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Protocol

PROTOCOL

A protocol for introduction of multiple genetic modifications in *Saccharomyces cerevisiae* using CRISPR/Cas9

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One sentence summary: Streamlined CRISPR-Cas9 protocols for genome editing in *Saccharomyces cerevisiae* executable by virtually any yeast molecular geneticist.

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ABSTRACT

Here, two methods are described for efficient genetic modification of *Saccharomyces cerevisiae* using CRISPR/Cas9. The first method enables the modification of a single genetic locus using *in vivo* assembly of a guide RNA (gRNA) expression plasmid without the need for prior cloning. A second method using *in vitro* assembled plasmids that could contain up to two gRNAs was used to simultaneously introduce up to six genetic modifications (e.g. six gene deletions) in a single transformation step by transforming up to three gRNA expression plasmids simultaneously. The method is not only suitable for gene deletion but is also applicable for *in vivo* site-directed mutagenesis and integration of multiple DNA fragments in a single locus. In all cases, the strain transformed with the gRNA expression plasmids was equipped with a genomic integration of *Spcas9,* leading to strong and constitutive expression of *Sp*Cas9. The protocols detailed here have been streamlined to be executed by virtually any yeast molecular geneticist.

Keywords: CRISPR/Cas9; *S. cerevisiae*; gRNA; genetic modification; webtool; plasmid

INTRODUCTION

Protocols for highly efficient transformation of *Saccharomyces cerevisiae* have been well established. However, prior to the emergence of CRISPR/Cas9 as a genetic engineering tool, most methods for gene deletion or integration relied on the simultaneous integration of a selection marker gene and thus required a subsequent marker-recycling step (Baudin *et al.* 1993; Güldener *et al.* 1996; Gietz and Woods 2002; Carter and Delneri 2010; Solis-Escalante *et al.* 2013, 2014). Furthermore, introduction of multiple genetic modifications has so far remained a time-consuming and labour-intensive process, as each individual alteration requires a cycle of transformation, selection and confirmation. This work describes the steps involved in two methods for CRISPR/Cas9-mediated transformation of *S. cerevisiae* (Mans *et al.* 2015). The first method (based on pMEL plasmids) makes use of *in vivo* assembly of plasmids encoding a single guide RNA (gRNA) via homologous recombination. This method enables fast modification of a single genetic locus without the need for prior cloning. The second method (based on pROS plasmids) is based

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Table 1. *Saccharomyces cerevisiae* strains deposited at EUROSCARF with a genomic integration of *cas9* (Mans *et al.* 2015).

Name (Accession no.)	Relevant genotype	Parental strain
IMX585 (Y40592)	MATa *can1Δ::Spcas9-natNT2 URA3 TRP1 LEU2 HIS3*	CEN.PK113–7D
IMX581 (Y40593)	MATa *ura3–52 can1Δ::Spcas9-natNT2 TRP1 LEU2 HIS3*	CEN.PK113–5D
IMX664 (Y40594)	MATa/MATα *CAN1/can1Δ::Spcas9-natNT2 URA3/URA3 TRP1/TRP1 LEU2/LEU2 HIS3/HIS3*	CEN.PK122
IMX672 (Y40595)	MATa *ura3–52 trp1–289 leu2–3112 his3Δ can1Δ::Spcas9-natNT2*	CEN.PK2–1C
IMX673 (Y40596)	MATa/MATα *ura3–52/ura3–52 CAN1/can1Δ::Spcas9-natNT2 TRP1/TRP1 LEU2/LEU2 HIS3/HIS3*	CEN.PK115

Table 2. *E. coli* strains deposited at EUROSCARF (https://tinyurl.com/y8xxzpr9) and at Addgene (https://www.addgene.org) containing the single gRNA (pMEL) and double gRNA (pROS) plasmid series (Mans *et al.* 2015).

Name	Accession n° Euroscarf	Accession n° Addgene	Relevant characteristics
p414-TEF1p-cas9-CYC1t		43802	*ARS4 CEN6 bla TRP1* $TEF1_p$::*Spcas9*::$CYC1_t$
pUG-natNT2		110922	*bla* $AgTEF1_p$::*nat*::$AgTEF1_t$
pMEL10	P30779	107916	2μm *bla KlURA3* gRNA-*CAN1.Y*
pMEL11	P30780	107917	2μm *bla* amdSYM gRNA-*CAN1.Y*
pMEL12	P30781	107918	2μm *bla* hphNT1 gRNA-*CAN1.Y*
pMEL13	P30782	107919	2μm *bla* kanMX gRNA-*CAN1.Y*
pMEL14	P30783	107920	2μm *bla KlLEU2* gRNA-*CAN1.Y*
pMEL15	P30784	107921	2μm *bla* natNT2 gRNA-*CAN1.Y*
pMEL16	P30785	107922	2μm *bla HIS3* gRNA-*CAN1.Y*
pMEL17	P30786	107923	2μm *bla TRP1* gRNA-*CAN1.Y*
pROS10	P30787	107924	2μm *bla URA3* gRNA-*CAN1.Y* gRNA-*ADE2.Y*
pROS11	P30788	107925	2μm *bla* amdSYM gRNA-*CAN1.Y* gRNA-*ADE2.Y*
pROS12	P30789	107926	2μm *bla* hphNT1 gRNA-*CAN1.Y* gRNA-*ADE2.Y*
pROS13	P30790	107927	2μm *bla* kanMX gRNA-*CAN1.Y* gRNA-*ADE2.Y*
pROS14	P30791	107928	2μm *bla KlLEU2* gRNA-*CAN1.Y* gRNA-*ADE2.Y*
pROS15	P30792	107929	2μm *bla* natNT2 gRNA-*CAN1.Y* gRNA-*ADE2.Y*
pROS16	P30793	107930	2μm *bla HIS3* gRNA-*CAN1.Y* gRNA-*ADE2.Y*
pROS17	P30794	107931	2μm *bla TRP1* gRNA-*CAN1.Y* gRNA-*ADE2.Y*

on *in vitro* pre-assembled plasmids containing two gRNA coding sequences, which enable the simultaneous modification of two genetic loci per transformed plasmid.

MATERIALS

This protocol offers a series of pMEL and pROS plasmids with a broad range of selection markers. The materials listed below are for application of these selection markers. When transforming with a subset of plasmid markers, not all listed materials might be required.

Saccharomyces cerevisiae and *Escherichia coli* strains

- Chemical- or electrocompetent *E. coli* XL1-Blue cells for plasmid transformation and propagation. Chemically competent cells can be prepared and transformed according to the protocol of Zymo Research (T3001, http://www.biocenter.hu/pdf/T3001.PDF). Electrocompetent cells can be prepared and transformed according to the protocol of Bio-Rad (165–2100, http://www.bio-rad.com/webroot/web/pdf/lsr/literature/4006174B.pdf).
- *Saccharomyces cerevisiae* strain(s) with a genomic integration of *cas9*. The yeast strains in Table 1 can be obtained via EUROSCARF (https://tinyurl.com/y8xxzpr9). *cas9* is flanked by the *TEF1* promoter and the *CYC1* terminator and is equipped with the SV40 nuclear localisation signal for targeting to the nucleus (DiCarlo *et al.* 2013; Mans *et al.* 2015).
- Note: The transfer of *Streptococcus pyogenes cas9* in a *S. cerevisiae* strain can be achieved via assembly and simultaneous integration of a cassette carrying *cas9* and another containing the natNT2 marker into the *CAN1* locus. The *cas9* cassette was PCR amplified from p414-TEF1p-Cas9-CYC1t (Addgene plasmid #43802) (DiCarlo *et al.* 2013) (Table 2), using primers 2873 & 4653 (Table 3). The natNT2 cassette was PCR amplified from pUG-natNT2 (Addgene plasmid #110922) (de Kok *et al.* 2012) with primers 3093 & 5542. 2.5 μg *cas9* and 800 ng natNT2 cassette were pooled and used for each transformation as described in (Mans *et al.* 2015).
- Plasmids for gRNA expression. The singe gRNA (pMEL) and double gRNA (pROS) plasmids can be obtained via EUROSCARF (Table 2).

Equipment

- Thermocycler
- Gel electrophoresis equipment for DNA separation
- UV transilluminator
- Safe Imager 2.0 Blue-Light Transilluminator
- Incubators with a temperature range of at least 30°C and 42°C
- Water bath or heat block with a temperature range of at least 30°C to 95°C
- Nanodrop or Qubit fluorometer with Qubit dsDNA BR Assay Kit (Thermo Fisher Scientific (Waltham, MA), Q32853) for DNA quantification
- Computer with internet access and DNA manager software (e.g. Clone Manager (Scientific & Educational Software, Denver, CO) Snapgene (Chicago, IL, (http://www.snapgene.com/))
- Optional: 2 mm cuvette (Bio-Rad, 165–2086) and MicroPulser Electroporator (Bio-Rad, 165–2100)

Table 3. Primers used to construct and verify insertion of CRISPR-Cas9 gRNA in pMEL and pROS plasmid series.

Name	Sequence 5′ → 3′	Purpose
6005	GATCATTTATCTTTCACTGCGGAGAAG	pMEL + pROS plasmid construction
6006	GTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTC	pMEL plasmid construction
2873	TCAGACTTCTTAACTCCTGTAAAAACAAAAAAAAAAAAAAGGCATAGCAATAAGCTGGAGCTCATAGCTTC	Construction *Spcas9* integration cassette
4653	GTGCCTATTGATGATCTGGCGGAATGTCTGCCGTGCCATAGCCATGCCTTCACATATAGTCCGCAAATTA AAGCCTTCGAG	Construction *Spcas9* integration cassette
3093	ACTATATGTGAAGGCATGGCTATGGCACGGCAGACATTCCGCCAGATCATCAATAGGCACCTTCGTACGC TGCAGGTCGAC	Construction of natNT2 integration cassette
5542	CTATGCTACAACATTCCAAAATTTGTCCCAAAAAGTCTTTGGTTCATGATCTTCCCATACGCATAGGCCA CTAGTGGATCTG	Construction of natNT2 integration cassette
Primer α	CACCTTTCGAGAGGACGATG	Confirmation pROS plasmids
Primer β	GCTGGCCTTTTGCTCACATG	Confirmation pROS plasmids
Ptarget FW[a]	TGCGCATGTTTCGGCGTTCGAAACTTCTCCGCAGTGAAAGATAAATGATCN$_{20}$GTTTTAGAGCTAGAAATAGC AAGTTAAAATAAGGCTAGTCCGTTATCAAC	pMEL construction
Ptarget RV[b]	GTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACN$_{20c}$GATCATTTATCTTTCACTG CGGAGAAGTTTCGAACGCCGAAACATGCGCA	pMEL construction
Ptarget ROS	TGCGCATGTTTCGGCGTTCGAAACTTCTCCGCAGTGAAAGATAAATGATCN$_{20}$GTTTTAGAGCTAGAAATAGC AAGTTAAAATAAG	pROS construction
Ptarget$_{cas9}$ ROS[c]	TGCGCATGTTTCGGCGTTCGAAACTTCTCCGCAGTGAAAGATAAATGATCCTAGGCTGTCCAAATCCCGGGTT TTAGAGCTAGAAATAGCAAGTTAAAATAAG	Construction of a pROS targeting *cas9*
PrepairD FW	TTTCAGAGTTCTTCAGACTTCTTAACTCCTGTAAAAACAAAAAAAAAAAAAAGGCATAGCATATGAGGGTGAGA ATGCGAAATGGCGTGGAAATGTGATCAAAGGTAATAAAACGTCATAT	Repair DNA for *cas9* deletion leaving a *can1*Δ locus
PrepairD RV	ATATGACGTTTTATTACCTTTGATCACATTTCCACGCCATTTCGCATTCTCACCCTCATATGCTATGCCTTTT TTTTTTTTGTTTTTACAGGAGTTAAGAAGTCTGAAGAACTCTGAAA	Repair DNA for *cas9* deletion leaving a *can1*Δ locus
Prepair$_{CAN1}$ FW[d]	TTTCAGAGTTCTTCAGACTTCTTAACTCCTGTAAAAACAAAAAAAAAAAAAAGGCATAGCAATGACAAATTCAA AAGAAGACGCC	Repair DNA for *cas9* deletion restoring a *CAN1* locus
Prepair$_{CAN1}$ RV[d]	ATATGACGTTTTATTACCTTTGATCACATTTCCACGCCATTTCGCATTCTCACCCTCATATCTATGCTACAAC ATTCCAAAATTTG	Repair DNA for *cas9* deletion restoring a *CAN1* locus

[a]N_{20} corresponds to the chosen target sequence. This nucleotide sequence does not include the PAM sequence (NGG).
[b]N_{20c} corresponds to the reverse complement of the chosen target sequence found in Ptarget FW.
[c]Primer for the construction of pROS plasmid carrying a spacer targeting *cas9*, which can be used to remove chromosomal integration of the endonuclease expression cassette.
[d]Primer suitable to PCR amplify the *CAN1* ORF.

Chemicals

- Bacto tryptone (Difco-Thermo Fisher Scientific; 211705)
- Bacto yeast extract (Difco-Thermo Fisher Scientific; 212750)
- Sodium chloride (J.T.Baker (Center Valley, PA); 0278)
- Bacto agar (Difco-Thermo Fisher Scientific; 214010)
- Bacto peptone (Difco-Thermo Fisher Scientific; 211677)
- Ammonium sulphate (Merck (Kenilworth, NJ); 101211)
- Monopotassium phosphate (Merck; 104877)
- Magnesium sulphate heptahydrate (J.T.Baker; 2500)
- Potassium hydroxide (J.T.Baker; 0222)
- L-glutamic acid monosodium salt (Sigma Aldrich (Saint-Louis, MO); G1626)
- 100% Glycerol (Merck; 8.18709)
- 100% Ethanol (Merck; 1.00983)
- Sodium dodecyl sulphate (Sigma Aldrich; L4390)
- Polyethylene glycol 3350 (Sigma Aldrich; P4338)
- Deoxyribonucleic acid sodium salt (Sigma Aldrich; D1626)
- Trizma base (Sigma Aldrich; T6066)
- Hydrochloric acid (Sigma Aldrich; 30721)
- Ethylene diamine tetra-acetic acid (EDTA) solution (Sigma Aldrich; E7889)
- Lithium acetate dehydrate (Sigma Aldrich; L6883)
- Acetamide (Sigma Aldrich; A0500)
- Potassium sulphate (Merck; 105153)

Molecular biology reagents

- DNA purification kits:
  a. GenElute Plasmid Miniprep Kit (Sigma Aldrich; PLN350)
  b. ZymoClean Gel DNA Recovery Kit (Zymo Research; D4002)
  c. Optional: YeaStar Genomic DNA Kit (Zymo Research: D2002)
- PCR reagents:
  a. DreamTaq PCR Master Mix (2x) (Thermo Fisher Scientific; K1071)
  b. Phusion High Fidelity DNA polymerase (2 U μL^{-1}) and 5x Phusion HF buffer (Thermo Fisher Scientific; F530)
  c. dNTPs (10 mM) (Thermo Fisher Scientific; R0181)
  d. MilliQ or RNase-free water.
- Primers (Table 3):
- FastDigest DpnI (Thermo Fisher Scientific; FD1704)
- Optional: FastDigest enzymes and FastDigest Green Buffer (10x) for confirmation of correct assembly pROS plasmids.
- GeneRuler DNA Ladder Mix (Thermo Fisher Scientific; SM0331)
- SERVA DNA stain G (SERVA (Heidelberg, Germany); 39803.02)
- 50X TAE Buffer (Thermo Fisher Scientific; B49)
- TopVision Agarose (Thermo Fisher Scientific; R0492)
- NEBuilder HiFi DNA Assembly Master Mix (New England Biolabs (Ipswich, MA); E2621)
- 0.2M lithium acetate 1% SDS solution; dissolve 5 g sodium dodecyl sulphate in 50 mL distilled water, add 10.2 g lithium acetate dehydrate and fill up to 500 mL with distilled water.
- TE buffer (pH 8.0); prepare 1.0M Tris by dissolving 60.57 g Trizma base in 500 mL distilled deionised water and set pH to 8.0 using HCl. Mix 5 mL 1M Tris pH 8.0 with 1 mL 0.5M EDTA and fill up to 500 mL with deionised water. When necessary, heat sterilise for 20 min at 121°C.
- Transformation reagents:
  a. 50% PEG. Prepare a 50% w/v solution of polyethylene glycol 3350 in distilled deionised water and autoclave at 121°C for 20 min.

b. 1.0M Lithium acetate. Prepare in distilled deionised water and filter sterilise. Final pH should be between 8.4 and 8.9.
c. ssDNA. Dissolve deoxyribonucleic acid sodium salt in TE buffer (pH 8.0) to a concentration of 2 mg L^{-1}. Mix vigorously on a magnetic stirrer for 2–3 h or until fully dissolved, aliquot and store at –20°C. Prior to use, boil for 5 min at 100°C and cool down on ice.

Growth medium

- LB medium: For preparation of 2 L LB medium, mix 20 g Bacto tryptone, 10 g Bacto yeast extract and 20 g sodium chloride and fill up to 2 L with deionised water. For solid medium also add 2% (w/v) Bacto agar. Heat sterilise for 20 min at 121°C. After sterilisation, antibiotics are added separately.
- YP (yeast peptone) medium: For 2 L YP medium, mix 10 g Bacto yeast extract, 20 g Bacto peptone and fill up to 2 L with demineralised water. Split the volume over five Schott bottles and, for solid medium, add 2% (w/v) Bacto agar to each bottle and heat sterilise for 20 min at 121°C. After sterilisation, the carbon source and antibiotics of choice are sterilised and added separately.
- SM (synthetic medium): For 2 L synthetic medium, start with 1.5 L of demineralised water and add 10 g ammonium sulphate [$(NH_4)_2SO_4$], 6 g monopotassium phosphate [KH_2PO_4] and 1 g magnesium sulphate heptahydrate [$MgSO_4.7{\cdot}H_2O$] and add 2 mL trace element solution (Verduyn *et al.* 1992). When these salts are dissolved, set the pH to 6.0 with 2M potassium hydroxide [KOH] and add demineralised water to reach a final volume of 2 L. For solid medium, add 2% (w/v) Bacto agar to each bottle and heat sterilise for 20 min at 121°C. The carbon source, auxotrophic growth requirements and antibiotics of choice are sterilised and added separately after sterilisation.
- SMglut (synthetic medium with glutamate as nitrogen source): SMglut is prepared similar to SM with the exception that the ammonium sulphate is replaced by 1 g L^{-1} L-glutamic acid monosodium salt and 6.6 g L^{-1} potassium sulphate to maintain a stable pH during cell growth, which improves selection when using antibiotic resistance selection markers (kanMX, natNT2, hphNT1).
- SMace (synthetic medium with acetamide as nitrogen source): SMace is prepared similar to SM, with the exception that ammonium sulphate is replaced by 0.6 g L^{-1} acetamide and 6.6 g L^{-1} potassium sulphate. SMace is used when using the amdSYM selection marker (Solis-Escalante *et al.* 2013).

Supplements for selection

Depending on marker usage, the following compounds are supplemented to the growth medium (Pronk 2002).

- 100 mg L^{-1} Ampicillin (Sigma Aldrich; A9518)
- 150 mg L^{-1} Uracil (Sigma Aldrich; U0750)
- 125 mg L^{-1} L-Histidine (Sigma Aldrich; H8000)
- 150 mg L^{-1} L-Leucine (Sigma Aldrich; 61819)
- 750 mg L^{-1} L-Tryptophan (Sigma Aldrich; T0254)
- 100 mg L^{-1} Nourseothricin (Jena Bioscience (Jena, Germany); AB-101)
- 200 mg L^{-1} G418 (Invivogen, Toulouse, France; ant-gn-1)
- 200 mg L^{-1} Hygromycin B (Thermo Fisher Scientific; 10687010)

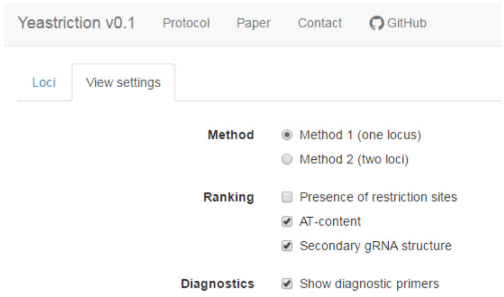
Protocol

Method (1): *In vivo* assembly of CRISPR-Cas9 gRNA plasmid using the pMEL series. Method (1) can be used to introduce marker-free and scarless genetic modifications. This method is highly efficient for editing a single locus and has the advantage of a very simple workflow prior to yeast transformation (steps 1–4). When aiming for multiple simultaneous genetic modifications, Method (2), *In vitro* assembly of single and double gRNA plasmids using the pROS, is recommended. The pROS method can be used to introduce multiple marker-free and scarless genetic modifications in a single transformation step. This method makes use of gRNA plasmids containing two gRNA coding sequences, facilitating restriction at two loci (steps 1–6).

1. Design of the guideRNA (gRNA) primer(s)
   a. Use the Yeastriction tool at http://yeastriction.tnw.tudelft.nl (Fig. 1)
   b. Alternatively, design the primer(s) manually (**Box S4**):
      i. For the pMEL method (1)
      Ptarget FW (5′ → 3′, N_{20} is the target sequence without the PAM) (Table 3): TGCGCATGTTTCGGCGTTCGAAACTTCTCCGCAGTGAAAGATAAATGATCN$_{20}$GTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAAC
      Ptarget RV (5′ → 3′, N_{20c} is the complementary target sequence without the PAM):
      GTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACN$_{20c}$GATCATTTATCTTTCACTGCGGAGAAGTTTCGAACGCCGAAACATGCGCA
      ii. For the pROS method (Fig. 2) (2)
      Forward & Reverse primer (Ptarget ROS (Table 3)) (5′ → 3′, where N_{20} is the target sequence without the PAM sequence, choosing a target site that contains a restriction site facilitates confirmation by restriction enzymes later on):
      TGCGCATGTTTCGGCGTTCGAAACTTCTCCGCAGTGAAAGATAAATGATCN$_{20}$GTTTTAGAGCTAGAAATAGCAAGTTAAAATAAG
      NOTE: When aiming to construct a pROS plasmid targeting two distinct loci, two Ptarget ROS primers with different target sequences should be designed.
   c. Order the primers PAGE-purified.

2. Construction of the gRNA insert fragment
   a. For the pMEL method (1)
      i. Dissolve the primers designed in **1.a** or **1.b.i** in deionised water to a final concentration of 10 μM
      ii. Mix the two complementary primers in a 1:1 molar ratio
      iii. Heat the mixture to 95°C for 5 min and subsequently cool down the mixture to room temperature on the bench to anneal both primers
      iv. Optional: Confirm efficient primer dimerisation using the Qubit fluorometer with Qubit dsDNA BR Assay Kit, repeat the process (**2.a**) when a concentration of over 200 ng μL^{-1} is observed.
   b. For the pROS method (2)
      i. Use the primer(s) designed in **1.a** or **1.b.ii** to set up the following PCR mixture:

Box 1: Yeastriction settings



Method: Method (1) designs primers for use with the pMEL method with two complementary gRNA primers used for *in vivo* plasmid assembly. Method (2) designs primers for use with the pROS method with one primer per gRNA, used for PCR amplification and subsequent *in vitro* plasmid assembly.

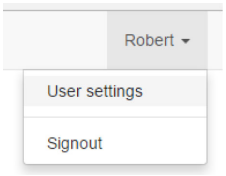
Ranking: By default, Yeastriction selects gRNA sequences based on the following criteria:

- Sequences containing a stretch of six or more Ts are discarded (this can terminate transcription)
- Sequences with off-targets are discarded (an off-target is defined as a sequence with either the NGG or NAG PAM sequence and 17 or more nucleotides identical to the original 20 bp target sequence)

When AT-content is enabled, Yeastriction ranks the remaining target sequences based on AT-content (20/20 ATs gets the maximal score of 1.0 and 0/20 ATs get the lowest score of 0.0)

When secondary gRNA structure is enabled, Yeastriction ranks the remaining target sequences based on the predicted secondary structure (using the RNAfold library, essentially with the parameters -MEA -noLP -temp=30). The number of target sequence nucleotides (20 in total) that are predicted to be involved in secondary structures determine the score (where 0/20 nucleotides get the maximal score of 1.0 and 20/20 nucleotides get the lowest score of 0).

When presence of restriction sites is enabled, Yeastriction ranks the remaining target sequences based on the presence of restriction sites pre-defined by the user. To define the restriction enzymes, log in to Yeastriction and select *User settings*. Target sequences containing an appropriate restriction site get a score of 1.0, target sequences without an appropriate restriction site get a score of 0.0. Note that Yeastriction does not check for the presence of the restriction site in the final plasmid backbone.



The final score for the gRNA sequences is calculated via addition of the scores for AT-content, secondary gRNA structure and presence of restriction sites.

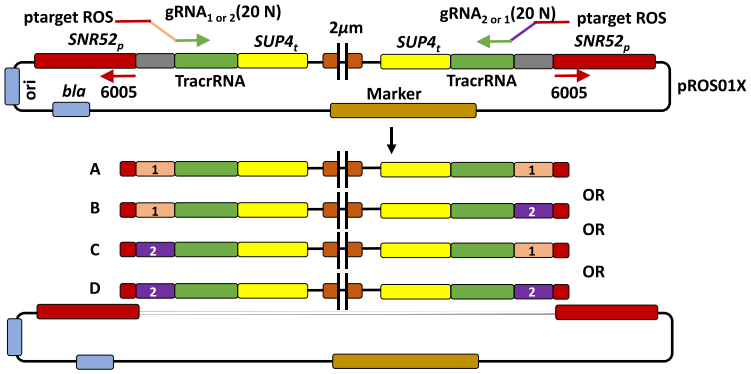
Diagnostics: When show diagnostic primers is enabled, the output of Yeastriction will contain two primers that can be used to amplify the region surrounding the coding region of the target gene to confirm deletion. Primers to check correct assembly of the gRNA plasmid have to be designed manually

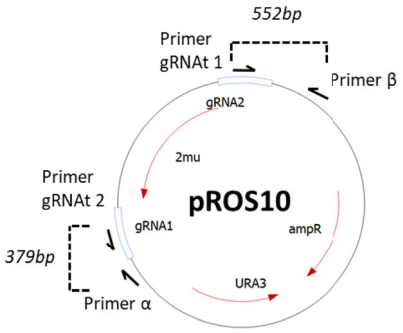
For a tutorial on Yeastriction we refer the reader to Mans *et al.* (2015).

Figure 1. Yeastriction settings.

Box 2: Confirming correct assembly of pROS plasmids using PCR

The introduction of one or two gRNA target sequences in pROS plasmids is accomplished by PCR amplification of a 2µm containing fragment using one or two 'ptarget ROS' primers containing the gRNA target(s) of choice. Using two different ptarget ROS primers, amplification of the gRNA insert fragment (see protocol **2.b**) can result in 4 different products, since the primers containing the gRNA sequences can anneal on both sides on the fragment. Therefore, Gibson assembly of the plasmid backbone, amplified with primer 6005, and the 2μm fragment containing the gRNA(s) can result in 4 different plasmid configurations (A-D):

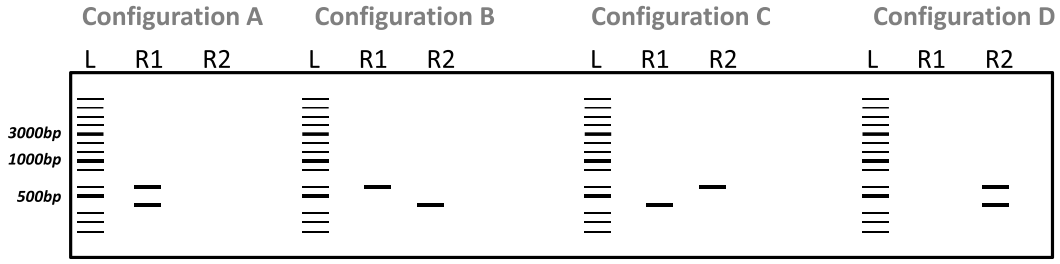




Plasmids assembled using 2μm fragment B or C contain only one of the two target sequences. Plasmids assembled using 2μm fragment A or D contain both target sequences.

Primer α and Primer β bind on the plasmid backbone and can be used to verify which version of the plasmid is obtained via PCR using a primer specific for each gRNA.

After both PCR reactions are performed (see protocol **6.a.ii**), the following results can be obtained (expected bands correspond with the plasmid configurations shown above the gel):



Where "L" denotes the ladder (GeneRuler DNA Ladder Mix), "R1" the result of PCR reaction 1 (primer α, primer β and primer gRNAt 1) and "R2" the result of PCR reaction 2 (primer α, primer β and primer gRNAt 2). Plasmids for which a single band is obtained in each of the reaction mixtures will contain both gRNA coding sequences and are stocked.

NOTE: The orientation of the 2μm fragment (after plasmid assembly) can be in either direction. This will impact the results of the PCR when primers binding inside the 2μm fragment are used.

Figure 2. Confirming correct assembly of pROS plasmids using PCR.

Component	Amount (μL)
DreamTaq Master Mix (2x)	50
pROS template*	1–100 ng
gRNA (Ptarget ROS1)(10 μM)	2
gRNA (Ptarget ROS2) (10 μM)**	2
MilliQ	X
Total volume	100

*This can be any pROS plasmid
**When aiming to construct a pROS plasmid targeting a single distinct locus, only gRNA primer 1 is used.

Note: The primer Ptarget$_{cas9}$ ROS (Table 3) can be used to construct a pROS plasmid carrying a gRNA targeting *cas9* gene.

ii. Divide the reaction mixture over two PCR tubes (50 μL each).
iii. Run the PCR with the following conditions:

Step	Temperature (°C)	Time (s)
1	95	240
2	95	30
3	55	30 40x*
4	66	120
5	66	600

*Steps 2–4 are repeated 40 times sequentially (2→3→4→2→3→4 etc.)

iv. After the PCR is finished, combine the PCR reactions and add 1 μL FastDigest DpnI and incubate for 30 min at 37°C.
v. Load the complete digested PCR reaction mixtures on a 1% agarose 1x TAE gel with SERVA staining (10 μL L^{-1}) and run the gel at 100 V for 30 min.
vi. Excise the 1589 bp PCR product using a transilluminator and purify using the ZymoClean Gel DNA Recovery Kit according to manufacturer's instructions.

3. Construction of the linearised gRNA plasmid backbone
   a. Amplify the plasmid backbone via PCR
      i. For the pMEL method (**1**)
         1. Set up the following PCR mixture:

Component	Amount (μL)
5x Phusion HF buffer	10
dNTPs (10 mM)	1
pMEL template*	1–5 ng
Primer 6005 (10 μM)	1
Primer 6006 (10 μM)	1
Phusion polymerase	0.75
MilliQ	X
Total volume	50

*This can be any pMEL plasmid

2. Run the PCR with the following conditions:

Step	Temperature (°C)	Time (s)
1	98	30
2	98	10
3	67	20 40x*
4	68	180
5	68	300

*Steps 2–4 are repeated 40 times sequentially (2→3→4→2→3→4 etc.)

ii. For the pROS method (**2**)
   1. Set up the following PCR mixture:

Component	Amount (μL)
5x Phusion HF buffer	10
dNTPs (10 mM)	1
pROS template*	1–100 ng
Primer 6005 (10 μM)	2
Phusion polymerase	0.75
MilliQ	X
Total volume	50

*This can be any pROS plasmid

2. Run the PCR with the following conditions:

Step	Temperature (°C)	Time (s)
1	98	300
2	98	30
3	63	30 40x*
4	68	120
5	68	600

*Steps 2–4 are repeated 40 times sequentially (2→3→4→2→3→4 etc.)

   b. Digest and purify the PCR product as described in **2.b. iv–vi**.
4. Construction of the double-stranded repair fragment(s)
   a. When aiming for deletions:
      i. Use the Yeastriction tool at http://yeastriction.tnw.tudelft.nl to design complementary primers which, when used as repair fragment, will delete the gene ORF.
      ii. Alternatively, manually design two complementary 120 bp primers. These primers are comprised of two adjacent 60 bp sequences, homologous to the up- and downstream region of the chromosomal locus containing the target site. The sequence between the selected up- and downstream regions will be removed from the chromosome during transformation (**Box S5**).
      iii. Order the primers DST- or PAGE-purified.
      iv. Anneal both primers as described in **2.a.i–iii**.
   b. When aiming for mutations:
      i. Design two complementary 120 bp primers. These primers are comprised of two adjacent 60 bp sequences, homologous to the up- and downstream region of the chromosomal target site. The desired mutation needs to prevent restriction by Cas9 and can therefore be introduced in the 20 bp gRNA recognition sequence or the adjacent 3 bp PAM sequence (changing the sequence from GG to any other sequence besides AG) (**Box S6**).

ii. Order the primers PAGE-purified.
iii. Anneal both primers as described in **2.a.i–iii**.

c. When aiming for DNA integration
i. Design primers for amplification of the desired DNA fragment for integration and add 60 bp 5′ tails in such a way that the resulting PCR product is flanked by 60 bp sequences homologous to the up- and downstream regions of the chromosomal target site (**Box S7**).
1. When aiming for deletion of the integration locus, design the 60 bp homologous sequences as described in **4.a.ii**.
2. To minimise alterations in the genomic DNA, the 60 bp homologous sequences can be designed in such a way that the integration event occurs in the 20 bp gRNA recognition sequence or the adjacent 3 bp PAM sequence, leaving the surrounding DNA intact.
ii. Order the primers PAGE-purified.
iii. PCR amplify the insert fragment using Phusion DNA polymerase according to manufacturer's instructions.
iv. Purify the PCR product as described in **2.b.v–vi**.

5. Assembly of the gRNA expression plasmid (pROS method (2) only)
a. Prepare the following NEBuilder reaction mixture:

Component	Amount (μL)
NEBuilder HiFi DNA Assembly Master Mix (2x)	2.5
gRNA insert fragment (**2.b**)	50 ng
Plasmid backbone (**3.a.ii** & **3.b**)	50 ng
MilliQ	X
Total volume	5

b. Incubate the reaction mixture at 50°C for 1 h.
c. Transform 2 μL of reaction mixture to 50 μL chemically competent *E. coli* cells according to the protocol of Zymo Research (T3001). Alternatively, transform 1 μL of the reaction mixture to 40 μL electro competent *E. coli* cells according to the protocol of Bio-Rad (165–2100).
d. Plate the transformed *E. coli* cells on a pre-warmed (37°C) LB plate containing 100 mg L^{-1} ampicillin.
e. Incubate the plate overnight at 37°C. The next day colonies are present on the plate.

6. Confirmation and storage of the constructed plasmid (pROS (2) method only)
a. Via colony PCR (Fig. 2)
i. Design primers that are complementary to the N20 introduced target sequences (gRNAt 1 and 2).
ii. Set up the following two PCR mixtures (for each plasmid, we recommend testing 4 to 8 colonies):

Mixture 1

Component	Amount (μL)
DreamTaq Master Mix (2x)	5
Template	*
Primer gRNAt 1 (10 μM)	0.5
Primer α (10 μM)	0.25
Primer β (10 μM)	0.25
MilliQ	4
Total volume	10

Mixture 2

Component	Amount (μL)
DreamTaq Master Mix (2x)	5
Template	*
Primer gRNAt 2 (10 μM)	0.5
Primer α (10 μM)	0.25
Primer β (10 μM)	0.25
MilliQ	4
Total volume	10

*Pick a small portion of the same colony (**5.e**) into both reaction mixtures using for example a sterile loop or sterile pipette tip.

iii. Run the PCR with the following conditions:

Step	Temperature (°C)	Time (s)
1	95	240
2	95	30
3	50–55**	30 35x*
4	72	30
5	72	600

*Steps 2–4 are repeated 35 times sequentially (2→3→4→2→3→4 etc.)
**When using AT-rich (>50%) gRNA sequences, a lower (50°C) annealing temperature could provide better results.

iv. Load 5–10 μL of the reaction mixture on a 1% agarose 1x TAE gel with SERVA staining (10 μL L^{-1}) and run the gel at 100 V for 30 min.
v. Confirm correct plasmid assembly by visualising bands using UV transillumination equipment. A double gRNA plasmid results in a single band (379 or 552 bp) for each reaction mixture.
vi. Pick one single *E. coli* colony (**5.e**) corresponding to a correct plasmid into a 15 mL Greiner tube containing 5 mL of liquid LB with 100 mg L^{-1} ampicillin, restreak the colony first if necessary.
vii. Incubate overnight at 37°C. Mix 3 mL of the grown culture with 1.5 mL 100% glycerol and divide over three 1.5 mL tubes and store at –80°C, the remainder or the culture can be used for isolation of the plasmid.

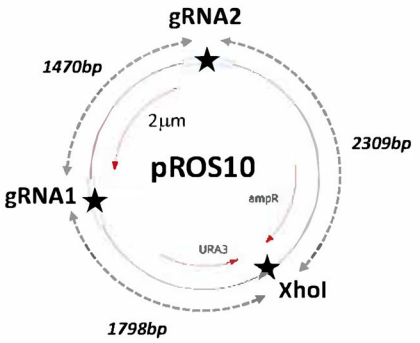
b. Via restriction analysis (when at least one of the gRNA primers designed in **1.b.ii** contains a restriction site) (Fig. 3)
i. Pick 4–8 colonies (**5.e**) with a sterile loop and transfer to 15 mL Greiner tubes containing 5 mL of liquid LB with 100 mg L^{-1} ampicillin.
ii. Incubate overnight at 37°C.
iii. Transfer 2 mL of the *E. coli* cultures to 2 mL Eppendorf tubes.
iv. Store the Greiner tubes with the remainder of the cultures at 4°C.
v. Extract the plasmids from the 2 mL of *E. coli* culture (**6.b.iii**) using the GenElute Plasmid Miniprep kit according to the supplier's manual, but elute the plasmid in 50 μL buffer or water instead of the recommended 100 μL.

Box 3: Confirming correct assembly of pROS plasmids using restriction

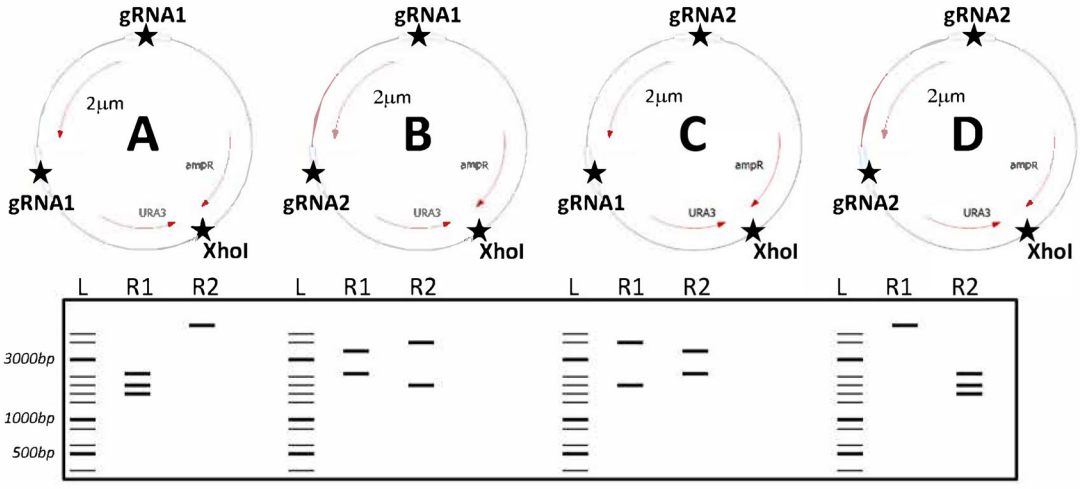
When a pROS plasmid is made to contain two different gRNA coding sequences, the presence of both gRNA sequences on the same plasmid has to be confirmed. Amplification of the double-gRNA insert fragment (see protocol **2.b**) can result in 4 different products, since the primers containing the gRNA sequences can anneal on both sides on the fragment. Therefore, assembly of the plasmid can result in 4 different plasmid configurations (**Box 2**).

When the gRNA coding sequences are designed such that at least one of the gRNA coding sequences contain a restriction site, the presence of both gRNA sequences can be confirmed via restriction analysis.

In this theoretical example, XhoI, an enzyme that only restricts a single location on the plasmid backbone is combined with restriction enzymes that cleave either gRNA sequence 1 or gRNA sequence 2 (and nowhere else on the plasmid). For actual experiments, the expected restriction sites and fragment lengths should always be confirmed using DNA analysis software.



After the two restriction reactions are performed (see protocol **6.b.vi**), the following results can be obtained:



Where "L" denotes the ladder (GeneRuler DNA Ladder Mix), "R1" the result of restriction reaction 1 (XhoI and a restriction enzyme cleaving gRNA1) and "R2" the result of restriction reaction 2 (XhoI and a restriction enzyme cleaving gRNA2). These expected results correlate with the plasmids configurations shown above the gel.

NOTE: The orientation of the 2μm fragment (after plasmid assembly) can be in either direction. This will impact the results of the restriction reaction when enzymes cutting inside the 2μm fragment are used. Also, the expected band sizes are heavily dependent on the choice of restriction enzymes and should always be checked prior to performing the experiment.

Figure 3. Confirming correct assembly of pROS plasmids using restriction.

vi. Set up the following digestion reaction:

Component	Amount (μL)
FastDigest Green Buffer (10x)	1
Plasmid (**6.b.v**)	250 ng
FastDigest Enzyme(s)**	0.5*
MilliQ	X
Total volume	10

*When multiple restriction enzymes are used, add 0.5 μL of each enzyme.
**When each gRNA coding sequence contains a restriction site, two separate reactions should be performed where only one of the restriction enzymes corresponding to the gRNA restriction sites is added.

vii. Incubate for 30–60 min at the temperature specified by the manufacturer.
viii. Load 5–10 μL of the reaction mixture on a 1% agarose 1x TAE gel with SERVA staining (10 μL L^{-1}) and run the gel at 100 V for 30 min.
ix. Confirm correct plasmid assembly by visualising bands using UV transillumination equipment.

c. Mix the culture containing the correct plasmid (**6.a.vii** or one of the cultures stored at **6.b.iv** corresponding to a correct plasmid) with 1.5 mL 100% glycerol.
d. Aliquot the culture in three 1.5 mL tubes and store at –80°C.

7. High concentration plasmid preparation using the GenElute Plasmid Miniprep kit. (**NOTE**: for most transformations, the protocol can be used as described under **6.b.v**)
   a. Inoculate a correct *E. coli* clone in 20 mL of liquid LB with 100 mg L^{-1} ampicillin in a 50 mL Erlenmeyer flask.
   b. Incubate overnight at 37°C.
   c. Transfer the whole *E. coli* culture to a 50 mL Greiner tube.
   d. Spin down the culture by centrifugation.
   e. Resuspend the cell pellet with 800 μL of resuspension solution and divide over four 1.5 mL Eppendorf tubes.
   f. Follow the rest of the GenElute Plasmid Miniprep Kit protocol, with the following modifications:
      i. Per *E. coli* culture, prepare four lysis reactions (see **7.e**).
      ii. When loading the DNA onto the column: load the cleared lysate of two reaction tubes, in two steps of 750 μL, onto a single column.
      iii. Elute the plasmid with 50 μL distilled water at 55°C. To obtain very high plasmid concentrations, use the eluate from the first column to elute the second column.
      iv. Load the eluate back onto the columns and elute again to increase the plasmid concentration.

8. Transformation to *S. cerevisiae*.
   a. *Saccharomyces cerevisiae* cells are prepared for transformation according to Gietz and Woods 2002.
   b. Prepare the following transformation mix (we recommend to include a negative control, which consists of the same mixture of which the double-stranded repair DNA is omitted). Comparing the number of transformants obtained with and without repair DNA should provide an indication on the targeting efficiency. In case of efficient editing, the number of transformants obtained in presence of the repair DNA should be significantly (>10-fold) higher than in its absence. You should also be aware that editing efficiency might be affected by strain ploidy (haploid, polyploid, aneuploid).
      i. For the pMEL method (**1**)

Component	Amount
pMEL plasmid backbone (**3.a.i.1–2** & **3b**)	100 ng
gRNA insert fragment (**2.a.3**)	500 ng
Double stranded repair DNA (**4**)	*
50% PEG	240 μL
Lithium acetate (1M)	36 μL
SSDNA	25 μL
Distilled water (sterile)	X μL
Total volume	351 μL

*When aiming for gene deletions or the introduction of SNPs, we recommend using 1 μg of repair DNA. When aiming for (multiple) gene integration, we recommend using at least 200 ng/kb for each fragment.

ii. For the pROS method (**2**)

Component	Amount
Double gRNA plasmid (**7f**)	1 μg#
Double stranded repair DNA (**4**)	*
50% PEG	240 μL
Lithium acetate (1M)	36 μL
SSDNA	25 μL
Distilled water (sterile)	X μL
Total volume	351

*When aiming for gene deletions or the introduction of SNPs, we recommend using 1 μg of repair DNA per target locus. When aiming for (multiple) gene integration, we recommend using at least 200 ng/kb for each fragment.
#When transforming multiple plasmids, we recommend using 2–5 μg of DNA per plasmid to increase the number of transformants.

   c. *Saccharomyces cerevisiae* cells are transformed according to Gietz and Schiestl (2007).
   d. Select for successful transformants by plating the transformation mixture on agar plates of the appropriate medium. For dominant markers, rich (YP) medium can be used.

Plasmid background	Marker gene	Selection medium
pMEL/pROS10	*URA3*	SM without uracil
pMEL/pROS11	amdSYM	SMAce
pMEL/pROS12	hphNT1	SMglut/YP + 200 mg L^{-1} hygromycin B
pMEL/pROS13	kanMX	SMglut/YP + 200 mg L^{-1} G418
pMEL/pROS14	*LEU2*	SM without leucine
pMEL/pROS15	natNT2	SMglut/YP + 100 mg L^{-1} nourseothricin
pMEL/pROS16	*HIS3*	SM without histidine
pMEL/pROS17	*TRP1*	SM without tryptophan

   e. Incubate the plates at 30°C until colonies are clearly visible.

9. Selection of successful transformants.
   a. Design and order diagnostic primers
      i. Use the Yeastriction tool at http://yeastriction.tnw.tudelft.nl.

ii. Alternatively, design forward and reverse primers, of which the resulting PCR products cover relevant homologous recombination sites to confirm correct DNA integration events during transformation (**Box S8**).
iii. Order the primers DST-purified.

b. Isolate genomic DNA.
i. According to Lõoke, Kristjuhan and Kristjuhan (2011)
1. Inoculate a single colony in 200 μL YP medium in a sterilised Eppendorf tube and incubate overnight at 30°C. Spin down 200 μL of the grown yeast culture (this can be done directly when starting from a liquid culture (**9f**)), remove the supernatant and resuspend the pellet in 100 μL 0.2 M lithium acetate and 1% SDS.
2. Alternatively (faster but less efficient), pick a large portion of a yeast colony into an Eppendorf tube containing 100 μL 0.2M lithium acetate and 1% SDS.
3. Heat to 75°C for 10 min.
4. Add 300 μL 100% ethanol and vortex.
5. In a benchtop centrifuge, spin down at ≥13 000 *g* for 1 min and remove the supernatant.
6. Resuspend the pellet in 150 μL 70% ethanol.
7. Spin down at ≥13 000 *g* for 1 min and remove the supernatant.
8. Dry the pellet at 37°C for 15–60 min by leaving the tube open until the pellet is completely dry.
9. Add 10–50 μL distilled water and vortex thoroughly.
10. Spin down at ≥13 000 *g* for 1 min.
11. Use the supernatant directly as template for PCR or transfer to a new Eppendorf tube and store at –20°C for later use.
ii. Alternatively, use the YeaStar Genomic DNA kit following the manufacturer's protocol.

c. Prepare the colony PCR reactions using the following PCR mix.

Component	Amount (μL)
DreamTaq Master Mix (2x)	5
Template DNA (**9b**)	0.25
Forward primer (10 μM)	0.25
Reverse primer (10 μM)	0.25
MilliQ	4.25
Total volume	10

d. Run the PCR with the following conditions:

Step	Temperature (°C)	Time (s)
1	95	240
2	95	30
3	55	30 40x*
4	72	60/kb
5	72	600

*Steps 2–4 are repeated 40 times sequentially (2→3→4→2→3→4 etc.)

e. Isolate one correct colony by steaking to a new plate and incubate at 30°C until single colonies are obtained.
f. Inoculate a single colony in liquid medium, when the culture is grown, repeat the confirmation PCR (**9b–d**).
g. Mix 10 mL of the grown culture with 5 mL 100% glycerol, aliquot in 1.5 mL tubes and store at –80°C.

10. Plasmid removal (up to four plasmids simultaneously in a single round) (**Box S9**)
a. Inoculate the plasmid bearing yeast strain (**9f or 9g**) in 25 mL of non-selective liquid medium.
i. When using auxotrophic markers this can be achieved by growing the cells on rich medium such as YP medium or via addition of the appropriate nutrients to SM.
ii. When using dominant markers this can be achieved by omitting or replacing the components associated with the dominant marker (**8d**) from the medium.
b. Incubate the culture at 30°C until the exponential growth phase is finished. The time required to achieve depletion of the carbon source heavily varies based on the strain background and medium composition.
c. Transfer 1 μL of the grown culture to 100 mL non-selective medium and incubate at 30°C until depletion of the carbon source. **NOTE**: This additional incubation step increases the fraction of cells that have lost the plasmid and can be repeated several times to increase the fraction of cells without plasmid(s).
d. Streak part of the grown culture on a non-selective agar plate and incubate at 30°C until single colonies are clearly visible.
e. Re-steak the obtained single colonies on non-selective plates and selective plates to confirm removal of the gRNA plasmid.
f. Transfer colonies (from the non-selective plate **10e**) that grow on non-selective, but not on selective medium agar plates to 20 mL non-selective liquid medium.
g. After sufficient cell growth, the culture is stored at –80°C as described in **9e - g** and can be used for subsequent round(s) of transformation.

11. Troubleshooting

Step	Solutions
2.a/4.ab: Low efficiency annealing of primers (measured by Qubit)	When low primer annealing efficiencies are obtained, repeating this step usually results in a higher efficiency.
2.b: Low PCR efficiency for amplification of the gRNA insert fragment for pROS plasmids	The gRNA insert fragment used in the construction scheme of the pROS plasmids (expression of two identical or different gRNA) might be difficult to PCR amplify. Empirically, the DreamTaq polymerase (Thermo Fischer Scientific) showed the best performance, and is routinely used for this purpose. Alternatively, the 2μm fragment might be split in two fragments that might be assembled with the yeast replication origin through a 25 to 60 bp overlap included in the primers sequences. This option would facilitate the construction of plasmid expressing two different gRNAs as each gRNA would be inserted in an independent PCR reaction, avoiding the need for screening for the correct plasmid (Figs 2 and 3). As a trade-off the subsequent Gibson assembly reaction will include three parts instead of two that might lower the assembly efficiency.
3.a: Low PCR efficiency for amplification of the plasmid backbone	DNA fragments containing a selection marker such as nourseothricin resistance marker can be difficult to PCR, probably due to higher GC content. When low PCR yields are observed, we recommend increasing the denaturation time (step 2) to 1–2 min.
5: Low transformation efficiency of Gibson assembly mix	If you observe a high rate of false-positive *E. coli* transformants after transformation of the Gibson assembled gRNA expression pMEL or pROS plasmids, we recommend to: 1) lower the concentration of template DNA for amplification of the plasmid backbone and gRNA insert fragment to 0.1–1 ng per 50 μL; 2) increase the duration of the DpnI digestion step; 3) if possible increase the gel separation of the PCR product by increasing the run time.
8c: Low yeast transformation efficiency	If transformation is performed with the LiAc protocol, we recommend to check the troubleshooting section described in Gietz and Schiestl (2007). In addition to issues directly related to the transformation itself, causes for low efficiency might have diverse origins such as: 1) Misassembled gRNA plasmid, resulting in no active gRNA expression. This can be confirmed via Sanger sequencing of the transformed plasmid and solved by picking a different *E. coli* transformant or repeating plasmid construction. 2) A mutation in *cas9*. This can be confirmed by transforming another gRNA plasmid which is known to be highly active. This can be solved by picking a different yeast transformant from the previous transformation. 3) A mutation in the gRNA sequence. Especially for yeast strains of which no whole genome sequence is available, SNPs compared to the used reference genome can reduce efficiency of Cas9 editing leading to imperfect annealing of the gRNA. This can be confirmed by Sanger sequencing of a PCR fragment which amplified the region of interest. This can be solved by picking a new gRNA targeting sequence or modifying the gRNA design to account for the mutation identified after sequencing. 4) A non-functional gRNA. For reasons not well understood, some gRNA target sequences exhibit low restriction activity. This is solved by designing a new gRNA. 5) A notoriously difficult loci to modify. Depending on the target locus, different transformation efficiencies are obtained. This is solved by increasing the gRNA expression plasmid concentration in the transformation step, resulting in more colonies.
9c and 9d: Low colony PCR efficiency	Some genetic loci are notoriously difficult to amplify via PCR. When low efficiencies are obtained in colony PCR of yeast colonies, we suggest to try the following: 1) Repeat the PCR, varying the concentration of template DNA (0,1x and 10x). 2) Repeat the DNA extraction from an exponentially growing liquid culture, preferably using a commercial high-quality DNA extraction kit. 3) Design new primers for amplification of the region of interest, paying attention to design the primers such that the length of the resulting PCR fragment is short (~250 bp) to increase PCR efficiency.
10: Low efficiency plasmid removal	When experiencing difficulties in plasmid removal, we suggest to increase the number of generations in non-selective medium. When working with counterselectable markers such as *URA3* or *amdS*, counterselection can help improve the efficiency of plasmid loss.

SUPPLEMENTARY DATA

Supplementary data are available at *FEMSYR* online.

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