Method for CRISPR/Cas9 Mutagenesis in *Candida albicans*

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[Abstract] *Candida albicans* is the most prevalent and important human fungal pathogen. The advent of CRISPR as a means of gene editing has greatly facilitated genetic analysis in *C. albicans*. Here, we describe a detailed step-by-step procedure to construct and analyze *C. albicans* deletion mutants. This protocol uses plasmids that allow simple ligation of synthetic duplex 23mer guide oligodeoxynucleotides for high copy gRNA expression in *C. albicans* strains that express codon-optimized Cas9. This protocol allows isolation and characterization of deletion strains within nine days.

Keywords: *Candida albicans*, Cas9, CRISPR, Fungal genetics, gRNA, Yeast

[Background] *C. albicans* is a difficult organism to manipulate genetically. Since it normally exists as a diploid that does not readily undergo sexual reproduction, homozygous recessive mutations require sequential modification of each locus. The development and application of Clustered Regularly Interspaced Short Palindromic Repea ts (CRISPR) mutagenesis in *C. albicans* facilitates genetic manipulations because it allows simultaneous mutation of both alleles (Vyas *et al.*, 2015; Min *et al.*, 2016; Ng and Dean, 2017). CRISPR gene editing involves recruitment of an RNA-guided nuclease to a complementary target site adjacent to an NGG protospacer adjacent motif (PAM) (Jinek *et al.,* 2012; Cong *et al.,* 2013; Mali *et al.*, 2013). CRISPR associated (Cas) nuclease is targeted with high specificity through complementary base pairing between a guide RNA associated with *trans*-activating CRISPR RNA (tracrRNA), which binds Cas9 (Gasiunas *et al.*, 2012). Since the chromosomal target sequence is only ~20 nucleotides, expression of a single guide RNA (sgRNA) fused to a ~80 nucleotide tracrRNA, along with Cas9, is sufficient for targeted double-strand DNA cleavage. Since chromosomal breaks are lethal, there is a strong selective pressure for double stranded break repair. In *C. albicans*, co-expression of a donor repair fragment, containing homology to regions flanking the break, oligonucleotides into pre-cut vectors allows repair of the break by homologous recombination. Thus, appropriate design of the donor repair fragment allows introduction of sequence deletions, replacements or other chromosomal alterations.

Our previous studies demonstrated that a key factor contributing to high efficiency CRISPR mutagenesis of *C. albicans* genes relies on optimal gRNA expression (Ng and Dean, 2017). Toward this end, we created gRNA expression vectors that permit high levels of gRNA expression. The basis for this high-level expression is in part due to the presence of a strong, RNA polymerase II promoter (P_{ADH1}). This promoter drives the expression of an sgRNA flanked by a 5' tRNA and a 3' hepatitis delta

virus (HDV) ribozyme RNA. The presence of these 5' and 3' flanking RNA sequences serve to promote efficient post-transcriptional processing to produce a mature sgRNA with precise ends. In the presence of an appropriate donor repair fragment, this increased sgRNA expression dramatically improves CRISPR/Cas mutagenesis in *C. albicans*. In practice, execution of mutagenesis is quite simple. The gRNA expression plasmid cloning relies on the annealing and ligation of two short gRNA encoding oligonucleotides into pre-cut vectors. Mutagenesis involves co-transformation of an sgRNA plasmid and the donor repair fragment into *C. albicans* strains that express codon-optimized Cas9 nuclease. Described below are detailed protocols for the design, synthesis, and cloning of gRNA oligonucleotides, healing fragment construction, yeast transformations, and mutant verification.

Materials and Reagents

1. 1.5 ml microfuge tubes (acceptable as sold by any science distributor)
2. Glass or disposable round bottom sterile tubes for growing 2-5 ml cultures of yeast and bacteria (acceptable as sold by any science distributor)
3. Plastic Petri dishes (acceptable as sold by any science distributor)
4. PCR tubes
5. Toothpicks
6. Yeast strains (Table 1)

Table 1. Strain list

Strain	Genotype	Auxotrophies	Reference
BWP17	*ura3Δ::λimm434/ura3Δ::λimm434 his1Δ::hisG/his1Δ::hisG arg4Δ::hisG/arg4Δ::hisG*	Ura⁻His⁻Arg⁻	Wilson *et al.,* 1999
RJY54	BWP17 *RPS1::P$_{TEF1}$CaCas9-HA-ARG4-RPS1*	Ura⁻His⁻	Ng and Dean, 2017
HNY30	BWP17 *eno1Δ*::CaCas9	Ura⁻His⁻Arg⁻	Ng and Dean, 2017
HNY31	BWP17 *eno1Δ*::CaCas9 *leu2Δ/leu2Δ*	Ura⁻His⁻Arg⁻Leu⁻	Ng and Dean, 2017

7. Plasmids (Table 2)

Table 2. Plasmids

Number	Name	Relevant features	Reference/Source
pND425	CIpArg4-P$_{TEF}$CaCas9	Codon-optimized *Cas9* driven by *AgTEF1* promoter in CIp-Arg	Ng and Dean, 2017
pV1025	pV1025	CaCas9/*SAT* flipper *ENO1* integrative plasmid	Vyas *et al.,* 2015
pND494	CIp10-P$_{ADH1}$ tA SapI HDV	CIP10 based cloning vector for ligating gRNA P$_{ADH1}$tA-SapI2x *HDV* in CIp10	Ng and Dean, 2017
pND501	CIp-dpl- P$_{ADH1}$ tA SapI HDV	pND494 but contains *ura3-dpl200* instead of *URA3*	Ng and Dean, 2017

8. *E. coli* competent cells (DH5α) (made as described, *e.g.*, https://www.neb.com/protocols/2012/06/21/making-your-own-chemically-competent-cells or can be purchased from New England Biolabs)

   Note: We use CaCl$_2$-treated DH5α but these can be purchased.

9. Glycerol (any source) (50% in H$_2$O, autoclaved)

10. Salmon sperm DNA (Sigma-Aldrich, catalog number: D1626; 10 mg/ml, sonicated and boiled)

11. *Sap*I (New England Biolabs, catalog number: R0569S)

12. *Cla*I (New England Biolabs, catalog number: R0197S)

13. Calf Intestinal Phosphatase (CIP) (New England Biolabs, catalog number: M0290L)

14. Custom oligonucleotides (between 18-60 nucleotides in length, Eurofins MWG Operon, Alabama)

15. T4 polynucleotide kinase (New England Biolabs, catalog number: M0201S)

16. T4 ligase (New England Biolabs, catalog number: M0202L)

17. 2x YT (see https://www.elabprotocols.com/protocols/#!protocol=5436 for recipe) (+ 100 μg/ml ampicillin) liquid media and plates (bacterial selection)

18. Ampicillin (any source)

19. *Taq* polymerase (any source)

20. Polyethylene glycol (PEG) 3350 (Sigma-Aldrich, catalog number: P4338)

21. Lithium acetate (any source)

22. *Nco*I or *Stu*I

23. Uridine (any source)

24. 0.2% SDS

25. Agarose (any source)

26. Qiaquick Gel Extraction Kit (QIAGEN, catalog number: 28706)

27. LB
    a. Yeast extract (BD, Bacto^{TM}, catalog number: 212750)
    b. Tryptone (BD, Bacto^{TM}, catalog number: 211705)
    c. Sodium chloride (NaCl, certified ACS grade ≥ 99.0%) (Fisher Scientific, catalog number: S271)

28. YPD
    a. Yeast extract (BD, Bacto^{TM}, catalog number: 212750)
    b. Peptone (BD, Bacto^{TM}, catalog number: 211677)
    c. Dextrose (Fisher Scientific, catalog number: D16)
       Note: Make 20% stock in double distilled water.

29. Synthetic dextrose lacking uracil (SD-URA) liquid media and plates (uracil prototrophic yeast selection) (see http://cshprotocols.cshlp.org/content/2015/2/pdb.rec085639.short for recipe)
    a. Bacto-Agar (for plates)
    b. Dextrose (Fisher Scientific, catalog number: D16)
       Note: Make 20% stock in double distilled water.

  c. Yeast Nitrogen base w/o amino acids w/ammonium sulfate (BD, catalog number: 291940)

  d. All amino acids, purines and pyrimidines (any source)

30. SD-complete + 5' FOA (*ura3Δ* yeast selection plates) (see http://cshprotocols.cshlp.org/content/2016/6/pdb.rec086637.short for recipe).

  a. Bacto-Agar

  b. Dextrose (Fisher Scientific, catalog number: D16)

   Note: Make 20% stock in double distilled water.

  c. Uracil

  d. Yeast Nitrogen base w/o amino acids w/ammonium sulfate (BD, catalog number: 291940)

  e. 5-Fluoroorotic acid (5-FOA) (Oakwood Products, catalog number: 003234)

Equipment

1. Pipettes (capable of accurately pipetting from 1 µl-10 ml) (any source)

2. Thermocycler (MJ Research, model: PTC-200)

3. Vortex (any source)

4. Micro centrifuge capable of 14 kG (Eppendorf)

5. Heating blocks (37 °C, 44 °C)

6. Incubators (30 °C, 37 °C)

7. DNA gel electrophoresis apparatus

8. UV transilluminator (or handheld UV lamp)

9. Shaker incubator (or rolling drum) (30 °C, 37 °C)

10. Apparatus for media sterilization (autoclave or pressure cooker)

Procedure

The overall procedure entails identification of appropriate target site in the chromosome, design of the gRNA oligonucleotides and their ligation into the gRNA expression plasmid, design and construction of the donor repair fragment, co-transformation of gRNA plasmid and repair fragment into yeast, and finally, screening and verification of mutant strains.

Detailed protocols for each of these steps are described following the timeline outlined below.

Basic protocol/time line for single gene knockout

Day 1-2. Design and order oligonucleotides (see Procedure A)

1. 3 pairs of different gRNA targets (if possible, target one PAM in promoter region up to 150 bases upstream the initiating ATG) (~23 bases/oligonucleotide).

2. 2 oligonucleotides for donor repair fragment (~60 bases/primer).

3. 2 oligonucleotides (forward and reverse) for genotyping.

Day 3-4. Make gRNA plasmid and repair fragment (see Procedure B, C, and D)

1. Anneal and fill in donor repair fragment.

2. Anneal and ligate gRNA-encoding oligonucleotides into *Sap*I digested and dephosphorylated vector.

3. Transform into *E. coli* competent cells.

Day 4-5. Confirm plasmid inserts (see Procedure B)

1. Grow bacterial culture and prepare gRNA plasmid (6-8 h incubation at 37 °C).

2. Check gRNA plasmid for loss of *Cla*I site.

3. Digest w/*Stu*I for yeast transformation.

4. Put up overnight yeast culture for next day transformation.

Day 6-7. *C. albicans* transformation (see Procedure E)

1. Dilute yeast culture 1:20 for transformation (5 h incubation at 30 °C).

2. Transform into *C. albicans.*

Day 9-10. Genotype mutants (see Procedure F and G)

1. Assay Ura$^+$ transformants by PCR

2. Streak single colonies to avoid mixture of mutants, recheck by PCR, then stock 2-3 mutant strains.

A. Design and order gRNA, knock-out verification oligonucleotides

1. Identify a gene or DNA region you wish to target for mutagenesis and find an appropriate gRNA target sequence. The requirement for Cas9 cleavage is the presence of a PAM sequence (5'-NGG-3') immediately downstream of the ~20 base pair target. This 5'-NGG-3' can be on either DNA strand. This sequence must be unique in the *C. albicans* genome to prevent off-target cleavage. A useful web site for identification and ranking of appropriate gRNA is CHOP CHOPv2 (http://chopchop.cbu.uib.no/) (Labun *et al.,* 2016). Generally, we adhere to the ranking of PAM sites obtained using this web site to guide the choice of target site.

2. Two complementary oligonucleotides corresponding to the 20 bp double stranded sequence are required. Each oligonucleotide is 23 bases in length and must also contain appropriate sequences at their 5' end to provide sticky ends for direct ligation of annealed dsDNA into pND494 or pND501 cut with *Sap*I (Figure 1). The choice of which vector to use is based on whether or not subsequent experiments require a uracil prototrophic or auxotrophic strain. If a uracil auxotroph is required, pND501 allows recycling of the *Ura-* genotype (see Procedure H). The cloning cassette (see Figure 1 below), into which these annealed oligonucleotides are ligated, has been designed to allow precise fusion to both an upstream tRNA sequence and a downstream tracrRNA (Ng and Dean, 2017). This entire tRNA-sgRNA-HDV sequence is driven by the *ADH1* promoter in both pND494 and pND501 vectors (Figure 1A). The end result is the

expression of an sgRNA flanked with tRNA and ribozymes for precise post-transcriptional processing to produce appropriate 5' and 3' ends.

3. Figure 1 depicts gRNA expression vector maps (A) and an example of the design of the two complementary gRNA oligonucleotides (B). The top sequence in Figure 1B depicts the *Cla*I-containing *Sap*I cassette present in the pND494/501 expression vectors. The lower sequence depicts the gRNA-encoding oligonucleotides after annealing and ligation into this cassette. This example shows the ligation of the duplex encoding *LEU2* gene-specific gRNA, with the boxed sequences indicating each of the oligonucleotides that were synthesized.

A
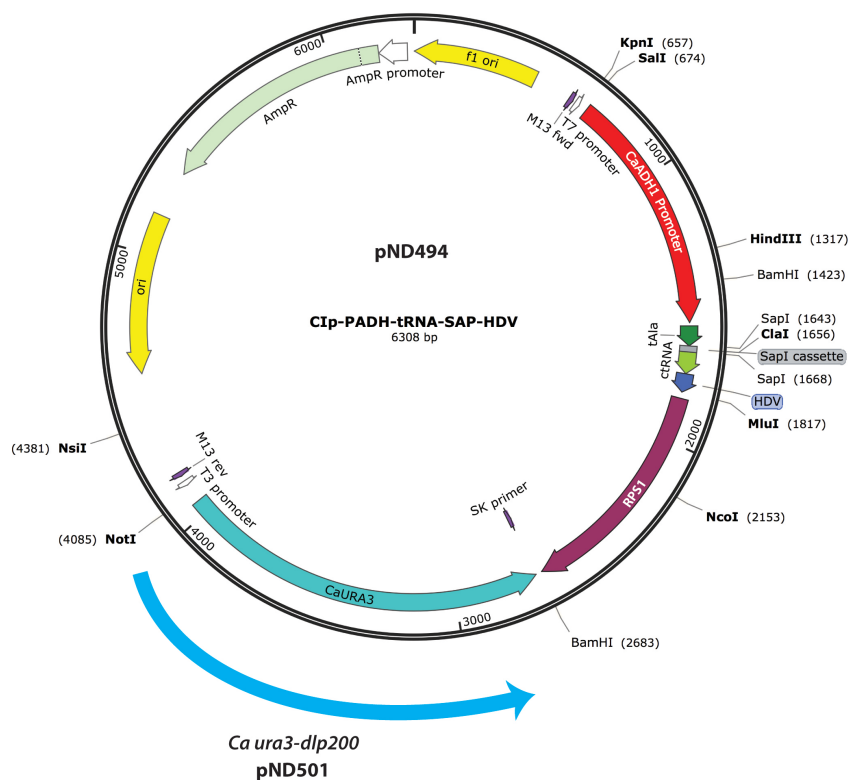
B

Figure 1. How to design gRNA-encoding oligonucleotides for expression cloning. The pND494 and pND501 cloning vectors (Panel A) contain a *Sap*I cloning cassette designed for

direct ligation of annealed gRNA oligonucleotides (Panel B). The DNA sequence of the *Sap*I cloning cassette is indicated in the upper DNA duplex (B). Shown below is an example of the ligation of double-stranded annealed oligonucleotides encoding a *LEU2* targeting gRNA (from Ng and Dean, 2017). The boxed in region indicates the sequence of each oligonucleotide. Note that each 23 bases oligonucleotide includes an overhang sequence that results in a sticky end cohesive with that of the vector digested with *Sap*I. Since *Sap*I cleavage is non-palindromic, re-ligation of the vector is not possible thus greatly reducing the background of false-positive *E. coli* transformants (C4).

4. When ordering gRNA oligonucleotides, we generally target 3 different PAM sites (which requires 6 different gRNA oligonucleotides) because the efficiency of gRNA-dependent CRISPR mutagenesis varies in a sequence dependent way. Targeting 3 different PAM sites maximizes the probability of obtaining a correct mutant. Experimental evidence suggests that targeting 5' proximal regions, near the start site of transcription results in higher mutagenesis frequency (Radzisheuskaya *et al.*, 2016). Thus if possible, choose one target within 150 bases upstream (5') the first initiating ATG methionine codon as one of target sites.
   *Note: **DO NOT INCLUDE PAM SITE in gRNA oligonucleotide.***

5. Design primers for verification of correct chromosomal mutation
   Order two ~18-30 bp oligonucleotides, forward and reverse, for checking chromosomal gene deletion, by PCR amplification of genomic DNA. If possible, the product of this PCR amplification should be less than 500 bp. This allows PCR extension time to be less than 30 sec (using *Taq* polymerase), thereby minimizing the time required for verification.

B. Create an appropriate repair template

A repair template is a double stranded DNA fragment that spans the cut site, has sufficient homology for homologous recombination, and creates appropriate mutation. In addition to introducing the desired mutation, repair templates **must lack the PAM site**. Otherwise the target break site will be re-introduced into the chromosome after its repair.

The simplest repair template for creating deletion allele uses a pair of 60 bp oligonucleotides with ~47 bp of homology (at 5' ends) to sequences flanking the chromosomal break site, ~20 bp overlap at 3' end, and a unique restriction site within the overlap confirmation of correct mutation (*e.g.*, see Vyas *et al.,* 2015; Ng and Dean, 2017). An example that shows repair strategy for deleting the *LEU2* gene (from Ng and Dean, 2017) and the sequence of repair oligonucleotides is illustrated in Figure 2.

A

B
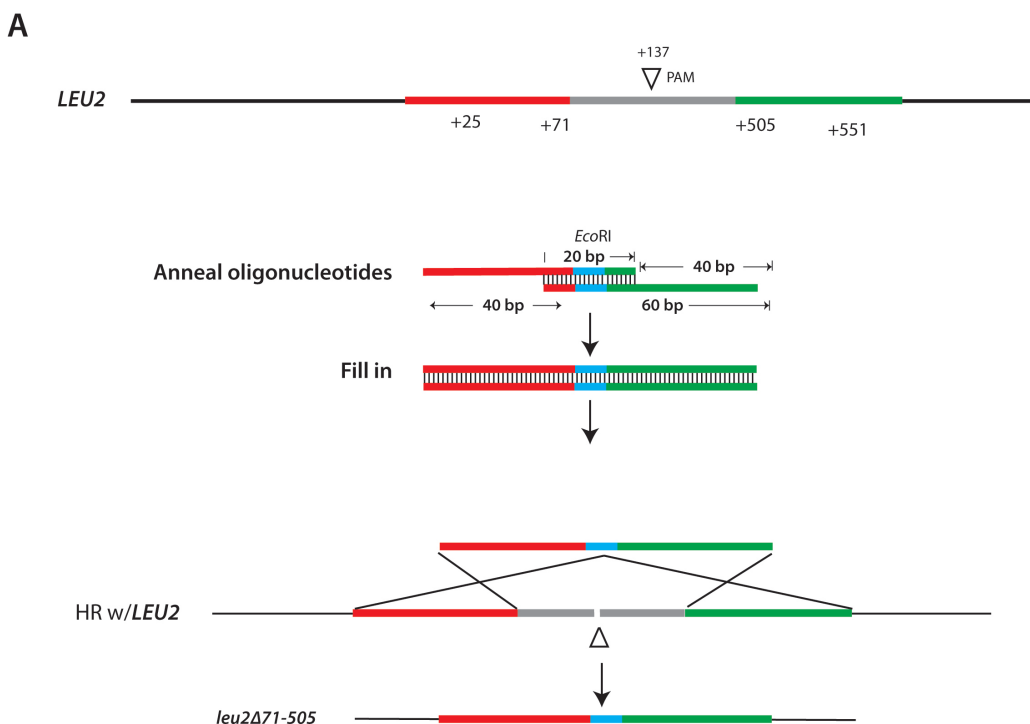
5'ACTGTCTTGCCTGGTGACCATGTCGGTACCGAAATTGTCAATGAAGCGAATTCCTGTTGA–>3'
                                  3'<– TACTTCGCTTAAGGACAACTGCTTCAATGAGCATAGTGAGCATACCGTCGTAAATACCGA 5'

Figure 2. Design of a repair template for gene deletion. Schematic diagram of donor repair fragment synthesis used for deletion of the *LEU2* locus, targeting break at the PAM site at position 137 **(Panel A)** using the oligonucleotides depicted in Figure 1B. Chromosomal sequences homologous to the repair template are colored in red and green. Each 60-mer oligonucleotide ends with a 3' 20 bp sequence of complementarity, including a restriction site that is absent in *LEU2* (*Eco*RI), shown in blue. When annealed and extended, this repair fragment contains 47 bp of homology to sequence flanking the DSB. In this example, taken from Ng and Dean, 2017, homologous recombination results in a 434 deletion of *LEU2* and insertion of a unique *Eco*RI site, which simplifies genotyping of putative mutants. **Panel B** shows the sequence of each "repair fragment" oligonucleotide and their alignment. Sequences are color-coded red, blue, and green to indicate the location of each sequence as indicated in Panel A.

C. Cloning gRNA oligonucleotides into gRNA vector

Both pND494 or pND501 can be used for gRNA expression in any *ura3Δ CAS9 C. albicans* strain. The only difference in these two vectors is that pND501 has a recyclable *URA3* allele (Wilson *et al.,* 2000) if further phenotypic analyses require the loss of *URA3* (see below).

1. Digest 2 µg vector (pND494 or pND501) with *Sap*I

DNA	2 µg
10x buffer	5 µl
*Sap*I	1 µl
H_2O	to 50 µl

   a. Incubate at 37 °C for 5-15 min for Fast Digest/High Fidelity enzyme.

   b. Spin down.

   c. Add 1 µl calf intestinal phosphatase (CIP) (or Antarctic phosphatase).

   d. Incubate at 37 °C for 1 h.

   e. Purify using a QIAquick Gel Column (no need to run it on a gel); elute in 30 µl elution buffer or TE, pH 8.0.

2. Phosphorylation and annealing sgRNA oligos (from Vyas *et al.,* 2015).

   a. Add to a PCR tube:

100 µM Oligo 1 (top)	1 µl
100 µM Oligo 2 (bottom)	1 µl
10x T4 ligase buffer	5 µl
T4 polynucleotide kinase	1 µl
H_2O	42 µl

   Note: Do a negative control with no oligos.

   b. Incubate in a thermocycler:

   37 °C for 30 min

   95 °C for 5 min

   Cool to 16 °C, at the slowest ramp rate your machine can do (ours does 0.1 °C/sec).

3. Ligation of annealed sgRNA oligonucleotides

   a. Assemble in a PCR tube:

10x T4 ligase buffer	1 µl
T4 ligase	0.5 µl
Annealed Oligo mix	0.5 µl (include a negative control w/no oligos)
Vector	20-40 ng
H_2O	to 10 µl

   b. Incubate tubes in a thermocycler or cool/heat blocks:

   16 °C for 30 min

   65 °C for 10 min

   Cool to 25 °C

4. Transform 5 µl into 50-100 µl competent bacterial DH5α cells.

   Plate cells on 2x YT (or LB) + ampicillin plates. Incubate overnight at 37 °C; prepare plasmid DNA using any published protocol.

5. Screening colonies

   This protocol usually results in ~10-20 colonies, with no background from cut vector alone. Identify correct clones by restriction analysis, screening for loss of the *Cla*I site that is predicted by its replacement in the *Sap*I cassette with gRNA oligonucleotides (note that pND494 contains a single *Cla*I site, in the cassette, while pND501 contains two *Cla*I sites). Any potentially correct clone should be verified by DNA sequence analysis. The following primers are useful as for sequencing gRNA ligations especially if these vectors will be used for multiple gene knockouts. These primers (see sequences below) are homologous to the flanking 5' P_{ADH1} promoter and 3' HDV ribozyme sequences.

   5' within P_{ADH1} upstream of tRNA (-247 relative to +1)
   5' GCTTATTCAGAATTTTCAGA 3'

   3' within HDV (includes the *Mlu*I site at 3' end of HDV)
   5'-CGAGATGACGCGTGTCCCATTCGCCAT-3'

D. Preparation of repair template

   To create the repair template using ~60 bp oligonucleotides with ~20 bp overlap at 3' end, subject mixed oligonucleotides to annealing and DNA replication to generate a product of ~100 bp (see Figure 2).

   Per 25 µl reaction:

Each oligonucleotide (100 mM stock)	3 µl
Taq polymerase	0.5 µl
dNTP mix (final 0.2 mM)	2.5 µl
10x buffer	2.5 µl
H$_2$O	to 25 µl

   Incubate tubes in a thermocycler for 30 cycles of 20 sec at 94 °C, 30 sec at 55 °C, and 30 sec at 72 °C.

   Transform 20 µl of this reaction per yeast transformation.

E. *C. albicans* transformation

   gRNA vectors are marked with *URA3* for selection, and *RPS1* to allow integration at the chromosomal *RPS1* locus after linearization with *Stu*I. Plasmids are linearized with *Stu*I (or *Nco*I if gRNA sequence contains a *Stu*I site), transformed into a Cas9-expressing yeast strain (*i.e.*, those in Table 1), and selected on SD (–Ura) plates.

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The transformation should include controls lacking gRNA plasmid, as well as those containing just the parental vector (lacking the gRNA sequences). Co-transformation of the donor repair fragment allows for double stranded break repair. Since a break that cannot be repaired is lethal, there is generally at least a two-fold increase in the number of transformants obtained in the presence of repair template compared to its absence.

A modified lithium acetate protocol (Walther and Wendlund, 2003), outlined below, is used for transformation.

Transformation (TF) mix (per transformation)

50% PEG (sterile)	240 µl
1 M LiOAc (sterile)	32 µl
Salmon sperm DNA (10 mg/ml boiled; keep on ice)	5 µl
Plasmid DNA (~10 µg, digested with *Nco*I or *Stu*I)	33 µl
Donor repair fragment	25 µl
Yeast cell suspension	50 µl
Total	360 µl

Yeast transformation protocol (for ~2-3 transformation reactions)

1. Inoculate fresh colony into YPD + 75 µg uridine/ml. Grow overnight in a shaker at 30 °C.
2. Dilute 100 µl from overnight into 2 ml of YPD + uridine, shake at 30 °C for 4-6 h.
3. Spin down 1 ml of cells (about 5 OD units), wash with H_2O, then wash with 1 ml 0.1 M LiOAc.
4. Resuspend in 100 µl of 0.1 M LiOAc (should appear to be thick and concentrated).
5. Mix TF mix, 50 µl of yeast cells and 30 µl linearized plasmid DNA (~30 µg) or 15 µl PCR reaction (1-10 µg/µl) so that the total volume is 360 µl.
6. Incubate overnight (up to 24 h) at 30 °C.
7. Heat shock at 44 °C for 15 min.
8. Spread the entire mixture on SD-URA plates. Incubate for 2-3 days at 30 °C.

F. Mutant verification

To identify the desired mutants, use colony PCR to amplify the flanking region of the wild type gene and mutant alleles. Isolate genomic DNA as follows:

1. Inoculate a toothpick full of cells from a single colony in ~400 µl of LB media and culture for ~3 h at 37 °C, 220 rpm.
2. Spin down cells and resuspend in 30 µl of 0.2% SDS and boil for 4 min.
3. After centrifugation of cells, 3 µl of the supernatant (containing genomic DNA) is added to 18.25 µl water, 2.5 µl PCR buffer, 20 µl 2.5 mM dNTP, 1 µl of each primer (10 mM stock) and 0.25 µl *Taq* polymerase. The PCR conditions are 30 cycles of 0.5 min at 94 °C, 1 min at 55 °C, and 1 min at 72 °C.

Notes:

1. *PCR products can be analyzed by agarose gel electrophoresis. It is strongly recommended that prior to this PCR analysis, streak for single colonies to avoid the possibility of mixing colonies of differing genotypes. For an example of these analyses, see Ng and Dean, 2017, Figure 7.*

2. *The design of oligonucleotides used for verification (see **Procedure C** above) should consider the need to detect a size mobility shift if deletion or alteration produces a smaller PCR product than the wild type allele. As described in **Procedure B**, susceptibility to restriction digest with a unique enzyme introduced into the donor repair fragment is very useful for mutant verification. In cases where this is not possible, PCR products can be screened directly by DNA sequencing. In either case, genomic sequencing of the deletion alleles should be performed for any mutant strain that will be used for subsequent phenotypic analyses.*

3. *CRISPR-mediated mutation frequency varies depending on the locus and the nature of the mutation, but this procedure generally results in frequencies ranging from 30-95%. Once mutant strains have been obtained and verified, stock 2-3 strains from single colonies in 15% glycerol at -80 °C.*

G. Regeneration of *ura3* auxotrophic strains

1. When using strains transformed with pND501, *ura3* mutants that lose the *ura3dpl200* allele can be isolated by selection on 5-FOA containing plates (http://cshprotocols.cshlp.org/content/2016/6/pdb.rec086637.short). Prior to 5-FOA selection, streak out the mutant strain on fresh media for single colonies.

2. Pick 3-4 colonies and streak them on 5-FOA containing plates. Alternatively, use fresh liquid culture, dilute, and plate out (frequency of looping out is ~1 in 10^5).

3. Dilute overnight culture 1:100, 1:10,000, 1:100,000. Plate 100 µl of each dilution on FOA-containing plate. Screen FOA-resistant colonies on SD-URA plates to confirm the loss of *URA3* gene.

Data analysis

During yeast transformation, it is important to include the following controls: No plasmid DNA (to ensure that transformants are uracil prototrophs and that SD (–Ura) plates or transformation solutions are not contaminated); no gRNA plasmid (to ensure that transformants arise from gRNA vector uptake), no donor repair fragment. This last control provides an estimate of the number of colonies that are likely to arise from homologous recombination of the repair fragment at the target locus. The fold stimulation of colonies that arise in the presence versus absence of healing fragment may provide an estimate of mutation frequency. Generally, we have found that a high mutation frequency results in a large stimulation of colonies that arise in the presence versus absence of repair fragment. The frequency of mutation and repair by this method is about 30-90%.

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It should be noted that several other CRISPR-mediated systems for introducing mutations in *C. albicans* have been described (Vyas *et al.*, 2015; Min *et al.*, 2016; Nguyen *et al.*, 2017), each having its own strength and weakness. It is hoped that as these methods are used more frequently, facile genetic analyses of *C. albicans* will become the norm.

Acknowledgments

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