the OD_{600} of the pre-culture (OD_{pc}). 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 (OD_{tc}) to be 1.0 after the incubation step described in 5.2.3 (*ti*).

$$df = \frac{\mathrm{OD_{pc}}}{\mathrm{OD_{tc}}} \cdot 2^{\frac{\mathrm{ti}}{\tau}},$$

where *ti* and τ are the incubation time and doubling time, respectively.

5.2.2.1. Calculate the volume of the pre-culture (V_i) required for inoculation of the transformation culture (V_{tc}) based on the dilution factor.

$$V_{\mathrm{i}} = \frac{V_{\mathrm{tc}}}{df}$$

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.

5.2.4. Measure the OD_{600} of the transformation culture until an OD_{600} of 1.0 is reached.

5.2.5. Harvest the cells by centrifugation of the 20 mL broth at 2,500 x *g* for 5 min. Discard the supernatant and wash the cells in 20 mL of room temperature demineralized water. Repeat the centrifugation step and keep the cell pellet.

5.2.6. Resuspend the cells in 100 µL of LiAc-TE solution and transfer to a microcentrifuge tube.

5.2.7. Add 5 µL of single-stranded carrier DNA (10 mg/mL salmon sperm DNA) and mix by pipetting.

5.2.8. Pipette 1 µg of plasmid pCSN067 to the microcentrifuge tube.

NOTE: The total volume of the DNA mixture should not exceed 100 µL to prevent a lower transformation efficiency.

5.2.9. Add 600 µL of PEG-LiAc-TE solution and mix by pipetting. Incubate for 30 min at 30 °C while shaking at 450 rpm in a table top heat block.

5.2.10. Add 70 µL of DMSO (100%) to the transformation mixture and mix by pipetting. Perform heat-shock by incubating the transformation mixture at 42 °C for 15 minutes in a water bath.

5.2.11. Recover the cells by transferring the mixture to a 15 mL round bottom tube and add 10 mL of YEPD (2% glucose) to the tube. Incubate overnight at 30 °C with shaking at 250 rpm.

5.2.12. Centrifuge the transformation mix at 2,500 x *g* for 5 min. Discard the supernatant and resuspend the cell pellet in approximately 200 µL of the remaining solution.

5.2.13. Plate out 150 µL of the transformation mix and a 20x dilution in YEPD (2% glucose) of transformation mix on YEPD (2% glucose) agar plates supplemented with 0.2 g/L G418. Incubate the plates at 30 °C for 48 – 72 hours.

5.2.14. Pick a single transformant and re-streak on a YEPD (2% glucose) agar plate supplemented with 0.2 g/L G418 to obtain single colonies.

5.3. Second transformation round (perform multiplex genome editing with CRISPR/Cas12a).

5.3.1. Prepare a pre-culture by growing the strain pre-expressing Cas12a, created in the first transformation round (step 5.2), in a 100 mL shake flask containing 20 mL of YEPD (2% glucose) medium. Incubate overnight at 30 °C with shaking 250 rpm.

NOTE: For multiple transformations, adapt the volume of the pre-culture.

5.3.2. Follow the steps 5.2.2 to 5.2.7 for the first transformation round.

NOTE: For multiple transformations, adapt the volumes of required solutions and culture of the strain pre-expressing Cas12a.

5.3.3. 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 (step 4.3) in a microcentrifuge tube.

NOTE: The total volume of the DNA mixture should not exceed 100 µL to prevent a lower transformation efficiency.

5.3.4. Prepare the following controls for the transformation: negative control (ultrapure H_2O); positive control for determination of the transformation efficiency (1 µg of circular pRN1120); a control verifying if introduction of donor DNA is conducted via CRISPR editing (1 µg of circular pRN1120, 1 µg of all donor DNA expression cassettes and 1 µg of flanking regions but no single crRNA array); control verifying if donor DNA can be integrated outside of target (1 µg of linearized pRN1120, 1 µg of donor DNA expression cassettes and 1 µg of the single crRNA array but no flanking regions); a control verifying full linearization of pRN1120 (1 µg of linearized pRN1120).

5.3.5. Follow the steps 5.2.9 to 5.2.12 for the first transformation round.

5.3.6. Plate out 150 µL of the transformation mix and 20x dilution in YEPD (2% glucose) of transformation mix on YEPD (2% glucose) agar supplemented with 0.2 g/L G418 and 0.2 g/L NTC. Plate out controls on YEPD (2% glucose) agar supplemented with the appropriate selection (G418 and/or NTC or no selection). Incubate the plates at 30 °C for 48 – 72 hours.

5.3.7. Pick a single colored transformant and re-streak on a YEPD (2% glucose) agar plate to obtain single colored colonies.

6. Evaluation of the genome editing efficiency

6.1. Count the number of colored colonies and white colonies on the transformation plates.

6.2. Calculate genome editing efficiency by dividing the number of colored colonies by the total number of colonies (both white and colored), as shown in **Table 1**.

7. Confirmation of integration of donor DNA at the intended loci

7.1. Re-streak a colored single colony from a transformation plate on a YEPD (2% glucose) agar plate without G418 and NTC selection and incubate for 48 hours at 30 °C.

7.2. Pick a single colony and inoculate a 500 mL shake flask filled with 100 mL of YEPD (2% glucose) medium. Incubate for 48 hours at 30 °C and shaking at 250 rpm.

7.3. Isolate the genomic DNA as described in Section 4.1.

NOTE: Alternatively, use a protocol for preparation of yeast for colony PCR previously proposed by Looke et al.[32]. In this case, growth in liquid medium (Section 7.2) can be skipped.

7.4. Verify correct integration by amplification of two fragments per integrated expression cassette.

7.4.1. Design primers which anneal to genomic DNA outside of the transformed flanking regions and the gene of interest (see examples in **Supplementary Table 2**, KC-121 to KC-132). When using primers KC-121 to KC-132, set the annealing temperature in the PCR program to 62 °C.

7.4.2. Amplify region of interest as described in Section 4.4.2. Adapt the PCR program, specifically adjust the time of the extension step in PCR according to the length of the template and manufacturer's recommendations for the DNA polymerase.

7.5. Check the size of the PCR products by electrophoresis on an agarose gel (0.8%, 40 min, 5 V/cm) using a DNA loading dye and DNA ladder with DNA fragments in a range of 100 to 10,000 bp.

8. Creation of yeast pixel art using an acoustic liquid handler

8.1. Prepare a picture template for the yeast pixel art.

8.1.1. Resize the original RGB picture (220 × 280 pixels, see the representative results), e.g. using ImageJ to create a final 64 × 96 pixels (width × height) grey-scale image visualized in intended colors (Representative Results).

8.1.2. Convert the RGB picture into grey-scale using this formula:

$$I_{\mathrm{gr}} = \frac{(I_{\mathrm{r}}+I_{\mathrm{g}}+I_{\mathrm{b}})}{3},$$

where I_{gr}, I_r, I_g, I_b are the grey, red, green and blue intensities, respectively.

8.1.3. In order to categorize the pixels, develop an ImageJ plugin applying the following rules: (a) If I_{gr} is ≤ 64, use the dark orange yeast (strain 1, **Supplementary Table 3**) for this pixel. (b) If 64 < I_{gr} ≤ 128, use the orange yeast (strain 2, **Supplementary Table 3**) for this pixel. (c) If 128 < I_{gr} ≤ 192, use the yellow yeast (strain 3, **Supplementary Table 3**) for this pixel. (d) If I_{gr} > 192, use the white yeast (CEN.PK113-7D) for this pixel.

8.2. Spot yeast cells to create the yeast pixel art.

8.2.1. Inoculate 500 mL shake flasks containing 100 mL of YEPD (2% glucose) medium with three differently colored carotenoid producing *S. cerevisiae* strain and wild type CEN.PK113-7D. Incubate cultures overnight at 30 °C with shaking at 250 rpm.

8.2.2. Transfer 0.5 mL of the overnight culture to a tube filled with 0.5 mL of sterile non-ionic density gradient medium (see the **Table of Materials**). Mix by vortexing briefly.

8.2.3. Transfer the cell suspension to a qualified reservoir, 2 x 3 well. Perform spotting using an acoustic liquid handler instrument from a qualified reservoir source plate to a microplate (see the **Table of Materials**) containing 50 mL of YEPD (2% glucose) agar. To simplify plating, define wells on plate, e.g. use a microplate as a 6144 well plate (64 × 96).

8.2.4. Spot 25 nL of each *S. cerevisiae* strain from the 2x 3 well reservoir source plate using a .csv file with the fluid calibration setting **6RES_AQ_GPSA2** onto the destination microplate. Define each of these 25 nL droplets as a pixel in the 64 x 96 grid which is translated to the well positions (A01, B01, C01 *etc*.).

8.2.5. Incubate the microplate at 30 °C for 48 hours. To intensify the colors of the strains store the agar plate at 4 °C for at least 72 hours.

REPRESENTATIVE RESULTS:

The protocol for multiplex genome editing using CRISRP/Cas12a was demonstrated by constructing three carotenoid producing *S. cerevisiae* strains expressing the *crtE*, *crtYB* and *crtI* genes using heterologous promoters of high, medium and low strength: strain 1, 2 and, 3 respectively (**Supplementary Table 3**). Construction of these strains required generation of three donor DNA expression cassettes and six flanking regions per strain for targeting to three different loci in genomic DNA (shown in **Figure 2B**). As described herein, promoter, open reading frame, terminator and two contiguous 50-bp connectors sequences were assembled into an expression cassette via a Golden Gate Cloning reaction and the assembly was verified by PCR (**Figure 3A**). The single crRNA array was ordered as a synthetic DNA fragment and was amplified by PCR (**Figure 3B**). The recipient plasmid for the single crRNA array (plasmid pRN1120) was linearized with *Eco*RI-HF and *Xho*I and linearization was confirmed by electrophoresis (**Figure 3C**). The design and nucleotide sequences of the introduced donor DNA expression cassettes and flanking regions are shown in **Supplementary Table 3 and Supplementary Table 4**. The sequence of single crRNA array expression cassettes is provided in **Supplementary Table 1**. Functionality of the spacers included in the single crRNA array was tested beforehand by singleplex genome editing with individual crRNAs[19].

The efficiency of genome editing using Cas12a was firstly evaluated based on the number of colored colonies obtained after transformation (**Table 1, Figure 4**). The editing efficiency of the three constructed strains varied from 50% to 94%. Notably, introduction of expression cassettes used to generate strain 1 displayed the lowest editing efficiency, possibly caused by the nature of the donor DNA (i.e., these expression cassettes encode *crtE*, *crtYB* and *crtI* from three high strength promoters). Secondly, correct integration of the three donor DNA expression cassettes at the intended loci on the genomic DNA was confirmed by PCR (**Figure 5**). Primers were designed in such a way that PCR products were obtained when correct integration of donor DNA at the intended locus occurred. For each transformation experiment, eight colonies were picked from the transformation plate and tested (note that only three are presented in **Figure 5**). In general,

out of 8 colonies tested per donor DNA, correct integration of the *crtE* donor DNA at the INT1 locus, *crtYB* at the INT2 locus and *crtI* at the INT3 locus was confirmed in >90% of the transformants. These results demonstrate the CRISPR/Cas12a system in combination with a single crRNA array enables efficient multiplex editing of the *S. cerevisiae* genome at multiple loci simultaneously.

Additionally, we demonstrate the creation of "yeast pixel art" using the three carotenoid producing strains that were constructed together with a non-colored wild-type strain. Starting from a black and white picture of Rosalind Franklin (**Figure 6A**), a 4-color picture (**Figure 6B**) and spotting list was created which was then used to spot the four different yeast strains on an agar microplate using an acoustic liquid handler, resulting in a high-resolution "yeast painting" of Rosalind Franklin (**Figure 6C,D,E**).

FIGURE AND TABLE LEGENDS:

Figure 1: Workflow of the protocol for CRISPR/Cas12a multiplex genome editing in *S. cerevisiae*. The workflow includes crucial steps of the presented method. For details see the Protocol.

Figure 2: Scheme of CRISPR/Cas12a multiplex genome editing using a single crRNA array. (**A**) The single crRNA array is composed of three crRNAs units in their mature form, a 20-bp direct repeat specific for LbCas12a (grey squares) with a 23-bp guide sequence (colored diamonds). Expression of the crRNA array is enabled by the *SNR52* promoter and *SUP4* terminator. Transformation of *S. cerevisiae* with a linearized pRN1120 and the single crRNA array expression cassette containing homology with pRN1120 (diagonal stripes) allows for in vivo recombination into a circular plasmid in cells pre-expressing LbCas12a. The single crRNA array is subsequently processed by Cas12a. (**B**) Cas12a is directed to the intended INT1, INT2 and INT3 genomic target sites and creates double stranded breaks. In the transformation mixture, donor DNA consisting of flanking regions and the carotenoid gene expression cassette were included. Donor DNA assemblies were targeted to one stretch of DNA in genomic DNA around the INT1 (*crtE*), INT2 (*crtYB*) and INT3 (*crtI*) loci by in vivo recombination due to the presence of 50-bp homologous connectors sequences, indicated as 5, A, B, C, D or E. P1–P3, different promoters; T1–T3, different terminators. This Figure has been modified from Verwaal et al. 2018[19]. Genetic constructs shown using Synthetic Biology Open Language (SBOL) Visual symbols[40].

Figure 3: PCR verifying the genome editing experiments. (**A**) Verification of Golden Gate Cloning reactions of assembled donor DNA cassettes. Obtained results are in agreement with expected lengths. (**B**) PCR of the single crRNA array. (**C**) Linearization of plasmid pRN1120.

Figure 4: Plates of *S. cerevisiae* transformations using the multiplex genome editing approach. (**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).

Figure 5: PCR verifying integration of the donor DNA expression cassettes at the intended loci within the genomic DNA. (**A**) Verification of three colonies of the strain 1. (**B**) Verification of three colonies of the strain 2. (**C**) Verification of three colonies of the strain 3.

Figure 6: Yeast pixel art of Rosalind Franklin. (**A**) Black and white RGB photo of 220 × 280 pixels of Rosalind Franklin that was used as a template. (**B**) Computer conversion of the black and white photo of Rosalind Franklin into a 4-color 64 × 96 pixel list. (**C**) Photo of yeast pixel art with 64 × 96 yeast colonies with a zoomed-in section. (**D**) Photo of an acoustic liquid handler with two full grown plates. (**E**) Photo of a full grown microplate with 64 × 96 yeast colonies.

Table 1: Editing efficiency of the multiplex genome editing approach.

Supplementary Table 1: Single crRNA array for LbCas12a containing homology with plasmid pRN1120.

Supplementary Table 2: Primer sequences.

Supplementary Table 3: Design of constructed strains.

Supplementary Table 4: Sequences of donor DNA expression cassettes and flaking regions.

DISCUSSION:

The provided protocol describes multiplex genome editing of *S. cerevisiae* using Cas12a from *Lachnospiraceae bacterium* ND2006 in combination with a single crRNA array and donor DNA. Design of the single crRNA array and donor DNA is explained in detail. In contrast to the well-established CRISPR/Cas9 system, the CRISPR/Cas12a has the unique additional ability of processing multiple crRNAs expressed from a single crRNA array[13,33]. Due to this feature, simultaneous editing of multiple targets is easier to set up and can be achieved in a single transformation. This single crRNA array approach was demonstrated before by Zetsche et al.[34] who simultaneously edited up to four genes in mammalian cells using AsCas12a, and by Swiat et al.[35] for the introduction of four DNA fragments into a yeast genome using FnCas12a. To our knowledge, a higher number of simultaneous genomic modifications using a Cas12a system has not been reported and the maximal limit of targets per single array for Cas12a is yet to be determined. Further research utilizing single crRNA arrays in combination with Cas12a includes multiplex transcriptional regulation in a wide range of organisms[33,36,37].

There are some critical steps in the presented protocol. Carefully design all DNA sequences that are involved in the Cas12a genome editing experiment, especially in case when novel DNA sequences are introduced. Determine the functionality of new spacer sequences part of a crRNA, for example by a singleplex genome editing experiment as described by Verwaal et al.[19] before combining them into a single crRNA array. Follow the recommendations for the preparation of transformation buffer solutions used in the Cas12a editing experiment to achieve a good transformation efficiency of yeast.

There are some optional modifications of the technique. It is recommended to 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 a satisfactory transformation efficiency. Perform a test transformation to determine whether lower DNA amounts can be used. 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. (2007)[38]. The guide RNA recipient plasmid pRN1120 is suitable for the expression of a single crRNA and single crRNA array of different Cas12a variants (e.g., from *Acidaminococcus spp.* BV3L6 or *Francisella novicida* U112) as well as for expression of sgRNA in combination with Cas9[19]. The donor DNA does not need to be limited to carotenoid gene expression cassettes and flanking regions that target donor DNA to the described INT1, INT2 and INT3 sites in genomic DNA. Any DNA of interest can be introduced, in a multiplex manner, into genomic DNA of the host by the design principles described in this protocol, or alternatively donor DNA can be used to delete DNA from a host genome. The modular structure of single crRNA array facilitates easy adjustment of spacer and direct repeat sequences. Modification of spacer sequences allows for a change of the intended integration locus which can be designed by one of the tools for identification of a genomic target site, e.g. GuideScan software 1.0[39]. Instead of using large flanking sequences that contain connectors sequences, 50-bp of the flanking region can be included in the donor DNA sequences by incorporating these 50-bp flanking region sequences in the primers used in the PCR. In this case, in total just three instead of nine donor DNA fragments are required for a successful multiplex genome editing experiment.

In summary, this protocol provides step-by-step directions to perform multiplex genome editing in *S. cerevisiae* using Cas12a in combination with a single crRNA array approach. The protocol was demonstrated by multiplex genome editing using 9 donor DNA fragments and single crRNA array coding for three gRNAs. We show high overall editing frequencies between 50 and 94% for the three strain designs reported here. Concluding, the unique feature of Cas12a is the ability to process a single crRNA array into individual crRNAs in a cell, which makes Cas12a an excellent tool to enable multiplex genome editing and develop transcriptional regulation modules targeting multiple expression cassettes in one go. In the end, three strains were obtained producing carotenoids at a different level and colors in shades between yellow and orange. With those strains and a wild-type strain, we showed how an acoustic liquid handler can be used straightforwardly to make yeast pixel art – this in honor of Rosalind Franklin who contributed to the discovery of the DNA structure 65 years ago by her famous photo 51[23].

ACKNOWLEDGMENTS:

This project received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement no. 686070 (DD-DeCaf) and 764591 (SynCrop), and from the research programme Building Blocks of Life with project number 737.016.005 by the Netherlands Organisation for Scientific Research (NWO). T.E.G. was supported by the Royal Society (grant UF160357) and BrisSynBio, a BBSRC/EPSRC Synthetic Biology Research Centre (grant BB/L01386X/1). We thank Zi Di and Jeffrey van Wijk for their contribution to the yeast spotting experiments for creating the yeast pixel art.

DISCLOSURES:

The authors declare that there is a conflict of interest. The authors have filed IP related to presented methods.

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Figure 1

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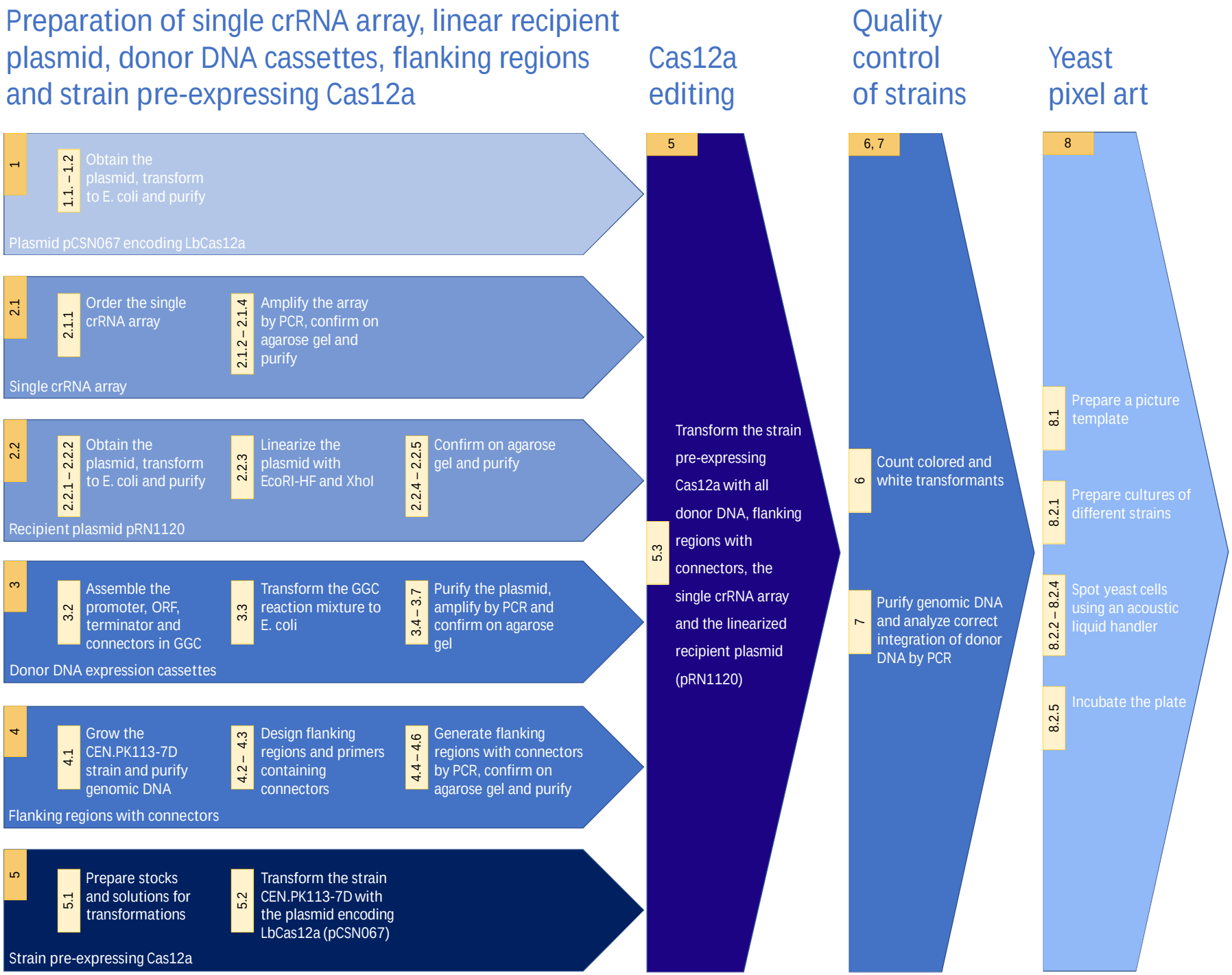
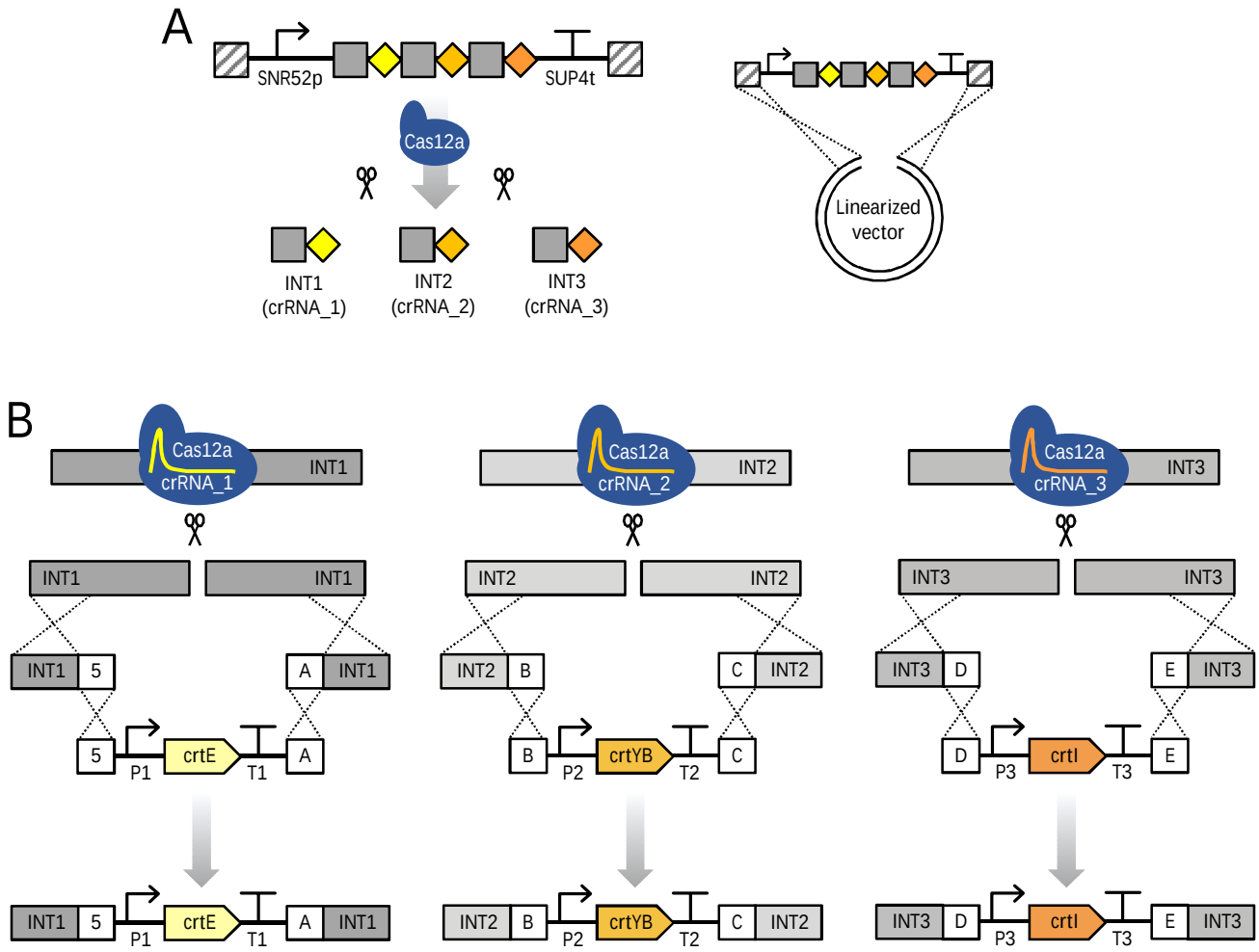


Figure 2



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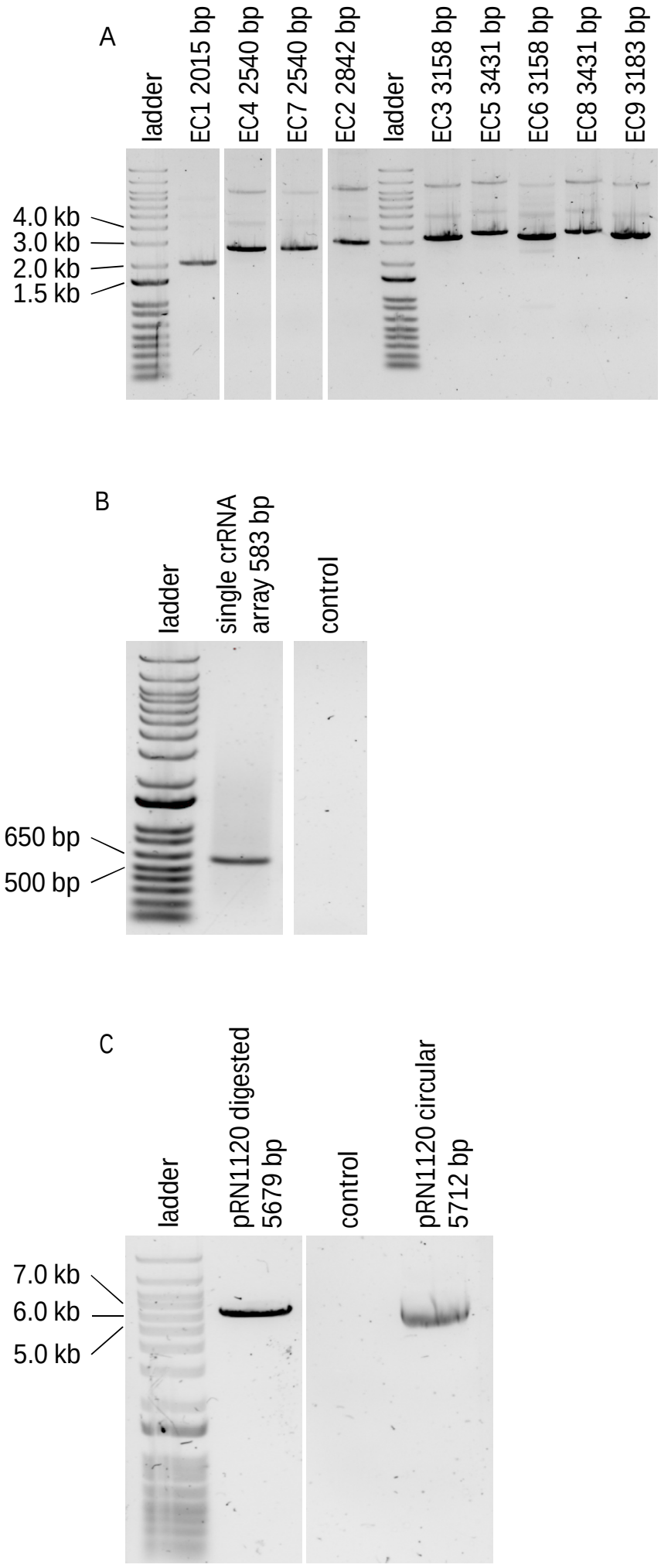

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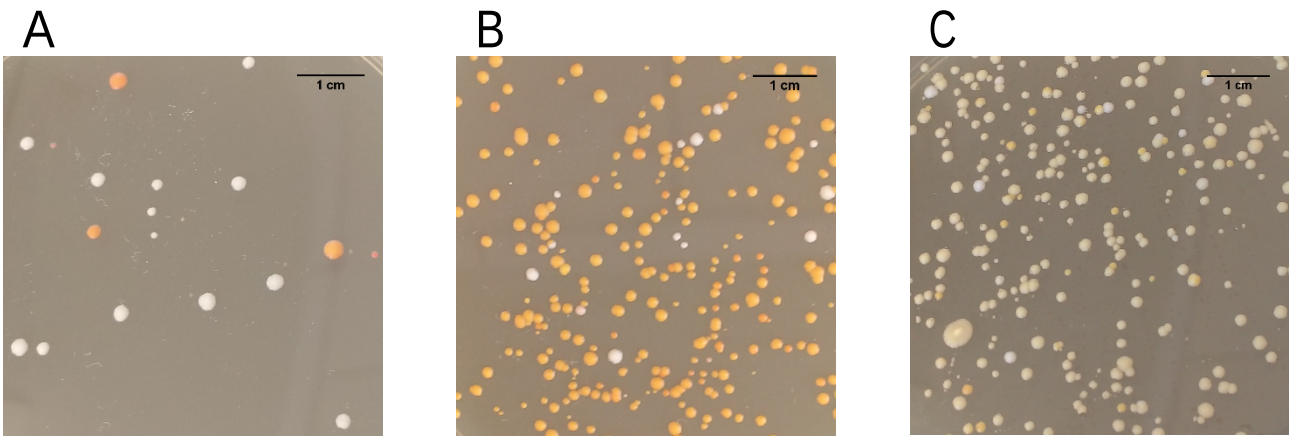

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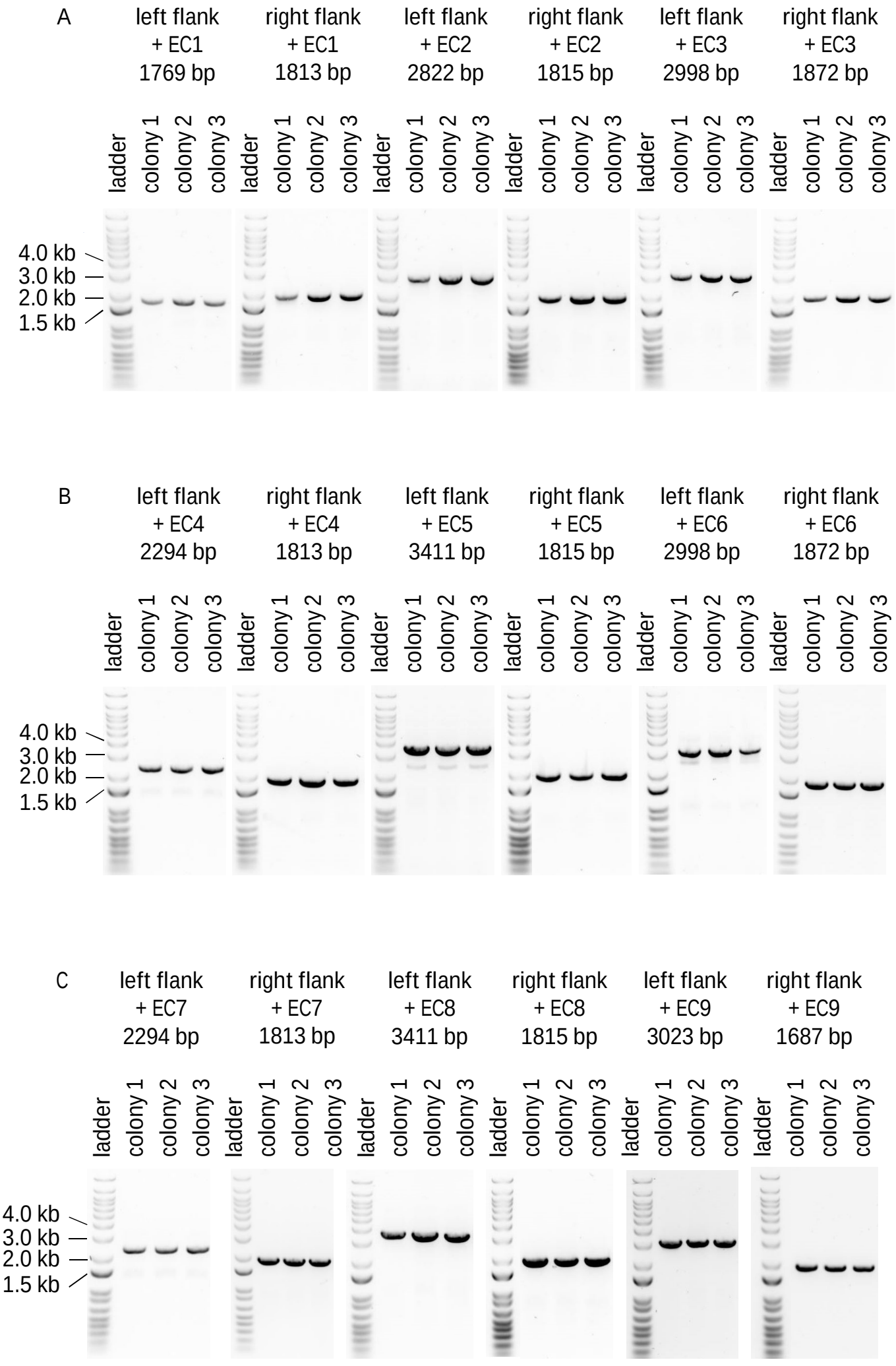

Figure 6

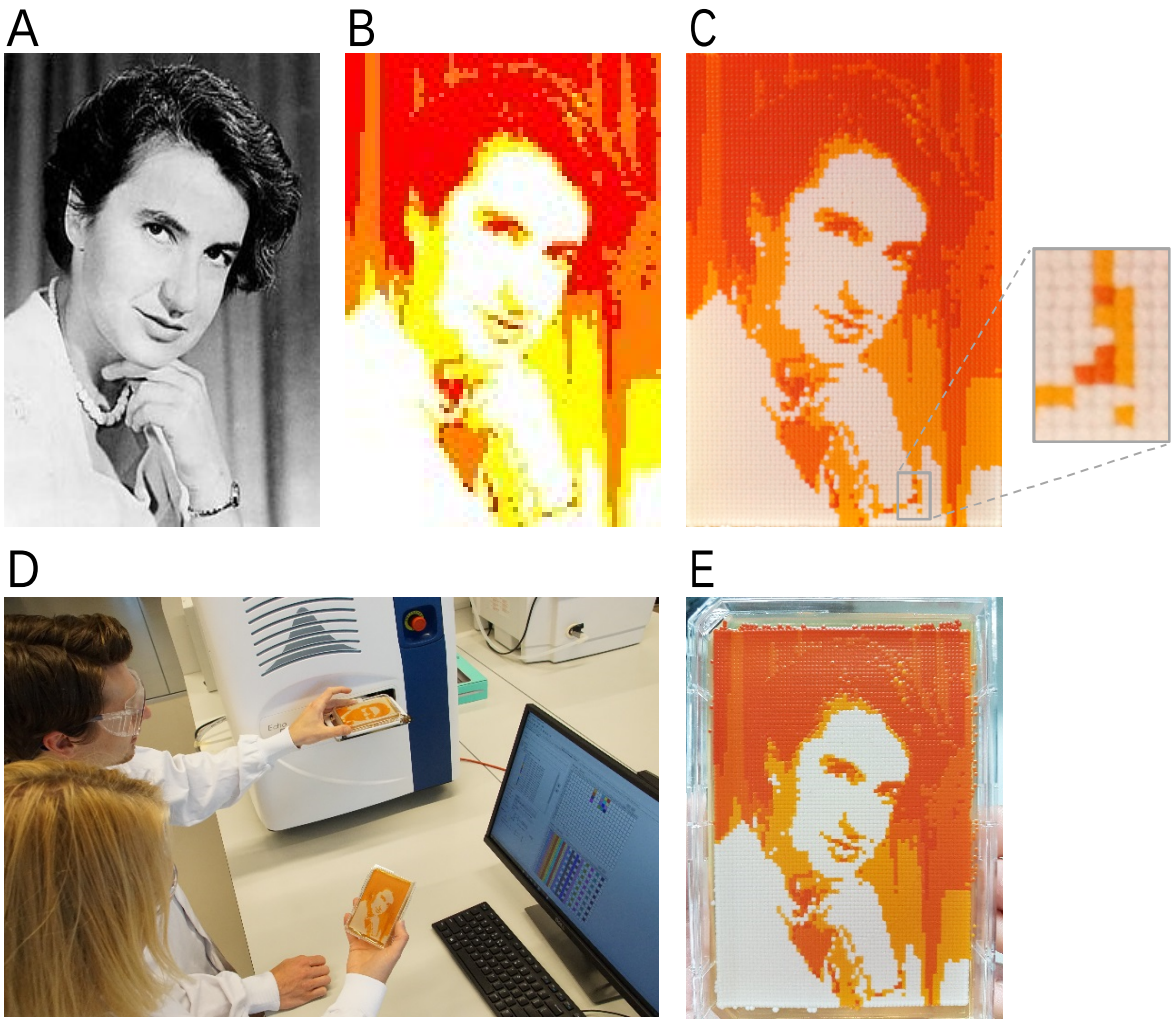

Table 1

	Strain 1	Strain 2	Strain 3
Colored colonies	16	279	220
White colonies	16	18	18
Total colonies	32	297	238
Efficiency	50%	94%	92%

Name of Material or Equipment	Company	Catalog Number	Comments
Chemicals specific for the protocol			
1 Kb Plus DNA Ladder	Thermo Fisher Scientific	10787018	Electrophoresis
Ampicillin sodium salt	Sigma Aldrich	A9518	Selection of *E. coli* transformants
BsaI-HF (20 U/µl)	New England BioLabs	R353L	Golden Gate Cloning
Cell Lysis Solution (from kit Puregene Yeast/Bact. Kit B)	QIAGEN	854016	Isolation of genomic DNA from *S. cerevisiae*
CutSmart Buffer	New England BioLabs	B7204S	Linearization of pRN1120
Deoxyribonucleic acid sodium salt from salmon testes	Sigma Aldrich	D1626	Transfromation of *S. cerevisiae* (carrier DNA)
dNTPs	Invitrogen	10297018	PCRs
EcoRI-HF	New England BioLabs	R3101S	Linearization of pRN1120
Ethanol absolute for analysis	Merck	100983	Isolation of genomic DNA from *S. cerevisiae*
Ethylenediamine-tetraacetic acid	Sigma Aldrich	ED	Transformation of *S. cerevisiae*
G418 disulfate salt	Sigma Aldrich	A1720	Selection of *S. cerevisiae* transformants
Histodenz	Sigma Aldrich	D2158	Yeast pixel art
Isopropanol	Merck	100993	Isolation of genomic DNA from *S. cerevisiae*
Lithium acetate dihydrate	Sigma Aldrich	L6883	Transformation of *S. cerevisiae*
Nancy-520 DNA Gel Stain	Sigma Aldrich	1494	Electrophoresis
NEB10 competent *E. coli* cells	New England BioLabs	C3019H	Transformation of *E. coli:* dx.doi.org/10.17504/protocols.io.nkvdcw6
Nourseothricin	Jena Bioscience	AB102	Selection of *S. cerevisiae* transformants
Phusion buffer	New England BioLabs	M0530L	PCRs
Phusion High-Fidelity DNA Polymerase	New England BioLabs	M0530L	PCRs
Polyethylene glycol 4000	Merck	7490	Transformation of *S. cerevisiae*
Protein Precipitation Solution (10 M NH4AC) (from kit Puregene Yeast/Bact. Kit B)	QIAGEN	854016	Isolation of genomic DNA from *S. cerevisiae*
Purple loading dye	New England BioLabs	B7024S	Electrophoresis
QIAprep Spin Miniprep Kit	QIAGEN	27106	Purification of plasmids
RNase coctail enzyme mix	Thermo Fisher Scientific	AM2286	Isolation of genomic DNA from *S. cerevisiae*

T4 DNA ligase buffer	Invitrogen	46300-018	Golden Gate Cloning
T4 DNA Ligase (1 U/µl)	Invitrogen	1705218	Golden Gate Cloning
UltraPure Agarose	Invitrogen	16500500	Electrophoresis
Wizard SV Gel and PCR Clean-Up System Kit	Promega	A9282	Purification of PCR products and linearized pRN1120
XhoI	New England BioLabs	R0146S	Linearization of pRN1120
Zymolyase 50 mg/ml (5 units/µL)	Zymo Research	E1006	Isolation of genomic DNA from *S. cerevisiae* (yeast lysis enzyme)
Zymolyase storage buffer	Zymo Research	E1004-B	Isolation of genomic DNA from *S. cerevisiae* (necessary for the preparation of yeast lysis enzyme)
Chemicals of general use			
2*Peptone-Yeast extract (PY) agar			Plate growth of *E. coli*
2*PY medium			Cultivation of *E. coli*
Demineralized water			Transformation of *S. cerevisiae*
ELFO buffer			Electrophoresis
MQ			Multiple steps
Physiological salt solution			Transformation of *S. cerevisiae*
TE buffer			Storage of DNA, transformation of *S. cerevisiae*
Yeast extract-peptone-dextrose (YEPD; 2% glucose) medium			Cultivation of *S. cerevisiae*
YEPD (2% glucose) agar			Plate growth of *S. cerevisiae*
Consumables			
Eppendorf tubes			
Falcon tubes (50 mL)			
Microplate 96 wells			
Petri dishes			
Pipette tips 0.5 - 10 µL			
Pipette tips 10 - 200 µL			
Pipette tips 100 - 1000 µL			
Shake flasks (500 mL)			
Sterile filters			
Equipment			
Centrifuge (Falcon tubes)			
Echo 525 acoustic liquid handler			
Incubator			

NanoDrop			
Set for eletrophoresis			
Spectrophotometer			
Table centrifuge (Eppendorfs tubes)			
Thermocycler			
Plasmids			
pCSN067	Addgene	ID 101748	https://www.addgene.org/
pRN1120	Addgene	ID 101750	https://www.addgene.org/
Strains			
S. cerevisiae strain CEN.PK113-7D	EUROSCARF collection		http://www.euroscarf.de

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Title of Article:	CRISPR/Cas12a multiplex genome editing of Saccharomyces cerevisiae and the creation of yeast pixel art
Author(s):	Klaudia Ciurkot, Brenda Vonk, Thomas E. Gorochowski, Johannes A. Roubos, René Verwaal

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Date:	29-10-15

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612542.6 For questions, please contact us at submissions@jove.com or +1.617.945.9051.

RV
29/10/15.

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*., 2018[19]" 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.

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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 1[10,11]” 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 subtypes[10]. 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 2[10,11]".

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 constructed[19] 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 described[26] 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 by Gietz *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.* 2007[37]”).

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 OD_{600} 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 OD_{600} of the pre-culture (OD_{pc}). 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 (OD_{tc}) to be 1.0 after the incubation step described in 5.2.3 (*ti*).

$$df = \frac{\mathrm{OD_{pc}}}{\mathrm{OD_{tc}}} \cdot 2^{\frac{\mathrm{ti}}{\tau}}$$

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.

$$V_{\mathrm{i}} = \frac{V_{\mathrm{tc}}}{df}$$

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”.

crRNA array sequence[a,b,c,d,e,f]
CATGTTTGACAGCTTATCATCGATAATCCGGAGCTAGCATGCGGCCGCTCTAGAACTAGTGGA TCCCCCGGGCTGCAG*TCTTTGAAAAGATAATGTATGATTATGCTTTCACTCATATTTATACAG AAACTTGATGTTTTCTTTCGAGTATATACAAGGTGATTACATGTACGTTTGAAGTACAACTCT AGATTTTGTAGTGCCCTCTTGGGCTAGCGGTAAAGGTGCGCATTTTTTCACACCCTACAATGT TCTGTTCAAAAGATTTTGGTCAAACGCTGTAGAAGTGAAAGTTGGTGCGCATGTTTCGGCGTT CGAAACTTCTCCGCAGTGAAAGATAAATGATC**AATTTCTACTAAGTGTAGAT**CTGGTGGGAGA GAAAGCTTATGA**AATTTCTACTAAGTGTAGAT**GTGCCGTACGCCGGAGCCGACGG**AATTTCTA CTAAGTGTAGAT**TGCCCCTCTTATACGATTATATT*TTTTTTTGTTTTTTATGTCT***GGGGGGCC CGGTACCCAGCTTTTGTTCCCTTTAGTGAGGGTTAATTCCGAGCTTGGCGTAATCATGGTCAT AGCTGTTTCCTGTGTG**

a. Homology to pRN1120 (bold).
b. SNR52 promoter (italics).
c. Genomic target sequences (underlined).
d. Guide direct repeats specific for LbCas12a (italics, bold).
e. SUP4 terminator (italics).
f. Homology to pRN1120 (bold).

Name	Sequence[a]	Description[b]	Used in point
KC-101	CATGTTTGACAGCTTATCATC	FW primer for amplification of single crRNA array	2.1.4
KC-102	CACACAGGAAACAGCTATGAC	RV primer for amplification of single crRNA array	2.1.4
KC-103	AAGCGACTTCCAATCGCTTTGC	FW primer for amplification of donor DNA with connector 5	3.6.1
KC-104	AAAGCAAAGGAAGGAGAGAAC	RV primer for amplification of donor DNA with connector A	3.6.1
KC-105	CGGATCGATGTACACAACCG	FW primer for amplification of donor DNA with connector B	3.6.1
KC-106	CAACAGGAGGCGGATGGATATAC	RV primer for amplification of donor DNA with connector C	3.6.1
KC-107	AACGTTGTCCAGGTTTGTATCC	FW primer for amplification of donor DNA with connector D	3.6.1
KC-108	AGGTACAACAAGCACGACCG	RV primer for amplification of donor DNA with connector E	3.6.1
KC-109	CACTATAGCAATCTGGCTATATG	FW primer for amplification of INT1 5' with connector 5	4.4
KC-110	**AAACGCCTGTGGGTGTGGTACTGGATA TGCAAAGCGATTGGAAGTCGCTT**GACT CCTCTGCCGTCATTCC	RV primer for amplification of INT1 5' with connector 5	4.4
KC-111	**TTGCCCATCGAACGTACAAGTACTCCT CTGTTCTCTCCTTCCTTTGCTTT**AAGC GTTGAAGTTTCCTCTTTG	FW primer for amplification of INT1 3' with connector A	4.4
KC-112	TGTCAACTGGAGAGCTATCG	RV primer for amplification of INT1 3' with connector A	4.4
KC-113	AGAAGATTTCTCTTCAATCTC	FW primer for amplification of INT2 5' with connector B	4.4
KC-114	**TGCTAAGATTTGTGTTCGTTTGGGTGC AGTCGGTTGTGTACATCGATCCG**CCCT TATCAAGGATACCTGGTTG	RV primer for amplification of INT2 5' with connector B	4.4
KC-115	**ACGCTTTCCGGCATCTTCCAGACCACA GTATATCCATCCGCCTCCTGTTG**GGCG ATTACACAAGCGGTGG	FW primer for amplification of INT2 3' with connector C	4.4
KC-116	TCTCCTCTTCGATGACCGGG	RV primer for amplification of INT2 3' with connector C	4.4
KC-117	GGTCGTTTTTGTGCAGCATATTG	FW primer for amplification of INT3 5' with connector D	4.4
KC-118	**GCGGAATATTGGCGGAACGGACACACG TGGATACAAACCTGGACAACGTT**TTCC AAGGAGGTGAAGAACG	RV primer for amplification of INT3 5' with connector D	4.4

KC-119	**AAATAACCACAAACATCCTTCCCATATGCTCGGTCGTGCTTGTTGTACCT**GATGGGACGTCAGCACTGTAC	FW primer for amplification of INT3 3' with connector E	4.4
KC-120	GAGCTTACTCTATATATTCATTC	RV primer for amplification of INT3 3' with connector E	4.4
KC-121	GTTACTAAACTGGAACTGTCCG	FW primer for verification of integration of con5-*crtE* -conA to INT1 5'	7.4.1
KC-122	CACTGCTAACTACGTTTACTTC	FW primer for verification of integration of con5-*crtE* -conA to INT1 3'	7.4.1
KC-123	CACTGGAACTTGAGCTTGAG	FW primer for verification of integration of conB-*crtYB* -conC to INT2 5'	7.4.1
KC-124	GTCTCCAGCTGAATTGGTCC	FW primer for verification of integration of conB-*crtYB* -conC to INT2 3'	7.4.1
KC-125	CTCTCATGAAGCAGTCAAGTC	FW primer for verification of integration of conD-*crtI* -conE to INT3 5'	7.4.1
KC-126	GATCGGTCAATTAGGTGAAG	FW primer for verification of integration of conD-*crtI* -conE to INT3 3'	7.4.1
KC-127	CCTTGTCCAAGTAGGTGTCC	RV primer for verification of integration of con5-*crtE* -conA to INT1 5'	7.4.1
KC-128	GCTGTCATGATCTGTGATAAC	RV primer for verification of integration of con5-*crtE* -conA to INT1 3'	7.4.1
KC-129	CTGGCAATGTTGACCAATTGC	RV primer for verification of integration of conB-*crtYB* -conC to INT2 5'	7.4.1
KC-130	CCAACGTGCCTTAAAGTCTG	RV primer for verification of integration of conB-*crtYB* -conC to INT2 3'	7.4.1
KC-131	CCTTACCTTCTGGAGCAGCAG	RV primer for verification of integration of conD-*crtI* -conE to INT3 5'	7.4.1
KC-132	CTGGTTACTTCCCTAAGACTG	RV primer for verification of integration of conD-*crtI* -conE to INT3 3'	7.4.1

a. Bold sequences denote connector sequences.
b. Forward and reverse primers are designated as FW and RV, respectively.

Expression cassette	Integration site (left)	Left connector	Promoter	ORF	Terminator	Right connector	Integration site (right)
Strain 1							
EC 1	INT1	con5	Sbay_TDH3	crtE	Sc_TDH3	conA	INT1
EC 2	INT2	conB	Smik_TEF1	crtYB	Sc_PDC1	conC	INT2
EC 3	INT3	conD	Kl_ENO1	crtI	Sc_TAL1	conE	INT3
Strain 2							
EC 4	INT1	con5	Kl_PGK1	crtE	Sc_TDH3	conA	INT1
EC 5	INT2	conB	Kl_TEF2	crtYB	Sc_PDC1	conC	INT2
EC 6	INT3	conD	Kl_OLE1	crtI	Sc_TAL1	conE	INT3
Strain 3							
EC 7	INT1	con5	Kl_TDH2	crtE	Sc_TDH3	conA	INT1
EC 8	INT2	conB	Kl_YDR1	crtYB	Sc_PDC1	conC	INT2
EC 9	INT3	conD	Kl_LEU2	crtI	Sc_TAL1	conE	INT3

Integration flank DNA sequences	
Name	**Purpose**
INT1 5'-con5	Multiplex array
CACTATAGCAATCTGGCTATATGATATGCAGAAAATAGTGATTACTGTGCTCTTTGCTGCATC ATGCATCATATGTTTGGCATTCTGTCCTGTCAGATGAATGGGCGAAACAATTCGGGGAGCTTT GTTGCGACTTGGTACCCGGCAAGCCGCGACCTATATTTTGCATTAAACGAGTTCATCGTGGAT TTATCGCCATGAGCCTTAGCTTATCAAGCCTCTCACAGACAATTAAGCAATGAAAAAGGTATA CCATCGGCGCAGAATGGTTAACTAGTGGGTTCATACTGCTGTGTTATAGATTGTTACCTAAGT GATCACCAAAAAAAAGTGCAAAAAGGAAAAAAAAATAAGAGACAGGTAACTTCCACAAGCTTA TTCTTCCAAAAATCAATCTTATCTTCATGCCAGCAATAGTTGCGTGCTGAGCTCAACAGTGCC CAACCCTTGTGCACCGTAGAATTGTAGAATACAAATACATAAATAAGTGTGTTCCCGAAGGAC TAAGGAATGACGGCAGAGGAGTC**AAGCGACTTCCAATCGCTTTGCATATCCAGTACCACACCC ACAGGCGTTT**	
conA-INT1 3'	Multiplex array
TTGCCCATCGAACGTACAAGTACTCCTCTGTTCTCTCCTTCCTTTGCTTTAAGCGTTGAAGTT TCCTCTTTGTATATTTGAGATCTTCATTTTATCGGATTCTTTGTCATCAGACAACTTGTTGAG TGGTACTAAAGGAGTGCTTTTCATCATCCTTTTGGTGAACGATTTCAAATACGTTAGTGTTTT CTGAGCTAGTTTTGATCAATTCAGGTGATTCGTTATCAGAACTCTCAGGTTTGTATTCGTGTC CAGTTGTGTAGCATTCGCCTAACGTGTAAGCACGGATTTCTTCCTCAGAAATTTCACTGTATG GAATCATGCCCTTCTTTCTCGCTTCTTCGTCGGTAAATGCACCATAGTAATCTTTGTCATCAT GTCTAACAGTAATTTTGAATGGGAAGAAGACACATAGCCCCCAGTAAACGAAAAAAGAAATCA AAAAGGAGAAGAAAGAATCACCATAAAAGAATTTAACAATACCTGAGTCGTGGAAATAGTTAT TGTTGACTTCCCAAGCGATACCAGGTAGACCGGGAGCCATACCACACACCCAGGCAACGATAG CTCTCCAGTTGACA	
INT2 5'-conB	Multiplex array
AGAAGATTTCTCTTCAATCTCCTTCTCCTTAACACTTAATTTTGGCACTTCGTCAACTTTAAT TTTCTTGGAAGCCATTGCTATGTATTCTTAGTGTACTGCGACTTGGGTTTCTTTCTTGCTTTG TATAAAAGTGCGATGAGTTCAATCATCAAGTGAATTTTTTTTTTTCAGCCTGCATTGAAAAAA AAAAAATTGTATGCTCGAGCGATGGGAAGGTTTTTTCCTCTTCCCGTTCAAAGCTTCCTCGGA CCAACTCCACTTTTTCTCGGCCCGTTGTAAAGAAATGGGTAAGAGAACTAAATTGCAATAAGC TTCGAATTATCGTTTTGATCCAGACATAAAGAGATGTACAAACTACAAAACAAGAGTAAAATA ACTCGAACGAAGGGTACACCTTCTGTAATAAAACAAATTCACTATAACTGCCTTATTGGAGTG AAAAAACAAGATTATGTAGACCCCCTCTCGCGATAAAGAACATTAATGACTGCAACACCCGCA CACCACAAGGTGAAGGGCAAAGGTGAATTAACCAAAGTGAAGAGGACGACGTAGAATCAACCA GGTATCCTTGATAAGGG**CGGATCGATGTACACAACCGACTGCACCCAAACGAACACAAATCTT AGCA**	
conC-INT2 3'	Multiplex array
ACGCTTTCCGGCATCTTCCAGACCACAGTATATCCATCCGCCTCCTGTTGGGCGATTACACAA GCGGTGGCGTCTTGAAAAAAGTTGGGTTTCTTATCTGTACGGCGATCACTCCCCTCATATGAT AGCTGTGTAGAGTGACTTGCTCTTTGATTGGCCTTACTTCTTGCATCTATCATCCTTTCTTTT	

ACTCTCTCCAATCACCACAACTCATATCATTCTACATTTCATTTTATTGTTCTGTACAATTTT
TAGGTTTGTAAAATATATAAGTGATGTACATATCTTCTATATCCTTTCCGTTTGTGTCTATTT
TAGGGTTGGCTTATTACGCATTATTCCTACTTTATTTTTACTTGCTTACTTGAAAAAGAAGGT
ATACCACAGTGCTGCTCCACTGCAAACAATATAAAATATTATCAATTATTTTTTTTTTCTTC
TTTTGAATTAGATCACTAATAAACTCTTATAATGCTCAACCATCCCAGCCAAGGCTCAGATGA
TGCTCAGGACGAAAAGCAAGGCGACTTCCCGGTCATCGAAGAGGAGA

INT3 5’-conD	Multiplex array

GGTCGTTTTTGTGCAGCATATTGTCCTCTAGATGCAAACTCTGCAGGTCCATTTGCAGTAAAG
TGAGTTGCCTCTCGAAGAATCATTAATTTCGTATAACCGTCACTATTAAAGTCAGAAAATAAA
TTCTGTCGTAGACAATGTTACCATAATGTTCTTGTCCATTTTGCATACACTTTAAATATTCAT
TTGATTTCTCAGGGTTCATGATCATAATAAATTGCGCATTCGCAAGGCGGTAGTATTATAATG
GGGTCCATCATTCTGTAGCAAGAAGTTACAGTACGCTGTTCAAGCGTTAAACAAGATAAGTAA
TCTCGAATGAAACATTCATATTTCGCATGAGCCAACATACAGTTGCTGAGTAATCTTCATTGC
GCTTATTTATCGGCATTGAGATTGTAAAGGAAGTAAAACGCATTTTTGCAGATCTGTTCTCTT
ATGTATTTTTAATCGTCCTTGTATGGAAGTATCAAAGGGGACGTTCTTCACCTCCTTGGAA**AA**
CGTTGTCCAGGTTTGTATCCACGTGTGTCCGTTCCGCCAATATTCCGC

conE-INT3 3’	Multiplex array

AAATAACCACAAACATCCTTCCCATATGCTCGGTCGTGCTTGTTGTACCTGATGGGACGTCAG
CACTGTACTTGTTTTTGCGACTAGATTGTAAATCATTCTTTATTTAATCTCTTTCTTTAACTA
CTGCTTAAAGTATAATTTGGTCCGTAGTTTAATAACTATACTAAGCGTAACAATGCATACTGA
CATTATAAGCCTGAACATTACGAGTTTAAGTTGTATGTAGGCGTTCTGTAAGAGGTTACTGCG
TAAATTATCAACGAATGCATTGGTGTATTTGCGAAAGCTACTTCTTTTAACAAGTATTTACAT
AAGAATAATGGTGATCTGCTCAACTGATTTGGTGATAACTCTAACTTTTTTAGCAACAATTTA
AAAGATAATTCGAACATATATAACAGTAGGAAGAATTTGTGTACGTCAAATTAAGATAATTTA
GCATTACCAAAGTTATTAACCTAAACATAAAATATATATGAGACACATGTGGAAATCGTATGA
AACAACTGTTATGAAACTGACAAGAATGAATATATAGAGTAAGCTC

Donor DNA expression cassette sequences	
Name	**Purpose**
crtE expression cassette: con5-Sbay_TDH3p-crtE-Sc_TDH3t-conA	High strength promoter for expression of crtE, multiplex array

AAGCGACTTCCAATCGCTTTGCATATCCAGTACCACACCCACAGGCGTTTGTGCCATTCATCT
TTCACCTGCCATTAGTAACCCGACTTCTCATTGAGCGGGTTACGGCAGCCACAGGCCACATTC
CGAATGTCTGGGTGAGCGGTCCCTTTTCCAGCATCCACTAAATATCTCGGATCCCGCTTTTTA
ATCTGGCTTCCTGAAAAAAATCAATGGAGTGATGCAAACTGACTGGAGCAAAAAGCTGACACA
AGGCAATCGACCTACGTGTCTGTCTATTTTCTCACACCTTCTATTACCTTCTAACTCTCTGGG
TTGGAAAAAACTGAAAAAAAGGTTGTCTCCAGTTTCCACAAATCATCCCCCTGTTTGATTAAT
AAATATATAAAGACGACAACTATCGATCATAAACTCATAAAACTATAACTCCTTTACACTTCT
TATTTTATAGTTATTCTATTTTAATTCTTATTGATTTTAAAACCCCAAGAACTTAGTTTCGAA
AACACACACACACAAACAATTAAAAATGGACTACGCTAACATCTTGACTGCCATTCCTTTGGA
ATTCACCCCACAAGATGACATTGTCTTGTTGGAACCATACCACTACTTAGGTAAGAACCCAGG
TAAGGAAATCAGATCTCAATTGATTGAAGCTTTCAACTACTGGTTAGATGTCAAGAAGGAAGA

CTTGGAAGTTATCCAAAATGTTGTTGGTATGTTGCACACCGCTTCTTTGTTGATGGATGATGT
TGAAGATTCTTCCGTCTTGAGAAGAGGTTCTCCAGTTGCTCATTTGATCTACGGTATTCCACA
AACCATCAACACTGCTAACTACGTTTACTTCTTGGCTTACCAAGAAATCTTCAAATTGCGTCC
AACTCCAATTCCAATGCCAGTTATCCCACCATCTTCTGCTTCTTTGCAATCTTCTGTCTCCTC
CGCCTCCTCTTCCTCTTCTGCCTCCTCTGAAAACGGTGGTACCTCCACTCCAAACTCCCAAAT
CCCATTCTCCAAGGACACCTACTTGGACAAGGTTATCACTGACGAAATGTTGTCTTTGCACCG
TGGTCAAGGTTTGGAATTATTCTGGAGAGACTCTTTGACCTGTCCATCTGAAGAAGAATACGT
CAAGATGGTCTTGGGTAAGACCGGTGGTTTGTTCAGAATTGCTGTCAGATTGATGATGGCCAA
GTCTGAATGTGACATTGACTTTGTTCAATTGGTTAACTTGATTTCCATCTACTTCCAAATCAG
AGATGACTACATGAACTTGCAATCCTCTGAATACGCTCACAACAAGAACTTCGCTGAAGACTT
GACTGAAGGTAAGTTCTCCTTCCCAACCATTCACTCCATTCACGCTAACCCATCTTCCAGATT
GGTTATCAACACTTTACAAAAGAAGTCCACTTCTCCAGAAATCTTACATCACTGTGTCAACTA
CATGAGAACTGAAACCCACTCTTTCGAATACACTCAAGAAGTCTTGAACACTTTATCTGGTGC
TTTGGAAAGAGAATTGGGTAGATTACAAGGTGAATTTGCTGAAGCTAACTCCAAGATCGATTT
GGGTGACGTTGAATCTGAAGGTAGAACCGGTAAGAACGTCAAATTGGAAGCCATCTTGAAGAA
ATTGGCTGATATCCCTCTATAAAGTGAATTTACTTTAAATCTTGCATTTAAATAAATTTTCTT
TTTATAGCTTTATGACTTAGTTTCAATTTATATACTATTTTAATGACATTTTCGATTCATTGA
TTGAAAGCTTTGTGTTTTTTCTTGATGCGCTATTGCATTGTTCTTGTCTTTTTCGCCACATGT
AATATCTGTAGTAGATACCTGATACATTGTGGATGCTGAGTGAAATTTTAGTTAATAATGGAG
GCGCTCTTAATAATTTTGGGGATATTGGCTTTTTTTTTTAAAGTTTACAAATGAATTTTTTCC
GCCAGGATCCTC**TTGCCCATCGAACGTACAAGTACTCCTCTGTTCTCTCCTTCCTTTGCTTT**

crtE expression cassette: con5-Kl_PGK1p-crtE-Sc_TDH3t-conA	Medium strength promoter for expression of crtE, multiplex array

AAGCGACTTCCAATCGCTTTGCATATCCAGTACCACACCCACAGGCGTTTGTGCGTTCCTCAT
CACTAGAAGCCGAACTGTTGTCTTCAGTGGGGATTGGTTCGACATTTTGCCAATTGCTGTCGA
TGTACCCTTTCAAAGCCATGTACCTTAAATCTTCATCCTTGGCAAGTAGATTCATCGGGTGTG
TTTGAAGTAAGAATATTTGCTTGTTTTTATGGTATCAAAGGTATATGTTGTAGAAGACAATTT
CCGGTAATCCAATTGTCTGTCTGCTCAGTTTAGCACATGTATAGTACGTTGCACATAGTCTAC
AATATTCAGCATTCAGCATTCAGTATACAGCATATGGCTAAATGATCACAAATGTGATTGATG
ATTTGACACGACTAGAAAAGAGAACGAAAAAGGGAAATTCCATGTCACGTGCGTTGGCACGTG
ACATGGAATATCGAAGAAAGAAAAAAAAAACGATCTCGTCCTAGTGGAAGCCCAGAGTCTGGT
CCCCCCGGAGTCTTCCCAAAACAAGAAGCTGACACATGTTGACACAGAACACCCCACAGCAAA
TGCACCACGCTACGTAGATCAGGAAGCTTAACTCTAGCGACCTGTCGCTCGCCCCACAGAACC
TCACCCGAGAACCACACATTACACGCCGCCAGCTCCCACTATACTCATCTTGCTTCCCTTAAG
CGTTCTCACGATTCGTTCGCTGCCCTTCTTCAAGAGTCTTCTGATTCTAATTCTCATTCGAAA
TCCTCTACAGTTAATGAATTGCTTGACATGACATTCATTGTCTCATGGTTTTGGCTTTTTGGC
TTTTGTCTTTTTAAAGCTATATCAACTTTACATATAAATATACGTCAAAAGGGGATTCATTAAT
TAGAAAATTCTCTTTTTCAATAGTTGCTATTCATTATCAATCTATTCAACTCAATTGGTTATT
ATTTTCATCTTTTTGTCATCCTAAACCATCAACAATATTTAAATATATCTGTTGCTACATTAA
GAGTTACTTCAGAAATAACAAAAAAATCGATCAAGAATTAATAAAAATGGACTACGCTAACAT
CTTGACTGCCATTCCTTTGGAATTCACCCCACAAGATGACATTGTCTTGTTGGAACCATACCA
CTACTTAGGTAAGAACCCAGGTAAGGAAATCAGATCTCAATTGATTGAAGCTTTCAACTACTG
GTTAGATGTCAAGAAGGAAGACTTGGAAGTTATCCAAAATGTTGTTGGTATGTTGCACACCGC
TTCTTTGTTGATGGATGATGTTGAAGATTCTTCCGTCTTGAGAAGAGGTTCTCCAGTTGCTCA
TTTGATCTACGGTATTCCACAAACCATCAACACTGCTAACTACGTTTACTTCTTGGCTTACCA
AGAAATCTTCAAATTGCGTCCAACTCCAATTCCAATGCCAGTTATCCCACCATCTTCTGCTTC

TTTGCAATCTTCTGTCTCCTCCGCCTCCTCTTCCTCTTCTGCCTCCTCTGAAAACGGTGGTAC
CTCCACTCCAAACTCCCAAATCCCATTCTCCAAGGACACCTACTTGGACAAGGTTATCACTGA
CGAAATGTTGTCTTTGCACCGTGGTCAAGGTTTGGAATTATTCTGGAGAGACTCTTTGACCTG
TCCATCTGAAGAAGAATACGTCAAGATGGTCTTGGGTAAGACCGGTGGTTTGTTCAGAATTGC
TGTCAGATTGATGATGGCCAAGTCTGAATGTGACATTGACTTTGTTCAATTGGTTAACTTGAT
TTCCATCTACTTCCAAATCAGAGATGACTACATGAACTTGCAATCCTCTGAATACGCTCACAA
CAAGAACTTCGCTGAAGACTTGACTGAAGGTAAGTTCTCCTTCCCAACCATTCACTCCATTCA
CGCTAACCCATCTTCCAGATTGGTTATCAACACTTTACAAAAGAAGTCCACTTCTCCAGAAAT
CTTACATCACTGTGTCAACTACATGAGAACTGAAACCCACTCTTTCGAATACACTCAAGAAGT
CTTGAACACTTTATCTGGTGCTTTGGAAAGAGAATTGGGTAGATTACAAGGTGAATTTGCTGA
AGCTAACTCCAAGATCGATTTGGGTGACGTTGAATCTGAAGGTAGAACCGGTAAGAACGTCAA
ATTGGAAGCCATCTTGAAGAAATTGGCTGATATCCCTCTATAAAGTGAATTTACTTTAAATCT
TGCATTTAAATAAATTTTCTTTTTATAGCTTTATGACTTAGTTTCAATTTATATACTATTTTA
ATGACATTTTCGATTCATTGATTGAAAGCTTTGTGTTTTTTCTTGATGCGCTATTGCATTGTT
CTTGTCTTTTTCGCCACATGTAATATCTGTAGTAGATACCTGATACATTGTGGATGCTGAGTG
AAATTTTAGTTAATAATGGAGGCGCTCTTAATAATTTTGGGGATATTGGCTTTTTTTTTTAAA
GTTTACAAATGAATTTTTTCCGCCAGGATCCTC**TTGCCCATCGAACGTACAAGTACTCCTCTG**
TTCTCTCCTTCCTTTGCTTT

crtE expression cassette: con5-Kl_TDH2p-crtE-Sc_TDH3t-conA	Low strength promoter for expression of crtE, multiplex array

AAGCGACTTCCAATCGCTTTGCATATCCAGTACCACACCCACAGGCGTTTGTGCCGTAAAAAC
TAAAACGAGCCCCCACCAAAGAACAAAAAAGAAGGTGCTGGGCCCCCACTTTCTTCCCTTGCA
CGTGATAGGAAGATGGCTACAGAAACAAGAAGATGGAAATCGAAGGAAAGAGGGAGACTGGAA
GCTGTAAAAACTGAAATGAAAAAAAAAAAAAAAAAAAAAAACAAGAAGCTGAAAATGGAAGAC
TGAAATTTGAAAAATGGTAAAAAAAAAAAAGAAACACGAAGCTAAAAACCTGGATTCCATTTT
GAGAAGAAGCAAGAAAGGTAAGTATGGTAACGACCGTACAGGCAAGCGCGAAGGCAAATGGAA
AAGCTGGAGTCCGGAAGATAATCATTTCATCTTCTTTTGTTAGAACAGAACAGTGGATGTCCC
TCATCTCGGTAACGTATTGTCCATGCCCTAGAACTCTCTGTCCCTAAAAAGAGGACAAAAACC
CAATGGTTTCCCCAGCTTCCAGTGGAGCCACCGATCCCACTGGAAACCACTGGACAGGAAGAG
AAAATCACGGACTTCCTCTATTGAAGGATAATTCAACACTTTCACCAGATCCCAAATGTCCCG
CCCCTATTCCCGTGTTCCATCACGTACCATAACTTACCATTTCATCACGTTCTCTATGGCACA
CTGGTACTGCTTCGACTGCTTTGCTTCATCTTCTCTATGGGCCAATGAGCTAATGAGCACAAT
GTGCTGCGAAATAAAGGGATATCTAATTTATATTATTACATTATAATATGTACTAGTGTGGTT
ATTGGTAATTGTACTTAATTTTGATATATAAAGGGTGGATCTTTTTCATTTTGAATCAGAATT
GGAATTGCAACTTGTCTCTTGTCACTATTACTTAATAGTAATTATATTTCTTATTAACCTTTT
TTTTAAGTCAAAACACCAAGGACAAGAACTACTCTTCAAAGGTATTTCAAGTTATCATACGTG
TCACACACGCTTCACAGTTTCAAGTAAAAAAAAAGAATATTACACAATGGACTACGCTAACAT
CTTGACTGCCATTCCTTTGGAATTCACCCCACAAGATGACATTGTCTTGTTGGAACCATACCA
CTACTTAGGTAAGAACCCAGGTAAGGAAATCAGATCTCAATTGATTGAAGCTTTCAACTACTG
GTTAGATGTCAAGAAGGAAGACTTGGAAGTTATCCAAAATGTTGTTGGTATGTTGCACACCGC
TTCTTTGTTGATGGATGATGTTGAAGATTCTTCCGTCTTGAGAAGAGGTTCTCCAGTTGCTCA
TTTGATCTACGGTATTCCACAAACCATCAACACTGCTAACTACGTTTACTTCTTGGCTTACCA
AGAAATCTTCAAATTGCGTCCAACTCCAATTCCAATGCCAGTTATCCCACCATCTTCTGCTTC
TTTGCAATCTTCTGTCTCCTCCGCCTCCTCTTCCTCTTCTGCCTCCTCTGAAAACGGTGGTAC
CTCCACTCCAAACTCCCAAATCCCATTCTCCAAGGACACCTACTTGGACAAGGTTATCACTGA
CGAAATGTTGTCTTTGCACCGTGGTCAAGGTTTGGAATTATTCTGGAGAGACTCTTTGACCTG

TCCATCTGAAGAAGAATACGTCAAGATGGTCTTGGGTAAGACCGGTGGTTTGTTCAGAATTGC
TGTCAGATTGATGATGGCCAAGTCTGAATGTGACATTGACTTTGTTCAATTGGTTAACTTGAT
TTCCATCTACTTCCAAATCAGAGATGACTACATGAACTTGCAATCCTCTGAATACGCTCACAA
CAAGAACTTCGCTGAAGACTTGACTGAAGGTAAGTTCTCCTTCCCAACCATTCACTCCATTCA
CGCTAACCCATCTTCCAGATTGGTTATCAACACTTTACAAAAGAAGTCCACTTCTCCAGAAAT
CTTACATCACTGTGTCAACTACATGAGAACTGAAACCCACTCTTTCGAATACACTCAAGAAGT
CTTGAACACTTTATCTGGTGCTTTGGAAAGAGAATTGGGTAGATTACAAGGTGAATTTGCTGA
AGCTAACTCCAAGATCGATTTGGGTGACGTTGAATCTGAAGGTAGAACCGGTAAGAACGTCAA
ATTGGAAGCCATCTTGAAGAAATTGGCTGATATCCCTCTATAAAGTGAATTTACTTTAAATCT
TGCATTTAAATAAATTTTCTTTTTATAGCTTTATGACTTAGTTTCAATTTATATACTATTTTA
ATGACATTTTCGATTCATTGATTGAAAGCTTTGTGTTTTTTCTTGATGCGCTATTGCATTGTT
CTTGTCTTTTTCGCCACATGTAATATCTGTAGTAGATACCTGATACATTGTGGATGCTGAGTG
AAATTTTAGTTAATAATGGAGGCGCTCTTAATAATTTTGGGGATATTGGCTTTTTTTTTTAAA
GTTTACAAATGAATTTTTTCCGCCAGGATCCTC**TTGCCCATCGAACGTACAAGTACTCCTCTG**
TTCTCTCCTTCCTTTGCTTT

crtYB expression cassette: conB-Smik_TEF1p-crtYB-Sc_PDC1t-conC	High strength promoter for expression of crtYB, multiplex array

CGGATCGATGTACACAACCGACTGCACCCAAACGAACACAAATCTTAGCAGTGCATGCTTCAA
AAACGCACTGTACTCCTTTTTACTCTTCCGGATTTTCTCGCACTCTCCGCATCGCCGCACGAG
CCAAGCCACACCCACACACCTCATACCATGTTTCCCCTCTTTGTCTCTTTCGTGCGGCTCCAT
TACCCGCATGAAACTGTATAAAAGTAACAAAAGACTATTTCGTTTCTTTTTCTTTGTCGGAAA
AGGCAAAAAAAAAAAATTTTTATCACATTTCTTTTTCTTGAAAATTTTTTTTGGGATTTTTTCT
CTTTCGATGACCTCCCATTGATATTTAAGTTAATAAAAGCACTCCCGTTTTCCAAGTTTTAAT
TTGTTCCTCTTGTTTAGTCATTCTTCTTCTCAGCATTGGTCAATTAGAAAGAGAGCATAGCAA
ACTGATCTAAGTTTTAATTACAAAATGACCGCTTTGGCTTACTACCAAATCCACTTGATCTAC
ACTTTGCCAATCTTAGGTTTGCTAGGTTTGTTGACTTCTCCAATTTTGACCAAATTCGACATC
TACAAGATTTCTATCTTAGTCTTTATTGCTTTCTCTGCTACCACTCCATGGGACTCCTGGATC
ATCAGAAACGGTGCCTGGACCTACCCATCTGCTGAATCTGGTCAAGGTGTTTTCGGTACCTTT
TTGGATGTCCCATACGAAGAATACGCCTTCTTTGTTATCCAAACCGTCATCACCGGTTTGGTT
TACGTTTTGGCTACCAGACATTTGTTGCCATCTTTGGCTCTACCAAAGACCCGTTCTTCTGCC
TTGTCTCTAGCTTTGAAGGCTTTAATCCCATTGCCAATCATCTATTTGTTCACCGCTCATCCA
TCTCCATCCCCAGATCCTTTGGTTACTGACCACTACTTCTACATGAGAGCTTTGTCTTTGTTG
ATCACCCCACCAACCATGTTGTTGGCTGCTTTATCTGGTGAATACGCTTTCGACTGGAAATCT
GGTAGAGCTAAGTCCACCATTGCTGCCATCATGATCCCAACTGTCTACTTGATCTGGGTTGAC
TACGTTGCCGTTGGTCAAGACTCCTGGTCCATCAACGATGAAAAGATTGTCGGTTGGAGATTA
GGTGGTGTCTTGCCAATTGAAGAAGCTATGTTCTTCTTATTGACCAACTTGATGATCGTTTTG
GGTTTGTCTGCCTGTGACCACACTCAAGCCTTGTACTTGTTGCACGGTAGAACTATCTACGGT
AACAAGAAGATGCCATCTTCTTTCCCATTAATCACTCCACCAGTTTTGTCCTTGTTCTTCTCC
TCCAGACCATACTCCTCCCAACCAAAGAGAGATTTGGAATTGGCTGTCAAGTTGTTGGAAAAG
AAGTCCAGATCTTTCTTCGTTGCTTCTGCCGGTTTCCCATCTGAAGTCAGAGAAAGATTGGTT
GGTTTGTACGCTTTCTGTCGTGTCACCGATGACTTGATTGACTCTCCAGAAGTTTCCTCCAAC
CCACACGCTACCATTGACATGGTTTCCGATTTCTTGACTTTATTATTCGGTCCTCCATTGCAC
CCATCTCAACCAGACAAGATTTTGTCTTCTCCATTATTACCACCTTCCCACCCATCCAGACCA
ACTGGTATGTACCCATTACCACCACCTCCATCTTTGTCTCCAGCTGAATTGGTCCAATTCTTG
ACTGAACGTGTCCCAGTTCAATACCACTTCGCTTTCAGATTGTTGGCCAAATTGCAAGGTTTG
ATTCCAAGATACCCATTGGATGAATTATTGAGAGGTTACACCACTGACTTGATCTTCCCATTG

TCCACTGAAGCCGTCCAAGCTAGAAAGACCCCAATTGAAACTACTGCTGACTTGTTGGACTAC
GGTTTGTGTGTTGCCGGTTCTGTTGCTGAATTGTTGGTCTACGTTTCCTGGGCTTCCGCTCCA
TCCCAAGTTCCAGCTACTATTGAAGAAAGAGAAGCTGTTTTGGTCGCCTCTCGTGAAATGGGT
ACCGCTTTGCAATTGGTCAACATTGCCAGAGATATCAAGGGTGACGCTACTGAAGGTAGATTC
TACTTGCCATTGTCTTTCTTTGGTTTGAGAGATGAATCCAAATTGGCCATTCCAACTGACTGG
ACTGAACCAAGACCTCAAGATTTCGACAAATTGTTGTCTCTATCTCCATCTTCCACTTTACCA
TCCTCTAACGCTTCTGAATCCTTCAGATTCGAATGGAAGACCTACTCTTTGCCATTGGTTGCT
TACGCTGAAGATTTGGCTAAGCACTCTTACAAGGGTATTGACAGATTACCAACTGAAGTCCAA
GCTGGTATGAGAGCTGCTTGTGCTTCTTACTTGTTGATTGGTCGTGAAATCAAGGTTGTCTGG
AAGGGTGATGTCGGTGAAAGAAGAACCGTTGCTGGTTGGAGAAGAGTCAGAAAGGTTTTGTCT
GTTGTCATGTCCGGTTGGGAAGGTCAATAAAGCGATTTAATCTCTAATTATTAGTTAAAGTTT
TATAAGCATTTTTATGTAACGAAAAATAAATTGGTTCATATTATTACTGCACTGTCACTTACC
ATGGAAAGACCAGACAAGAAGTTGCCGACAGTCTGTTGAATTGGCCTGGTTAGGCTTAAGTCT
GGGTCCGCTTCTTTACAAATTTGGAGAATTTCTCTTAAACGATATGTATATTCTTTTCGTTGG
AAAAGATGTCTTCCAAAAAAAAAACCGATGAATTAGTGGAACCAAGGAAAAAAAAAGAGGTAT
CCTTGATTAAGGAACACCTC**ACGCTTTCCGGCATCTTCCAGACCACAGTATATCCATCCGCCT**
CCTGTTG

crtYB expression cassette: conB-Kl_TEF2p-crtYB-Sc_PDC1t-conC	Medium strength promoter for expression of crtYB, multiplex array

CGGATCGATGTACACAACCGACTGCACCCAAACGAACACAAATCTTAGCAGTGCGAGCCTGTC
CAAGCAAATGCCTTCTCATAAATGGTGCCAAAGACCCGCAAGCCCAAAGCAATTACCCCCCAA
AAAGAAATGATATAGTGCAAGATACGTATATGACCATGACTTGACTAGGTGAAACAGTGCAGA
AACAGCCGCACAAAAGCAGCCCTAACCCTCAGAGTCGATTTTACTCTTTCAGGTAATAAAGCC
TCGACATCAATTTTAGACAGAAGCCAGGCTGGCCTCGAGATTATAGCCATAGGCAAGCAAGAG
GAGAGAAGGGGAGGCCCCCCATGGGGGGCCTCCCCCCCGCTGTCAAGGTTTGGCAGAACCTAG
CTTCATTAGGCCACTAGCCCAGCCTAAAACGTCAACGGGCAGGAGGAACACTCCCACAAGACG
GCGTAGTATTCTCGATTCATAACCATTTTCTCAATCGAATTACACAGAACACACCGTACAAAC
CTCTCTATCATAACTACTTAATAGTCACACACGTACTCGTCTAAATACACATCATCGTCCTAC
AAGTTCATCAAAGTGTTGGACAGACAACTATACCAGCATGGATCTCTTGTATCGGTTCTTTTC
TCCCGCTCTCTCGCAATAACAATGAACACTGGGTCAATCATAGCCTACACAGGTGAACAGAGT
AGCGTTTATACAGGGTTTATACGGTGATTCCTACGGCAAAAATTTTTCATTTCTAAAAAAAAA
AAGAAAAATTTTTCTTTCCAACGCTAGAAGGAAAAGAAAAATCTAATTAAATTGATTTGGTGA
TTTTCTGAGAGTTCCCTTTTTCATATATCGAATTTTGAATATAAAAGGAGATCGAAAAAATTT
TTCTATTCAATCTGTTTTCTGGTTTTATTTGATAGTTTTTTTGTGTATTATTATTATGGATTA
GTACTGGTTTATATGGGTTTTTCTGTATAACTTCTTTTTATTTTAGTTTGTTTAATCTTATTT
TGAGTTACATTATAGTTCCCTAACTGCAAGAGAAGTAACATTAAAAATGACCGCTTTGGCTTA
CTACCAAATCCACTTGATCTACACTTTGCCAATCTTAGGTTTGCTAGGTTTGTTGACTTCTCC
AATTTTGACCAAATTCGACATCTACAAGATTTCTATCTTAGTCTTTATTGCTTTCTCTGCTAC
CACTCCATGGGACTCCTGGATCATCAGAAACGGTGCCTGGACCTACCCATCTGCTGAATCTGG
TCAAGGTGTTTTCGGTACCTTTTTGGATGTCCCATACGAAGAATACGCCTTCTTTGTTATCCA
AACCGTCATCACCGGTTTGGTTTACGTTTTGGCTACCAGACATTTGTTGCCATCTTTGGCTCT
ACCAAAGACCCGTTCTTCTGCCTTGTCTCTAGCTTTGAAGGCTTTAATCCCATTGCCAATCAT
CTATTTGTTCACCGCTCATCCATCTCCATCCCCAGATCCTTTGGTTACTGACCACTACTTCTA
CATGAGAGCTTTGTCTTTGTTGATCACCCCACCAACCATGTTGTTGGCTGCTTTATCTGGTGA
ATACGCTTTCGACTGGAAATCTGGTAGAGCTAAGTCCACCATTGCTGCCATCATGATCCCAAC
TGTCTACTTGATCTGGGTTGACTACGTTGCCGTTGGTCAAGACTCCTGGTCCATCAACGATGA

AAAGATTGTCGGTTGGAGATTAGGTGGTGTCTTGCCAATTGAAGAAGCTATGTTCTTCTTATT
GACCAACTTGATGATCGTTTTGGGTTTGTCTGCCTGTGACCACACTCAAGCCTTGTACTTGTT
GCACGGTAGAACTATCTACGGTAACAAGAAGATGCCATCTTCTTTCCCATTAATCACTCCACC
AGTTTTGTCCTTGTTCTTCTCCTCCAGACCATACTCCTCCCAACCAAAGAGAGATTTGGAATT
GGCTGTCAAGTTGTTGGAAAAGAAGTCCAGATCTTTCTTCGTTGCTTCTGCCGGTTTCCCATC
TGAAGTCAGAGAAAGATTGGTTGGTTTGTACGCTTTCTGTCGTGTCACCGATGACTTGATTGA
CTCTCCAGAAGTTTCCTCCAACCCACACGCTACCATTGACATGGTTTCCGATTTCTTGACTTT
ATTATTCGGTCCTCCATTGCACCCATCTCAACCAGACAAGATTTTGTCTTCTCCATTATTACC
ACCTTCCCACCCATCCAGACCAACTGGTATGTACCCATTACCACCACCTCCATCTTTGTCTCC
AGCTGAATTGGTCCAATTCTTGACTGAACGTGTCCCAGTTCAATACCACTTCGCTTTCAGATT
GTTGGCCAAATTGCAAGGTTTGATTCCAAGATACCCATTGGATGAATTATTGAGAGGTTACAC
CACTGACTTGATCTTCCCATTGTCCACTGAAGCCGTCCAAGCTAGAAAGACCCCAATTGAAAC
TACTGCTGACTTGTTGGACTACGGTTTGTGTGTTGCCGGTTCTGTTGCTGAATTGTTGGTCTA
CGTTTCCTGGGCTTCCGCTCCATCCCAAGTTCCAGCTACTATTGAAGAAAGAGAAGCTGTTTT
GGTCGCCTCTCGTGAAATGGGTACCGCTTTGCAATTGGTCAACATTGCCAGAGATATCAAGGG
TGACGCTACTGAAGGTAGATTCTACTTGCCATTGTCTTTCTTTGGTTTGAGAGATGAATCCAA
ATTGGCCATTCCAACTGACTGGACTGAACCAAGACCTCAAGATTTCGACAAATTGTTGTCTCT
ATCTCCATCTTCCACTTTACCATCCTCTAACGCTTCTGAATCCTTCAGATTCGAATGGAAGAC
CTACTCTTTGCCATTGGTTGCTTACGCTGAAGATTTGGCTAAGCACTCTTACAAGGGTATTGA
CAGATTACCAACTGAAGTCCAAGCTGGTATGAGAGCTGCTTGTGCTTCTTACTTGTTGATTGG
TCGTGAAATCAAGGTTGTCTGGAAGGGTGATGTCGGTGAAAGAAGAACCGTTGCTGGTTGGAG
AAGAGTCAGAAAGGTTTTGTCTGTTGTCATGTCCGGTTGGGAAGGTCAATAAAGCGATTTAAT
CTCTAATTATTAGTTAAAGTTTTATAAGCATTTTTATGTAACGAAAAATAAATTGGTTCATAT
TATTACTGCACTGTCACTTACCATGGAAAGACCAGACAAGAAGTTGCCGACAGTCTGTTGAAT
TGGCCTGGTTAGGCTTAAGTCTGGGTCCGCTTCTTTACAAATTTGGAGAATTTCTCTTAAACG
ATATGTATATTCTTTTCGTTGGAAAAGATGTCTTCCAAAAAAAAAACCGATGAATTAGTGGAA
CCAAGGAAAAAAAAAGAGGTATCCTTGATTAAGGAACACCTC**ACGCTTTCCGGCATCTTCCAG**
ACCACAGTATATCCATCCGCCTCCTGTTG

crtYB expression cassette: conB-Kl_YDR1p-crtYB-Sc_PDC1t-conC	Low strength promoter for expression of crtYB, multiplex array

CGGATCGATGTACACAACCGACTGCACCCAAACGAACACAAATCTTAGCAGTGCTTTTCTTTT
TTTGCGGTCACCCCCATGTGGCGGGGAGGCAGAGGAGTAGGTAGAGCAACGAATCCTACTATT
TATCCAAATTAGTCTAGGAACTCTTTTTCTAGATTTTTTAGATTTGAGGGCAAGCGCTGTTAA
CGACTCAGAAATGTAAGCACTACGGAGTAGAACGAGAAATCCGCCATAGGTGGAAATCCTAGC
AAAATCTTGCTTACCCTAGCTAGCCTCAGGTAAGCTAGCCTTAGCCTGTCAAATTTTTTTCAA
AATTTGGTAAGTTTCTACTAGCAAAGCAAACACGGTTCAACAAACCGAAAACTCCACTCATTA
TACGTGGAAACCGAAACAAAAAAACAAAAACCAAAATACTCGCCAATGAGAAAGTTGCTGCGT
TTCTACTTTCGAGGAAGAGGAACTGAGAGGATTGACTACGAAAGGGGCAAAAACGAGTCGTAT
TCTCCCATTATTGTCTGCTACCACGCGGTCTAGTAGAATAAGCAACCAGTCAACGCTAAGACA
GGTAATCAAAATACCAGTCTGCTGGCTACGGGCTAGTTTTTACCTCTTTTAGAACCCACTGTA
AAAGTCCGTTGTAAAGCCCGTTCTCACTGTTGGCGTTTTTTTTTTTTGGTTTAGTTTCTTAT
TTTTCATTTTTTTCTTTCATGACCAAAAACAAACAAATCTCGCGATTTGTACTGCGGCCACTG
GGGCGTGGCCAAAAAAATGACAAATTTAGAAACCTTAGTTTCTGATTTTTCCTGTTATGAGGA
GATATGATAAAAAATATTACTGCTTTATTGTTTTTTTTTTATCTACTGAAATAGAGAAACTTA
CCCAAGGAGGAGGCAAAAAAAAGAGTATATATACAGCAGGTACCATTCAGATTTTAATATATT
CTTTTCTCTTCTTCTACACTATTATTATAATAATTTTACTATATTCATTTTTAGCTTAAAACC

TCATAGAATATTATTCTTCAGTCACTCGCTTAAATACTTATCAAAAATGACCGCTTTGGCTTA
CTACCAAATCCACTTGATCTACACTTTGCCAATCTTAGGTTTGCTAGGTTTGTTGACTTCTCC
AATTTTGACCAAATTCGACATCTACAAGATTTCTATCTTAGTCTTTATTGCTTTCTCTGCTAC
CACTCCATGGGACTCCTGGATCATCAGAAACGGTGCCTGGACCTACCCATCTGCTGAATCTGG
TCAAGGTGTTTTCGGTACCTTTTTGGATGTCCCATACGAAGAATACGCCTTCTTTGTTATCCA
AACCGTCATCACCGGTTTGGTTTACGTTTTGGCTACCAGACATTTGTTGCCATCTTTGGCTCT
ACCAAAGACCCGTTCTTCTGCCTTGTCTCTAGCTTTGAAGGCTTTAATCCCATTGCCAATCAT
CTATTTGTTCACCGCTCATCCATCTCCATCCCCAGATCCTTTGGTTACTGACCACTACTTCTA
CATGAGAGCTTTGTCTTTGTTGATCACCCCACCAACCATGTTGTTGGCTGCTTTATCTGGTGA
ATACGCTTTCGACTGGAAATCTGGTAGAGCTAAGTCCACCATTGCTGCCATCATGATCCCAAC
TGTCTACTTGATCTGGGTTGACTACGTTGCCGTTGGTCAAGACTCCTGGTCCATCAACGATGA
AAAGATTGTCGGTTGGAGATTAGGTGGTGTCTTGCCAATTGAAGAAGCTATGTTCTTCTTATT
GACCAACTTGATGATCGTTTTGGGTTTGTCTGCCTGTGACCACACTCAAGCCTTGTACTTGTT
GCACGGTAGAACTATCTACGGTAACAAGAAGATGCCATCTTCTTTCCCATTAATCACTCCACC
AGTTTTGTCCTTGTTCTTCTCCTCCAGACCATACTCCTCCCAACCAAAGAGAGATTTGGAATT
GGCTGTCAAGTTGTTGGAAAAGAAGTCCAGATCTTTCTTCGTTGCTTCTGCCGGTTTCCCATC
TGAAGTCAGAGAAAGATTGGTTGGTTTGTACGCTTTCTGTCGTGTCACCGATGACTTGATTGA
CTCTCCAGAAGTTTCCTCCAACCCACACGCTACCATTGACATGGTTTCCGATTTCTTGACTTT
ATTATTCGGTCCTCCATTGCACCCATCTCAACCAGACAAGATTTTGTCTTCTCCATTATTACC
ACCTTCCCACCCATCCAGACCAACTGGTATGTACCCATTACCACCACCTCCATCTTTGTCTCC
AGCTGAATTGGTCCAATTCTTGACTGAACGTGTCCCAGTTCAATACCACTTCGCTTTCAGATT
GTTGGCCAAATTGCAAGGTTTGATTCCAAGATACCCATTGGATGAATTATTGAGAGGTTACAC
CACTGACTTGATCTTCCCATTGTCCACTGAAGCCGTCCAAGCTAGAAAGACCCCAATTGAAAC
TACTGCTGACTTGTTGGACTACGGTTTGTGTGTTGCCGGTTCTGTTGCTGAATTGTTGGTCTA
CGTTTCCTGGGCTTCCGCTCCATCCCAAGTTCCAGCTACTATTGAAGAAAGAGAAGCTGTTTT
GGTCGCCTCTCGTGAAATGGGTACCGCTTTGCAATTGGTCAACATTGCCAGAGATATCAAGGG
TGACGCTACTGAAGGTAGATTCTACTTGCCATTGTCTTTCTTTGGTTTGAGAGATGAATCCAA
ATTGGCCATTCCAACTGACTGGACTGAACCAAGACCTCAAGATTTCGACAAATTGTTGTCTCT
ATCTCCATCTTCCACTTTACCATCCTCTAACGCTTCTGAATCCTTCAGATTCGAATGGAAGAC
CTACTCTTTGCCATTGGTTGCTTACGCTGAAGATTTGGCTAAGCACTCTTACAAGGGTATTGA
CAGATTACCAACTGAAGTCCAAGCTGGTATGAGAGCTGCTTGTGCTTCTTACTTGTTGATTGG
TCGTGAAATCAAGGTTGTCTGGAAGGGTGATGTCGGTGAAAGAAGAACCGTTGCTGGTTGGAG
AAGAGTCAGAAAGGTTTTGTCTGTTGTCATGTCCGGTTGGGAAGGTCAATAAAGCGATTTAAT
CTCTAATTATTAGTTAAAGTTTTATAAGCATTTTTATGTAACGAAAAATAAATTGGTTCATAT
TATTACTGCACTGTCACTTACCATGGAAAGACCAGACAAGAAGTTGCCGACAGTCTGTTGAAT
TGGCCTGGTTAGGCTTAAGTCTGGGTCCGCTTCTTTACAAATTTGGAGAATTTCTCTTAAACG
ATATGTATATTCTTTTCGTTGGAAAAGATGTCTTCCAAAAAAAAAACCGATGAATTAGTGGAA
CCAAGGAAAAAAAAAGAGGTATCCTTGATTAAGGAACACCTC**ACGCTTTCCGGCATCTTCCAG**
ACCACAGTATATCCATCCGCCTCCTGTTG

crtI expression cassette: conD-Kl_ENO1p-crtI-Sc_TAL1t-conE	High strength promoter for expression of crtI, multiplex array

AACGTTGTCCAGGTTTGTATCCACGTGTGTCCGTTCCGCCAATATTCCGCGTGCCGTATCCCT
ATCTGGATTAACATCACTGCCACAGATCGAATTGCAAGAAGCCACACTTCACGTGATCCACTC
GTTCATCAGGTTTGTAGCTTCATGGCGCAGGACTTCTGATGATGAACTATCTGGCTCATCCGG
ATGGATCACAAGGATGGATACCCTCAGACAGTACGTTTCCGTATGGAGCGATCTTACAAGAAC
CAACAGTTGATCCTATTACTTTTTTTTTATTTTTTGTCCCTCCGGGATGGCAAGAGGGACAAA

GAAGAATCTTCGTTCTTCTTTCTTGTTCTCAACTTCCCAGCTTCCGTGTGATTACCCTCCGGG
ACAACAGAAAAACTGGCATTCGGTATCCCGGGAATCTGCTGAGAAGGAAAGAAAACGAAAAAA
AAATTGTACATTTGTGTCACATTATGAATTACAGGAAGTCAGAAAACAGGCAGCACATGTCTC
GCACATGCATGTCCATCAGACGAGACATTATGAGACATGCACGCGTGTGAGAGACATAGCAAA
AGTCTCTCCAGTACACACAGAAAGACACGTTCACAATCCAGGCACCCCACAGAGAAAAAAAAA
AGAAGAAGCCCGGAAGCTGGCACGCCATCATCAACCACCGCTCGGTTTACACGCATCCCAACT
GTCTTTTTTTTCTGGAATCCTATAATAACTGGCATCTGGAAATCACGTTGTATGTTGCACCAT
AGTGACTGGCTGTCTGACTAGCAAACATTGATTCCCTGATTCCCATTTGGCTCAATTTTGATG
AGAAACAGTTGATTGATTCTTGTCAATTTTTTTTTCTTTGGACCACCACCAACCAATTGACAT
TGAAGTACTTTCCCATGATTTGAGGTTATATAAAAGGACGTTCAAATCACTTTCAAGGTTAAT
TCAGTTTTGTCAATTGATTTAAGTTCAATTGTTAACAAATTTAATTTAATTCGAAACAAACCA
AACCAATTCATTTGAATTAACAAACCAACCCACAAAACAAAAAAAAATGGGTAAGGAACAAGA
CCAAGACAAGCCAACTGCCATCATCGTTGGTTGTGGTATCGGTGGTATTGCTACCGCTGCCAG
ATTAGCTAAGGAAGGTTTCCAAGTTACCGTCTTTGAAAAGAACGACTACTCCGGTGGTAGATG
TTCTTTGATTGAAAGAGATGGTTACAGATTCGACCAAGGTCCATCTTTGTTGCTATTACCAGA
CTTGTTCAAGCAAACCTTCGAAGATTTGGGTGAAAAGATGGAAGACTGGGTTGATTTGATCAA
GTGTGAACCAAACTACGTTTGTCACTTCCATGATGAAGAAACTTTCACCTTCTCCACTGACAT
GGCTTTATTGAAGAGAGAAGTCGAAAGATTTGAAGGTAAAGATGGTTTCGACAGATTCTTGTC
TTTCATCCAAGAAGCTCACAGACATTACGAATTGGCTGTTGTCCACGTCTTGCAAAAGAACTT
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TGAATCCATCTGGACCAGAGTTTGTCGTTACTTCAAGACTGACAGATTGAGAAGAGTCTTCTC
CTTTGCCGTTATGTACATGGGTCAATCTCCATACTCTGCTCCAGGTACCTACTCCTTGTTGCA
ATACACTGAATTGACTGAAGGTATCTGGTACCCAAGAGGTGGTTTCTGGCAAGTTCCAAACAC
TTTGTTGCAAATCGTCAAGAGAAACAACCCATCTGCTAAGTTCAACTTCAACGCTCCAGTTTC
TCAAGTTTTGTTGTCTCCAGCTAAGGACAGAGCTACCGGTGTCAGATTAGAATCTGGTGAAGA
ACACCACGCTGATGTTGTCATTGTCAATGCTGACTTGGTCTACGCTTCTGAACATTTGATTCC
AGATGATGCTAGAAACAAGATCGGTCAATTAGGTGAAGTTAAGCGTTCCTGGTGGGCTGATTT
GGTTGGTGGTAAGAAGTTGAAGGGTTCTTGTTCTTCTTTGTCTTTCTACTGGTCTATGGACAG
AATCGTTGACGGTTTGGGTGGTCACAACATCTTCTTGGCTGAAGACTTCAAGGGTTCCTTCGA
CACCATTTTCGAAGAATTGGGTTTGCCAGCTGACCCATCTTTCTATGTTAACGTTCCATCCAG
AATTGACCCTTCTGCTGCTCCAGAAGGTAAGGATGCCATTGTCATCTTAGTCCCATGTGGTCA
CATCGATGCTTCCAACCCTCAAGACTACAACAAATTGGTTGCCAGAGCCAGAAAGTTCGTCAT
CCAAACCTTGTCTGCCAAGTTGGGTCTACCAGATTTCGAAAAGATGATTGTTGCTGAAAAGGT
TCACGATGCTCCATCCTGGGAAAAGGAATTCAACTTGAAGGACGGTTCCATTTTGGGTTTGGC
TCACAACTTCATGCAAGTCTTGGGTTTCAGACCATCCACCAGACACCCAAAGTACGACAAATT
GTTCTTTGTCGGTGCTTCTACCCACCCAGGTACTGGTGTTCCAATTGTCTTGGCTGGTGCCAA
ATTGACTGCTAACCAAGTTTTGGAATCCTTCGATCGTTCTCCAGCTCCAGATCCTAACATGTC
TTTGTCTGTTCCATACGGTAAGCCATTGAAATCCAACGGTACTGGTATTGACTCTCAAGTCCA
ATTGAAATTCATGGACTTGGAACGTTGGGTTTACCTATTAGTCTTGTTGATTGGTGCTGTTAT
CGCCAGATCCGTCGGTGTCTTGGCCTTTTAAAGGAAGTATCTCGGAAATATTAATTTAGGCCA
TGTCCTTATGCACGTTTCTTTTGATACTTACGGGTACATGTACACAAGTATATCTATATATAT
AAATTAATGAAAATCCCCTATTTATATATATGACTTTAACGAGACAGAACAGTTTTTTATTTT
TTATCCTATTTGATGAATGATACAGTTTCTTATTCACGTGTTATACCCACACCAAATCCAATA
GCAATACCGGCCATCACAATCACTGTTTCGGCAGCCCCTAAGATCAGACAAAACATCCGGAAC
CACCTTAAATCAACGTCCCTC**AAATAACCACAAACATCCTTCCCATATGCTCGGTCGTGCTTG**
TTGTACCT

crtI expression cassette: conD-Kl_OLE1p-crtI-Sc_TAL1t-conE	Medium strength promoter for expression of crtI, multiplex array

AACGTTGTCCAGGTTTGTATCCACGTGTGTCCGTTCCGCCAATATTCCGCGTGCCAAAGGGGG
GGCAGGGACAGGGATACGACAAGGGCTGGGGAAAAAAAAAAAGATAGATACGATTGGCCGGGT
AAGCCTGGGGAAATGTAGCAAGTGCGGGTAAGTTAAAAGGTAACCACGTGACTCCGGAAGAGT
CACGTGGTTACGGACTTTTTTCTCTAGATCTCAGCTTTTTATCGGTCTTACCCTGCCCTCCTG
CCCCCTGCCCCTTCCCTTTGCCCCAAAAAGAAAGGAAATCTGTTGGATTTCGCTCAGGCCATC
CCTTTCGTTAATATCGGTTATCGCTTTACACACTGCACATCCTTCTGTCCAAAAGGAATCCAG
AAGTTTAGCTTTTCCTTCCTTTCCCACAGACATTAGCCTAGGCCCTCTCTCATCATTTGCATG
CCTCAGCCAATGTACCAAGAATAACGCAACGAGGTTGGGAAATTTTAACCCAACAATCGATGC
AGATGTGACAAGAGATTAGACACGTTCCAGATACCAGATTACACAGCTTGTGCTAGCAGAGTG
ACATATGGTGGTGTTGTGTCTCGTTTAGTACCTGTAATCGAGAGTGTTCAAATCAGTCGATTT
GAACACCCTTACTGCCACTGAATATTGATTGAATACCGTTTATTGAAGGTTTTATGAGTGATC
TTCTTTCGGTCCAGGACAATTTGTTGAGCTTTTTCTATGTAGAGTTCCGTCCCTTTTTTTTTT
TTTTTTGCTTTCTCGCACTTACTAGCACTATTTTTTTTTCACACACTAAAACACTTTATTTTA
ATCTATATATATATATATATATATATGTAGGAATGGAATCACAGACATTTGATACTCATCCTC
ATCCTTATTAATTCTTGTTTTAATTTGTTTGACTTAGCCAAACCACCAATCTCAACCCATCGT
ATTTCAGGTATTGTGTGTCTAGTGTGTCTCTGGTATACGGAAATAAGTGCCAGAAGTAAGGAA
GAAACAAAGAACAAGTGTCTGAATACTACTAGCCTCTCTTTTCATAATGGGTAAGGAACAAGA
CCAAGACAAGCCAACTGCCATCATCGTTGGTTGTGGTATCGGTGGTATTGCTACCGCTGCCAG
ATTAGCTAAGGAAGGTTTCCAAGTTACCGTCTTTGAAAAGAACGACTACTCCGGTGGTAGATG
TTCTTTGATTGAAAGAGATGGTTACAGATTCGACCAAGGTCCATCTTTGTTGCTATTACCAGA
CTTGTTCAAGCAAACCTTCGAAGATTTGGGTGAAAAGATGGAAGACTGGGTTGATTTGATCAA
GTGTGAACCAAACTACGTTTGTCACTTCCATGATGAAGAAACTTTCACCTTCTCCACTGACAT
GGCTTTATTGAAGAGAGAAGTCGAAAGATTTGAAGGTAAAGATGGTTTCGACAGATTCTTGTC
TTTCATCCAAGAAGCTCACAGACATTACGAATTGGCTGTTGTCCACGTCTTGCAAAAGAACTT
CCCAGGTTTCGCTGCTTTCTTGAGATTACAATTCATCGGTCAAATCTTAGCTTTGCACCCATT
TGAATCCATCTGGACCAGAGTTTGTCGTTACTTCAAGACTGACAGATTGAGAAGAGTCTTCTC
CTTTGCCGTTATGTACATGGGTCAATCTCCATACTCTGCTCCAGGTACCTACTCCTTGTTGCA
ATACACTGAATTGACTGAAGGTATCTGGTACCCAAGAGGTGGTTTCTGGCAAGTTCCAAACAC
TTTGTTGCAAATCGTCAAGAGAAACAACCCATCTGCTAAGTTCAACTTCAACGCTCCAGTTTC
TCAAGTTTTGTTGTCTCCAGCTAAGGACAGAGCTACCGGTGTCAGATTAGAATCTGGTGAAGA
ACACCACGCTGATGTTGTCATTGTCAATGCTGACTTGGTCTACGCTTCTGAACATTTGATTCC
AGATGATGCTAGAAACAAGATCGGTCAATTAGGTGAAGTTAAGCGTTCCTGGTGGGCTGATTT
GGTTGGTGGTAAGAAGTTGAAGGGTTCTTGTTCTTCTTTGTCTTTCTACTGGTCTATGGACAG
AATCGTTGACGGTTTGGGTGGTCACAACATCTTCTTGGCTGAAGACTTCAAGGGTTCCTTCGA
CACCATTTTCGAAGAATTGGGTTTGCCAGCTGACCCATCTTTCTATGTTAACGTTCCATCCAG
AATTGACCCTTCTGCTGCTCCAGAAGGTAAGGATGCCATTGTCATCTTAGTCCCATGTGGTCA
CATCGATGCTTCCAACCCTCAAGACTACAACAAATTGGTTGCCAGAGCCAGAAAGTTCGTCAT
CCAAACCTTGTCTGCCAAGTTGGGTCTACCAGATTTCGAAAAGATGATTGTTGCTGAAAAGGT
TCACGATGCTCCATCCTGGGAAAAGGAATTCAACTTGAAGGACGGTTCCATTTTGGGTTTGGC
TCACAACTTCATGCAAGTCTTGGGTTTCAGACCATCCACCAGACACCCAAAGTACGACAAATT
GTTCTTTGTCGGTGCTTCTACCCACCCAGGTACTGGTGTTCCAATTGTCTTGGCTGGTGCCAA
ATTGACTGCTAACCAAGTTTTGGAATCCTTCGATCGTTCTCCAGCTCCAGATCCTAACATGTC
TTTGTCTGTTCCATACGGTAAGCCATTGAAATCCAACGGTACTGGTATTGACTCTCAAGTCCA
ATTGAAATTCATGGACTTGGAACGTTGGGTTTACCTATTAGTCTTGTTGATTGGTGCTGTTAT
CGCCAGATCCGTCGGTGTCTTGGCCTTTTAAAGGAAGTATCTCGGAAATATTAATTTAGGCCA

TGTCCTTATGCACGTTTCTTTTGATACTTACGGGTACATGTACACAAGTATATCTATATATAT
AAATTAATGAAAATCCCCTATTTATATATATGACTTTAACGAGACAGAACAGTTTTTTATTTT
TTATCCTATTTGATGAATGATACAGTTTCTTATTCACGTGTTATACCCACACCAAATCCAATA
GCAATACCGGCCATCACAATCACTGTTTCGGCAGCCCCTAAGATCAGACAAAACATCCGGAAC
CACCTTAAATCAACGTCCCTC**AAATAACCACAAACATCCTTCCCATATGCTCGGTCGTGCTTG**
TTGTACCT

crtI expression cassette: conD-Kl_LEU2p-crtI-Sc_TAL1t-conE	Low strength promoter for expression of crtI, multiplex array

AACGTTGTCCAGGTTTGTATCCACGTGTGTCCGTTCCGCCAATATTCCGCGTGCGCTGTGAAG
ATCCCAGCAAAGGCTTACAAAGTGTTATCTCTTTTGAGACTTGTTGAGTTGAACACTGGTGTT
TTCATCAAACTTACCAAGGACGTGTACCCATTGTTGAAACTTGTATCACCATATATTGTTATC
GGACAACCTTCACTTGCATCTATCCGTTCTTTAATCCAAAAGAGATCTAGAATAATGTGGCAA
AGGCCAGAAGATAAAGAACCAAAAGAGATAATCTTGAATGACAACAATATCGTTGAAGAGAAA
TTAGGTGATGAAGGTGTCATTTGTATCGAGGATATCATCCATGAGATTTCGACGTTGGGCGAA
AATTTCTCGAAATGTACTTTCTTCCTATTACCATTCAAATTGAACAGAGAAGTCAGTGGATTC
GGTGCCATCTCCCGTTTGAATAAACTGAAAATGCGCGAACAAAACAACAAGACACGTCAAATT
TCAAACGCTGCCACGGCTCCAGTTATCCAAGTAGATATCGACACAATGATTTCCAAGTTGAAT
TGATTAACTATAAAAGGAAAATATCTGTACAATAGACATCGGGCTCCCATTGGCCCTACCCAC
ATATGTAGAAATACATTACTCTATTCACTACTGCATTTAGTTATGTTTAACATTTGATATAGC
AGACTACCGCCAGGCACAATATATTCCCCTTCCCTCTTGCCATTCGCTGTACTTGTGGTGGAT
TCCAATTCAGCGCAGTCACGTGCTAGTAATCACCGCATTTTTTTCTTTTCCTTTCAGGCTAAA
ACCGGTTCCGGGCCTGATCCCTGCACTCATTTTCTAACGGAAAACCTTCAGAAGCATAACTAC
CCATTCCAGTTTAGAGACATGACAGGTTCAACATCAGATGCTTCATATACTTTTATATATTGA
ATTATATAAATATATCTATGTACTCTAAGTAAGTACATCTGCTTTAACGCATTCCTACATTTG
CTTCGATTTATTTTTATTGTTGATACCTATTTGAAGAAGTAAAAAGTATCCCACACTACACAG
ATTATACAATGGGTAAGGAACAAGACCAAGACAAGCCAACTGCCATCATCGTTGGTTGTGGTA
TCGGTGGTATTGCTACCGCTGCCAGATTAGCTAAGGAAGGTTTCCAAGTTACCGTCTTTGAAA
AGAACGACTACTCCGGTGGTAGATGTTCTTTGATTGAAAGAGATGGTTACAGATTCGACCAAG
GTCCATCTTTGTTGCTATTACCAGACTTGTTCAAGCAAACCTTCGAAGATTTGGGTGAAAAGA
TGGAAGACTGGGTTGATTTGATCAAGTGTGAACCAAACTACGTTTGTCACTTCCATGATGAAG
AAACTTTCACCTTCTCCACTGACATGGCTTTATTGAAGAGAGAAGTCGAAAGATTTGAAGGTA
AAGATGGTTTCGACAGATTCTTGTCTTTCATCCAAGAAGCTCACAGACATTACGAATTGGCTG
TTGTCCACGTCTTGCAAAAGAACTTCCCAGGTTTCGCTGCTTTCTTGAGATTACAATTCATCG
GTCAAATCTTAGCTTTGCACCCATTTGAATCCATCTGGACCAGAGTTTGTCGTTACTTCAAGA
CTGACAGATTGAGAAGAGTCTTCTCCTTTGCCGTTATGTACATGGGTCAATCTCCATACTCTG
CTCCAGGTACCTACTCCTTGTTGCAATACACTGAATTGACTGAAGGTATCTGGTACCCAAGAG
GTGGTTTCTGGCAAGTTCCAAACACTTTGTTGCAAATCGTCAAGAGAAACAACCCATCTGCTA
AGTTCAACTTCAACGCTCCAGTTTCTCAAGTTTTGTTGTCTCCAGCTAAGGACAGAGCTACCG
GTGTCAGATTAGAATCTGGTGAAGAACACCACGCTGATGTTGTCATTGTCAATGCTGACTTGG
TCTACGCTTCTGAACATTTGATTCCAGATGATGCTAGAAACAAGATCGGTCAATTAGGTGAAG
TTAAGCGTTCCTGGTGGGCTGATTTGGTTGGTGGTAAGAAGTTGAAGGGTTCTTGTTCTTCTT
TGTCTTTCTACTGGTCTATGGACAGAATCGTTGACGGTTTGGGTGGTCACAACATCTTCTTGG
CTGAAGACTTCAAGGGTTCCTTCGACACCATTTTCGAAGAATTGGGTTTGCCAGCTGACCCAT
CTTTCTATGTTAACGTTCCATCCAGAATTGACCCTTCTGCTGCTCCAGAAGGTAAGGATGCCA
TTGTCATCTTAGTCCCATGTGGTCACATCGATGCTTCCAACCCTCAAGACTACAACAAATTGG
TTGCCAGAGCCAGAAAGTTCGTCATCCAAACCTTGTCTGCCAAGTTGGGTCTACCAGATTTCG

AAAAGATGATTGTTGCTGAAAAGGTTCACGATGCTCCATCCTGGGAAAAGGAATTCAACTTGA
AGGACGGTTCCATTTTGGGTTTGGCTCACAACTTCATGCAAGTCTTGGGTTTCAGACCATCCA
CCAGACACCCAAAGTACGACAAATTGTTCTTTGTCGGTGCTTCTACCCACCCAGGTACTGGTG
TTCCAATTGTCTTGGCTGGTGCCAAATTGACTGCTAACCAAGTTTTGGAATCCTTCGATCGTT
CTCCAGCTCCAGATCCTAACATGTCTTTGTCTGTTCCATACGGTAAGCCATTGAAATCCAACG
GTACTGGTATTGACTCTCAAGTCCAATTGAAATTCATGGACTTGGAACGTTGGGTTTACCTAT
TAGTCTTGTTGATTGGTGCTGTTATCGCCAGATCCGTCGGTGTCTTGGCCTTTTAAAGGAAGT
ATCTCGGAAATATTAATTTAGGCCATGTCCTTATGCACGTTTCTTTTGATACTTACGGGTACA
TGTACACAAGTATATCTATATATATAAATTAATGAAAATCCCCTATTTATATATATGACTTTA
ACGAGACAGAACAGTTTTTTATTTTTTATCCTATTTGATGAATGATACAGTTTCTTATTCACG
TGTTATACCCACACCAAATCCAATAGCAATACCGGCCATCACAATCACTGTTTCGGCAGCCCC
TAAGATCAGACAAAACATCCGGAACCACCTTAAATCAACGTCCCTC**AAATAACCACAAACATC**
CTTCCCATATGCTCGGTCGTGCTTGTTGTACCT

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Dear reader,

I am the first author of a paper we published last year in Yeast:
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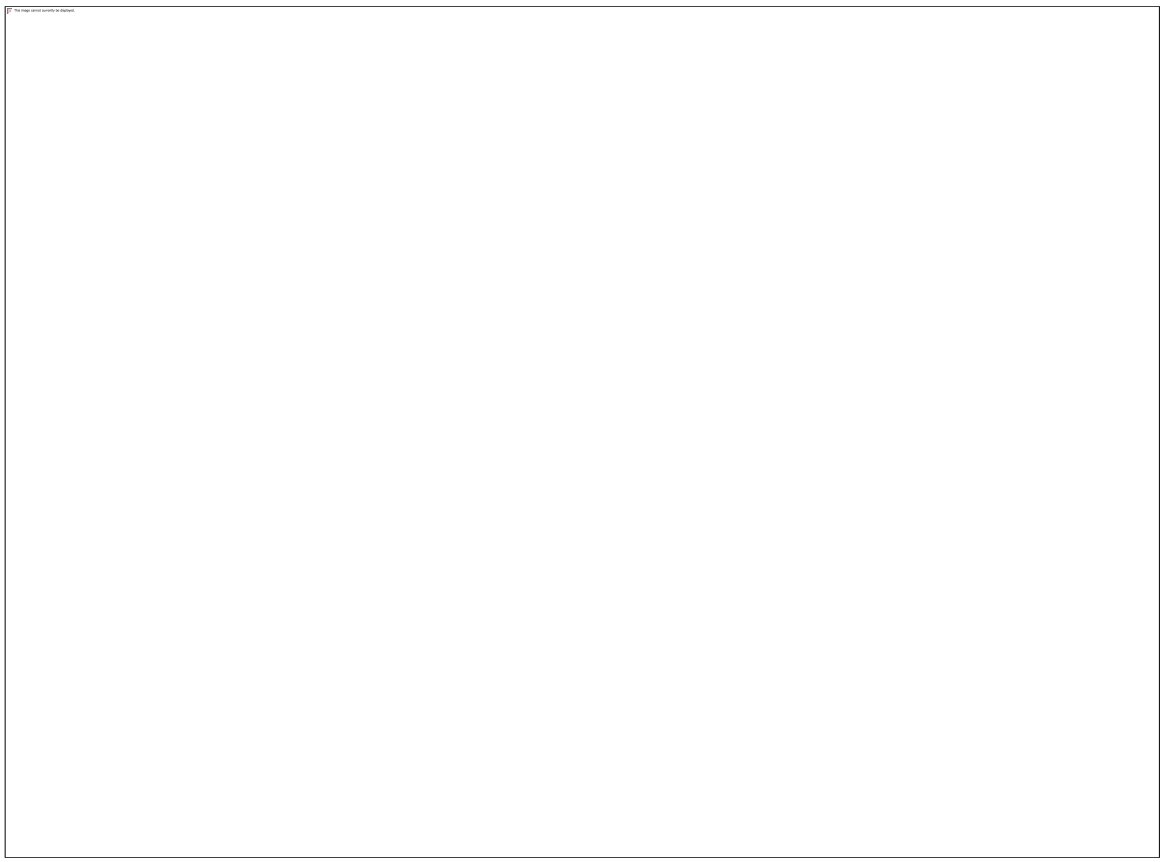
We were invited to write a “methods paper” about the experimental details of a part of the Yeast manuscript, i.e. multiplex genome editing experiments.
For clarification, we would like to re-use Figure 5a and figure 5b in the paper.



I hereby request permission to re-use Figures 5a and figure 5b from our Yeast paper.

Kind regards,

René Verwaal

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