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CRISPR mediated genome editing of the human fungal pathogen C. albicans

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Dear JoVE Editorial Staff,

We are pleased to resubmit our invited manuscript “CRISPR mediated genome editing in the human fungal pathogen *C. albicans*” with the edits that you recommended. Our paper provides protocols to edit the genome of the most prevalent human fungal pathogen *C. albicans*. Furthermore, our manuscript discusses a variety of applications and limitations of CRISPR mediated genome editing in *C. albicans*. If you have any questions regarding the manuscript, please feel free to contact me.

Sincerely,

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TITLE:

CRISPR-Mediated Genome Editing of the Human Fungal Pathogen *Candida albicans*

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KEYWORDS:

CRISPR, *Candida albicans*, genome editing, transformation, Cas9, pathogen, fungi

SUMMARY:

Efficient genome engineering of *Candida albicans* is critical to understanding the pathogenesis and development of therapeutics. Here, we described a protocol to quickly and accurately edit the *C. albicans* genome using CRISPR. The protocol allows investigators to introduce a wide variety of genetic modifications including point mutations, insertions, and deletions.

ABSTRACT:

This method describes the efficient CRISPR mediated genome editing of the diploid human fungal pathogen *Candida albicans.* CRISPR-mediated genome editing in *C. albicans* requires Cas9, guide RNA, and repair template. A plasmid expressing a yeast codon optimized Cas9 (CaCas9) has been generated. Guide sequences directly upstream from a PAM site (NGG) are cloned into the Cas9 expression vector. A repair template is then made by primer extension *in vitro*. Cotransformation of the repair template and vector into *C. albicans* leads to genome editing. Depending on the repair template used, the investigator can introduce nucleotide changes, insertions, or deletions. As *C. albicans* is a diploid, mutations are made in both alleles of a gene, provided that the A and B alleles do not harbor SNPs that interfere with guide targeting or repair template incorporation. Multimember gene families can be edited in parallel if suitable conserved sequences exist in all family members. The *C. albicans* CRISPR system described is flanked by FRT sites and encodes flippase. Upon induction of flippase, the antibiotic marker (CaCas9) and guide RNA are removed from the genome. This allows the investigator to perform subsequent edits to the genome. *C. albicans* CRISPR is a powerful fungal genetic engineering tool, and minor alterations to the described protocols permit the modification of other fungal species including *C. glabrata, N.*

castellii, and *S. cerevisiae*.

INTRODUCTION:

Candida albicans is the most prevalent human fungal pathogen[1-3]. Understanding differences between *C. albicans* and mammalian molecular biology is critical to development of the next generation of antifungal therapeutics. This requires investigators to be able to quickly and accurately genetically manipulate *C. albicans*.

Genetic manipulation of *C. albicans* has historically been challenging. *C. albicans* does not maintain plasmids, thus all constructs must be incorporated into the genome. Furthermore, *C. albicans* is diploid; therefore, when knocking out a gene or introducing a mutation, it is important to ensure that both copies have been changed[4]. In addition, some *C. albicans* loci are heterozygous, further complicating genetic interrogation[5]. To genetically manipulate *C. albicans*, it is typical to perform multiple rounds of homologous recombination[6]. However, the diploid nature of the genome and laborious construct development have made this a potentially tedious process, especially if multiple changes are required. These limitations and the medical importance of *C. albicans* demand the development of new technologies that enable investigators to more easily manipulate the *C. albicans* genome.

Clustered regularly interspaced short palindromic repeats (CRISPR)-mediated genome editing is a powerful tool that allows researchers to change the sequence of a genome. CRISPR requires three components: 1) the Cas9 nuclease that cleaves the target DNA, 2) a 20 base guide RNA that targets Cas9 to the sequence of interest, and 3) repair template DNA that repairs the cleavage site and incorporates the intended change[7,8]. Once the guide brings Cas9 to the target genome sequence, Cas9 requires a protospacer adjacent motif (PAM) sequence (NGG) directly upstream of the guide sequence to cleave the DNA[9]. The requirement for both the 20 base guide and PAM sequences provides a high degree of targeting specificity and limits off-target cleavage.

CRISPR systems have been designed to edit the genomes of a diverse set of organisms and tackle a wide variety of problems[10]. Described here is a flexible, efficient CRISPR protocol for editing a *C. albicans* gene of interest. The experiment introduces a stop codon to a gene, causing translation termination. Other edits can be made depending on the repair template that is introduced. A fragment marked with nourseothricin (Nat^r) containing yeast codon-optimized Cas9 (CaCas9) and a guide RNA is incorporated into the *C. albicans* genome at a neutral site. Cotransformation with the repair template encoding the desired mutation leads to repair of the cleavage by homologous recombination and efficient genome editing. Described below is the editing of *TPK2*, but all *C. albicans* open reading frames can be targeted multiple times by CRISPR. The CRISPR system is flanked by FRT sites and can be removed from the *C. albicans* genome by induction of flippase encoded on the CaCas9 expression plasmid. The *C. albicans* CRISPR system enables investigators to accurately and quickly edit the *C. albicans* genome[11,12].

PROTOCOL:

1. Identification and Cloning of Guide RNA Sequence

1.1. Identification of guide RNA sequence

1.1.1. Identify a 5'-NGG-3' PAM sequence close to where the stop codon will be inserted. **(Figure 1 A)** Labeled are all PAM sequences found in the first 100 base pairs of *TPK2* **(Figure 1A)**.

Note: Guide sequences targeting each *C. albicans* open reading frame can be found at http://osf.io/ARDTX [11,12].

1.1.2. Identify the Forward Guide Primer_3 sequence, which will be the 20 bases directly upstream of a NGG PAM site and will not contain more than 5 Ts in a row. Left-click on the base directly upstream of the NGG and drag the cursor 20 bases, then left-click on the primer tab to add the primer.

1.1.3. Identify the Reverse Guide Primer_3 sequence, which will be the complement to the Forward Guide sequence.

Note: Shown are guides that use PAM_3 **(Figure 1B)**.

1.1.4 Right-click the primer and select "copy primer data". Paste the sequences into a text editing program.

1.2. Add overhang sequences to Forward and Reverse Guide oligos to facilitate cloning **(Table 1, Figure 1B)**.

1.2.1. Add the nucleotide sequence ATTTG to the 5' end of the Forward Guide Primer_3 before purchasing.

1.2.2. Add G to the 3' end of the Forward Guide Primer_3 before purchasing.

1.2.3. Add the nucleotide sequence AAAAC to the 5' end of the Reverse Guide Primer_3 before purchasing.

1.2.4. Add C to the 3' end of the Reverse Guide Primer_3 before purchasing.

1.3. Digest CaCas9 expression vector pV1524 with BsmBI.

Note: pV1524 contains an ampicillin (Amp^r) and nourseothricin (Nat^r) markers. Cas9 has been codon-optimized for *C. albicans*.

1.3.1. Digest the plasmid by adding: 2 µg of pv1524, 5 µL of 10x Buffer, 1 µL of BsmBI, and H_2O to 50 µL in a 1.5 mL tube. Incubate at 55 °C for 20 min. (Alternatively, digest pv1524 for 15 min with Esp3I, an isoschizomer of BsmBI, at 37 °C.)

1.3.2. Cool to room temperature (RT) and spin for 30 s at 2348 x g to bring condensation to the bottom of the tube. Proceed to step 1.4 or store the digested plasmid at -20 °C.

1.4. Phosphatase-treat the digested backbone.

1.4.1. Add 1 µL of calf intestinal phosphatase (CIP) to the digestion mixture and incubate at 37 °C for 1 h.

1.4.2. Purify the digested plasmid using a commercially available polymerase chain reaction (PCR) purification kit (instructions provided with kit) and elute it in 30 µL of elution buffer (EB).

1.5. Phosphorylate and anneal Forward Guide Primer_3 and Reverse Guide Primer_3.

1.5.1. Add 0.5 µL of 100 µM Forward Guide Primer_3, 0.5 µL of 100 µM Reverse Guide Primer_3, 5 µL of 10x T4 ligase buffer, 1 µL of T4 polynucleotide kinase, and 43 µL of H_2O to a PCR tube.

1.5.2. Add 5 µL of 10x T4 ligase buffer, 1 µL of T4 polynucleotide kinase, and 44 µL of molecular biology-grade H_2O in a second PCR tube.

Note: This will serve as the negative control.

1.5.3. Incubate the reaction mixtures in a thermocycler at 37 °C for 30 min, then at 95 °C for 5 min.

1.5.4. Cool the mixture at the slowest ramp rate to 16 °C to anneal the ,oligos. Then place the annealed oligo mixture at 4 °C.

1.6. Ligate the annealed oligos into digested pv1524 from step 1.4.3.

1.6.1. Add the following to a PCR tube: 1 µL of 10x T4 ligase buffer, 0.5 µL of T4 DNA ligase, 0.5 µL of annealed oligo mix, digested CIP-treated purified plasmid (20-40 ng), and H_2O to a 10 µL total volume.

1.6.2. Add the following to a PCR tube: 1 µL of 10x T4 ligase buffer, 0.5 µL of T4 DNA ligase, digested CIP-treated purified vector (20-40 ng), 1 µL of negative control mixture, and H_2O up to a 10 µL total volume.

1.6.3. Incubate both tubes in a thermocycler at 16 °C for 30 min, then at 65 °C for 10 min, then cool to 25 °C.

1.7. Transform 5 µL of the ligation mixtures into chemically competent *Escherichia coli* DH5α using a standard heat shock transformation protocol. Select on LB Amp/Nat media (200 µg/mL Amp, 50 µg/mL Nat).

Note: Failure to select pV1524 and its derivatives on double selection media will result in loss of Nat/CaCas9/guide module by FLP/FRT excision in bacteria.

1.8. Purify plasmids from four transformants by miniprep, and sequence the insertion sequence with sequencing primer **(Table 1)**.

Note: Most of the time, sequencing 4 transformants is sufficient to identify at least 1 correct clone.

1.9. Save plasmids that have the guide RNA sequence cloned a single time into the BsmBI cut site at -20 °C.

2. **Designing and Generation of Repair Template**

2.2. Insert a stop codon by left-clicking in the gene sequence and adding nucleotides that encode a stop codon and restriction digestion site **(Figure 1C, Table 1)**.

Note: The insertion will disrupt the PAM sequence.

Note: A restriction digestion site will be included in the repair template sequence to facilitate efficient screening of clones **(Figure 1C)**.

2.1. Left-click 10 bases downstream of where the mutation will be made and drag the cursor 60 bases upstream. Left-click on the primer tab to add the primer. This will add Repair Template Forward_3. Left-click 10 bases upstream of where the mutation will be made and drag the cursor 60 bases downstream. Left-click on the primer tab to add the primer. This will add Repair Template Reverse 3. **(Figure 1C)**

2.3. Perform primer extension to generate the repair template.

2.3.1. Add 1.2 µL of 100 µm repair template forward primer, 1.2 µL of 100 µm repair template reverse primer, 6 µL of deoxynucleotide triphosphates (dNTPs) (total concentration 40 mM), 6 µL of buffer, 0.6 µL of Taq polymerase (3 units), and 45 µL of H_2O to each of the 4 PCR tubes.

2.3.2. Perform primer extension by running between 20 and 30 rounds of PCR. Example extension conditions: 2 min at 95 °C, (30 s at 95 °C, 1 min at 50 °C, 1 min at 68 °C) x 34, 10 min at 68 °C.

2.3.3. Combine contents of all 4 PCR tubes in a 1.5 mL tube and use a PCR purification kit to purify products in 50 µL of H_2O.

2.3.4. Quantitate the primer extension products to ensure sufficient DNA by determining the absorbance at 260 nm.

Note: Typical final concentration of the primer extension product is ~200-300 ng/µL.

3. Transformation of *C. albicans* with Repair Template and Plasmid

3.1. Make ethylenediaminetetraacetic acid (EDTA)-Tris/lithium acetate.

3.1.1. Mix 10 mM Tris-Cl, 1 mM EDTA, 100 mM lithium acetate, and H_2O (all stock solution pH 7.5) to achieve 50 mL total volume.

3.2. Make PLATE polyethylene glycol (PEG), lithium acetate, Tris, and EDTA.

3.2.1. Mix 40% PEG 3350, 100 mM lithium acetate, 10 mM Tris-Cl, 1 mM EDTA, and H_2O (all stock solution pH 7.5) to achieve 50 mL total volume.

3.3. Digest correctly-cloned plasmids from step 1.9.

3.3.1. Add 10 µg of plasmid, 4 µL of 10x buffer, 0.4 µL of 10 mg/mL bovine serum albumin (BSA), 0.5 µL of KpnI, 0.5 µL of SacI, and H_20 to 40 µL total volume in a 1.5 mL tube. Incubate at 37 °C overnight **(Figure 1D)**.

3.4. Grow an overnight culture of *C. albicans* SC5314, wild-type prototroph, at 25 °C in yeast peptone dextrose supplemented with 0.27 mM Uridine (YPD + Uri), ideally to OD_{600} less than 6.

3.4.1. Pellet 5 OD_{600} units of cells per transformation by spinning for 5 min at 2348 x g and suspend the 5 OD_{600} of pelleted cells in 100 µL TE/lithium acetate.

3.5. Add the following to a 1.5 mL tube in the order listed: 1) 100 µL of cells from step 3.4.1, 2) 40 µL of boiled and quick-cooled salmon sperm DNA (10 mg/mL), 3) 10 µg of plasmid digestion from step 3.3.1, 4) 6 µg of purified repair template, and 5) 1 mL of PLATE.

3.6. Add the following to a 1.5 mL tube in the order listed: 1) 100 µL of cells, 2) 40 µL of boiled and quick-cooled salmon sperm DNA (10 mg/mL), 3) H_2O volume equal to that of transforming DNA in step 3.5, and 4) 1 mL of PLATE.

Note: This will serve as a negative control.

3.7. Mix the transformations gently by pipetting and let incubate at 25 °C overnight.

3.8. Heat shock the cells by placing them in a 44 °C water bath for 25 min.

3.9. Spin for 5 min at 2348 x g in a benchtop centrifuge and remove the PLATE mixture supernatant. Wash once by adding 1 mL of YPD + Uri and centrifuge again for 5 min at 2348 x g.

3.10. Suspend the cells in 0.1 mL of YPD + Uri and incubate on a roller drum or shaker at 25 °C overnight.

3.11. Plate on YPD + Uri with 200 µg/mL nourseothricin. Colonies will appear in 2-5 days.

4. Streaks for Single Colonies

4.1. Divide a 100 x 15 mm Petri dish into quarters, and label each quadrant.

4.1.1. Touch one of the colonies from the transformation plate with a sterile toothpick or applicator and streak across the longest side of the quadrant.

4.1.2. Streak for single colonies using an aseptic technique and allow the colonies to grow at 30 °C for 2 days.

5. Colony PCR

5.1. Design the forward check primer (FCP) ~200 base pairs upstream of the restriction site that was introduced and the reverse check primer (RCP) ~300 base pairs downstream.

5.1.1 Add 0.3 µL of FCP, 0.3 µL of RCP, 0.3 µL of thermostable polymerase (ExTaq 1.5 units), 3 µL of dNTPs (total concentration 40 mM), 3 µL of ExTaq Buffer, and 23 µL of H_2O to a 1.5 mL tube.

Note: Addition of 0.5 µL/reaction dimethyl sulfoxide (DMSO) can improve PCR efficiency.

5.1.2 Add 0.25 µL of a single yeast colony from step 4.1.2 to the mixture from step 5.1.1, using a P10 pipette tip and taking care not to disturb the agar.

5.1.3. Amplify DNA by PCR and run 5 µL of the PCR on a gel to ensure amplification is successful, taking care not to disturb the cell pellet at the bottom of the tube.

6. Restriction Digestion of Colony PCR

6.1. Add 10 µL of PCR product (taking care not to not disturb the cell pellet at the bottom of the tube), 3 µL of buffer, 1 µL of restriction enzyme, and 16 µL of H_2O, then incubate according to manufacturer's instructions and resolve on an agarose gel to identify correct genome edits.

Note: The restriction enzyme used here is the site encoded in the *TPK2* specific repair template.

7. Saving Strains

7.1. Grow an overnight culture from colonies that have been confirmed by restriction digestion in YPD + Uri at 30 °C.

7.2. Add 1 mL of culture and 1 mL of 50% glycerol (bringing the final concentration of glycerol to 25%) to a tube suitable for storage at -80 °C.

7.3. Store the correct clones at -80 °C.

Note: Correct strains can be stored at -80 °C for many years.

8. Removal of Nat^r Marker

8.1. Streak correct transformant onto yeast peptone maltose (YPMaltose) (2% maltose).

8.2. Pick a colony from the streaked plate and culture the yeast for 48 h at 30 °C in liquid YPMaltose 20 g/L maltose.

8.3. Plate 200-400 cells on YPMaltose 20 g/L maltose and incubate at 30 °C for 24 h.

8.4. Replicate the plate onto YPMaltose and YPMaltose 200 µg/mL Nat.

8.5. Incubate at 30 °C for 24 h.

Note: Colonies that no longer grow on YPMaltose 200 µg/mL nourseothricin but grow on YPMaltose have lost the Nat^r marker (CaCAS9) and guide RNA.

8.6. Save the strains that have lost the Nat^r marker *(CaCAS9)* and guide RNA as done in steps 7.1-7.3.

Note: A similar plasmid, pV1393, uses the *SAP2* as opposed to a *MAL2* promotor. Growth on YCB–BSA will induce flippase and removal of Nat^r if pV1393 is used for gene editing.

REPRESENTATIVE RESULTS:

Sequences of guide RNAs and repair templates that target *C. albicans TPK2,* a c-AMP kinase catalytic subunit, were designed according to the guidelines s gested above. Sequences are shown in **(Table 1, Figure 1)**. Guide RNAs were cloned into CaCas9 expression vectors and cotransformed with repair template in wild-type *C. albicans*. An *Eco*RI restriction digestion site and stop codons in the repair template disrupt the PAM site and facilitate screening for correct mutants **(Figure 1)**. Transformants were streaked for single colonies and screened by colony PCR and restriction digestion for incorporation of the repair template **(Figure 2)**. Restriction digestion quickly distinguishes wild-type from mutant sequences.

FIGURE AND TABLE LEGENDS:

Table 1: List of oligo nucleotides used for this study. Sites added for cloning purposes are capitalized and bolded in the guide primer sequences. Sequences in the repair template that mutate the genomic DNA are capitalized and bolded.

Figure 1: Diagram of guide RNA and repair template design. (A) Labeling of all the PAM sequences in the first 100 nucleotides of *TPK2*. PAM sequence 3 (PAM_3) is highlighted, as that

is the sequence used in this study. (B) Guide RNA design using PAM_3. 20 base primers designed using SnapGene are lowercase and blue. Additional bases required for cloning are uppercase and green shown offset. (C) Repair template primers that insert a TAA stop codon and *Eco*RI site are inserted into the *TPK2* reading frame. DNA that differs from the wild-type sequence is red and uppercase. (D) Example of how a guide is designed on the positive strand of DNA using PAM_4. (E) Schematic diagram of pV1524 after cloning of the guide RNA and digestion with KpnI and SacI. Neut5L-5' and Neut5L-3' target the vector to the Neut5L site in the *C. albicans* genome. *CaENO1p* is the promotor that drives expression of the yeast-optimized *CaCas9*. Nat^r is the nourseothricin resistance cassette. CaSNR52p is the promotor driving guide RNA expression (sgRNA). FRT sites are cleaved and recombined by flippase (FLP) removing the CRISPR cassette upon flippase expression. A schematic similar to (E) was published by Vyas *et al*.[11].

Figure 2: Introduction and confirmation of a stop codon and *Eco*RI restriction site to *TPK2*. Primers used for amplification are listed in **Table 1**. Wild-type and mutant sequences are shown below the gel. This figure has been modified from Vyas *et al.*[11].

Figure 3: Cartoon description of repair templates that will generate (A) deletions and (B) insertions. Grey dashes in (A) depict intervening sequences not present in the repair template primers.

DISCUSSION:

C. albicans CRISPR efficiently edits the *C. albicans* genome. pV1524 encodes a yeast codon-optimized Cas9 and is designed such that investigators can easily clone guide RNA sequences downstream of the *CaSNR52* promoter **(Figure 1)**[11]. It must be ensured that only a single copy of the guide sequence has been cloned into CaCas9 expression vectors by sequencing, as extra copies will impede genome editing. If multiple copies of the guide are introduced consistently, one should lower the concentration of the annealed guide used in ligation. The vector and protocol described allow targeting of any *C. albicans* gene. Although *C. albicans* is diploid, only a single transformation is required to target both alleles of a gene. Furthermore, the processive nature of CRISPR-CaCas9 genome editing enables researchers to target multiple members of gene families. Many gene families such as the secreted aspartyl proteases (SAPS) and agglutinin-like sequence proteins (ALS) are important for *C. albicans* virulence. CRISPR genome editing will facilitate investigation of these gene families.

The protocols described above introduce a stop codon to an open reading frame, resulting in the phenotypic equivalent of a null **(Figure 2)**. A wide variety of genetic alternations can be made by varying the repair template. Nonsense, missense, and silent mutations can be inserted via recombination with an appropriate repair template. Incorporation of a restriction site streamlines transformant screening, as those without must be screened by sequencing[12,13]. In addition, *C. albicans* CRISPR enables researchers to generate insertions and deletions, making it an ideal system to insert affinity tags, perform promotor swaps, and generate knockouts **(Figure 3)**. Screening for correct transformants for these mutations is more laborious, as it is necessary to sequence the edits to confirm correct incorporation of the repair templates. Furthermore, Southern blot may be necessary to ensure additional copies of a gene have not been inserted at

additional locations in the genome. The requirement of the NGG PAM site places slight limitations on the regions of the genome that can be targeted. The development of alternative CRISPR systems that use alternative nucleases such as Cpf1 or variations on the Cas9 system have/will alleviate many of these limitations[14]. To the investigators' knowledge at this time, these systems have not yet been applied to *C. albicans*.

The CRISPR system described in the above protocol has been developed such that it can be applied in a wide variety of species including *Saccharomyces cerevisiae*, *Naumouozyma castellii*, and the human pathogen *Candida glabrata*[11]. Transformation and efficient editing of these yeast requires slight changes to the described protocol, but the framework for editing these alternate genomes is remarkably similar to that described for *C. albicans*[12]. Furthermore, yeast provide an excellent mechanism to develop genome editing procedures. In yeast, when *ADE2* is mutated, a precursor to the adenine biosynthesis pathway accumulates, turning the cells red. This easily observable phenotype allows investigators to identify edited cells and quickly troubleshoot genome editing protocols. Combined with the extensive molecular biology toolbox available for fungi, protocols for editing numerous yeast species have been developed[15,16]. Such a broad application of genome editing technology in fungi has the potential to significantly impact a wide variety of scientific disciplines.

CRISPR has greatly improved the efficiency of genome engineering in *C. albicans*, but to date CRISPR has not been used to perform genome wide screens in *C. albicans*. Current protocols require a repair template to introduce mutations, as the nonhomologous end joining pathway in *C. albicans* is inefficient[12]. The generation of repair template oligos for every gene is a significant barrier to the execution of genome-wide screens. The confluence of decreased costs of DNA synthesis and advances to CRISPR technologies will make development of deletion libraries more feasible. For instance, expression of a repair template from the CaCas9 vector paves the way for the development of sustainable plasmid libraries that target every gene[11]. Furthermore, transient *Candida* CRISPR protocols that do not require CaCas9 expression vector incorporate into the *C. albicans* genome have been developed[17]. In addition, increased guide expression increases genome editing efficiency[18]. These, and other advances to CRISPR technologies, are crucial to the development of genome-wide screens in *C. albicans*[19-22].

The *C. albicans* genome is diploid, but A and B alleles are not always identical[5]. Such heterozygosity provides both challenges and opportunities. If one aims to target both alleles, a PAM site, guide sequence, and repair template that will act on both copies of the gene must be used. However, depending upon single nucleotide polymorphisms present in a gene, the *C. albicans* CRISPR system enables investigators to target a single allele. Such precision has the potential to allow investigators to examine functional differences between alleles. Targeting specific alleles must be done carefully, as loss of heterozygosity (LOH) at an allele or of an entire chromosome has been observed. When editing single *C. albicans* alleles, one must examine adjacent DNA sequences to determine if a clone has maintained a diploid SNP profile. In addition, off-target effects are quite low for *C. albicans* CRISPR, but whole genome sequencing can be considered for key strains.

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DISCLOSURES:

The authors have nothing to disclose.

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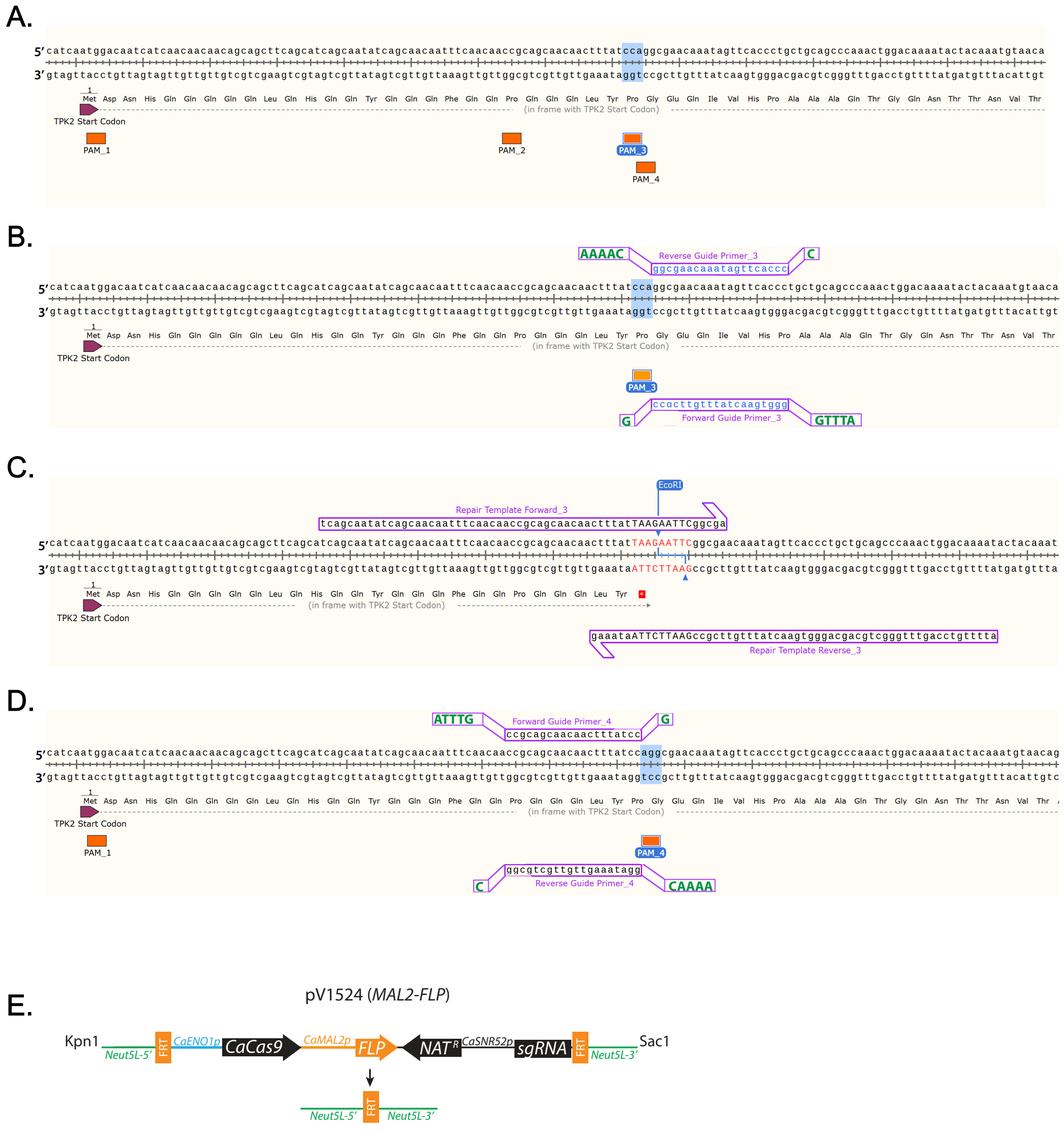


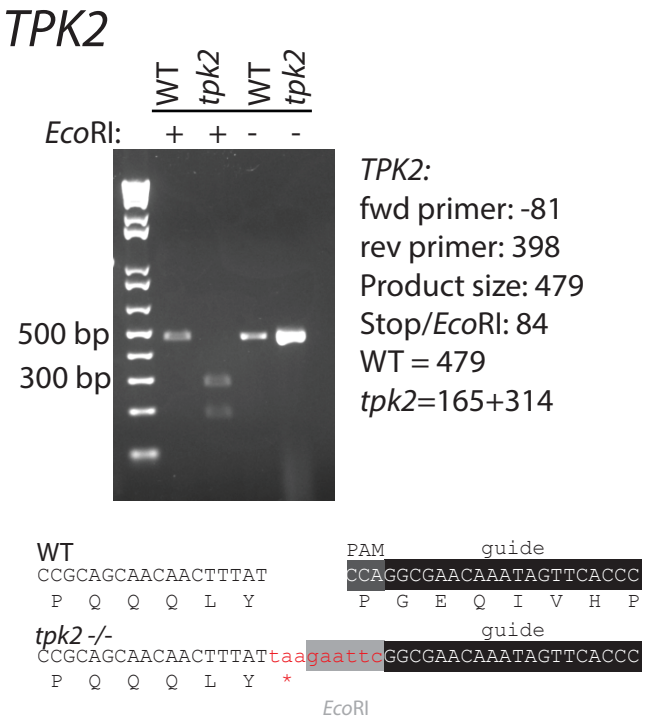
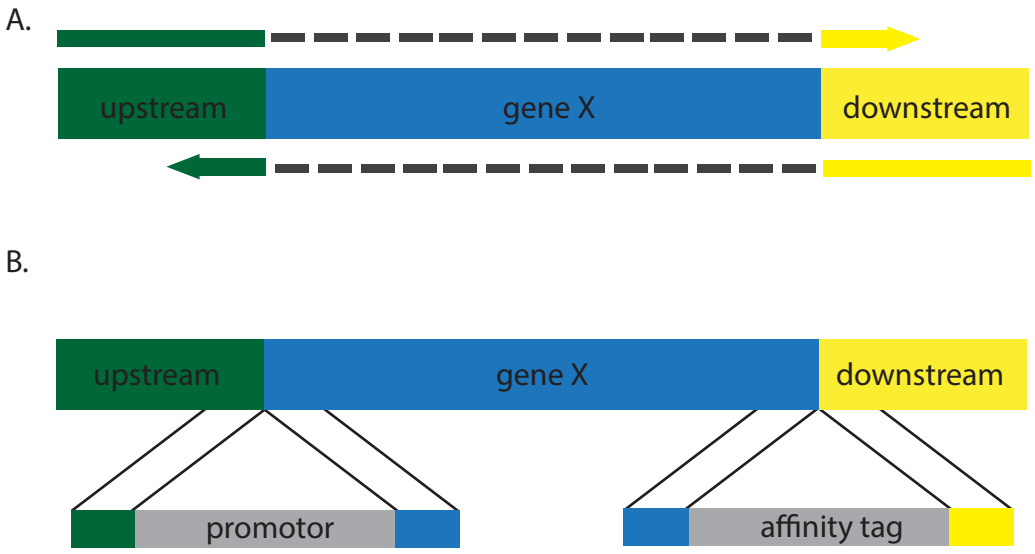


Figure_3

Click here to access/download;Figure;Figure_3.ai



Table

Oligonucleotide Name
Forward Guide Primer_3
Reverse Guide Primer_3
Repair Template Forward_3
Repair Template Reverse_3
Forward Check Primer
Reverse Check Primer
Sequencing Primer

Oligonucleotide Sequence
ATTTGgggtgaactatttgttcgcc**G**
AAAACggcgaacaaatagttcaccc**C**
tcagcaatatcagcaacaatttcaacaaccgcagcaacaactttat**TAAGAATTC**ggcga
attttgtccagtttgggctgcagcagggtgaactatttgttcgcc**GAATTCTTA**ataaag
ttaaagaaacttcacatcaccaag
actttgatagcataatatctaccat
ggcatagctgaaacttcggccc

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Chemicals			
Agar	BD Bacto	214010	
agarose	amresco	0710-500G	
Ampicillan	Sigma-Aldrich	A9518	
Bacto Peptone	BD Bacto	211677	
Bsmb1	New England Biolabs	R0580L	
Calf intestinal phosphatase (CIP)	New England Biolabs	M0290L	
Cut Smart Buffer	New England Biolabs	B7204S	
Dimethyl Sulfoxide (DMSO)	Sigma-Aldrich	D8418	
dNTPs	New England Biolabs	N0447L	
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich	3609	
Glacial Acetic Acid	Sigma-Aldrich	2810000ACS	
Glucose	BDH VWR analytical	BDH9230	
Glycerol	Sigma-Aldrich	49767	
Kpn1	New England Biolabs	R3142L	
LB-Medium	MP	3002-032	
Lithium Acetate	Sigma-Aldrich	517992	
L-Tryptophan	Sigma-Aldrich	T0254	
Maltose	Sigma-Aldrich	M5885	
Molecular Biology Water	Sigma-Aldrich	W4502	
NEB3.1 Buffer	New England Biolabs	B7203S	
Nourseothricin	Werner Bioagents	74667	
Poly(ethylene glycol) PEG 3350	Sigma-Aldrich	P4338	
Sac1	New England Biolabs	R3156L	
Salmon Sperm DNA	Invitrogen	AM9680	

T4 Polynucleotide kinase	New England Biolabs	M0201S
T4 DNA ligase	New England Biolabs	M0202L
Taq polymerase	New England Biolabs	M0267X
Tris HCl	Sigma-Aldrich	T3253
uridine	Sigma-Aldrich	U3750
Yeast Extract	BD Bacto	212750

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Author(s): Ben A. Evans, Ethan S. Pickerill, Valmik K. Vyas, Douglas A. Bernstein

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CORRESPONDING AUTHOR

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Institution:	Ball State University
Title:	Assistant Professor of Biology
Signature:	[signature]
Date:	7/9/18

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8. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.). **We have done this.**

9. 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. **We have done this although safety should be done with accordance with the local rules of the University or place doing the experiments.**

10. 1.1.1-1.1.3, 2.1-2.3, 5.1: Unclear what we can show here, please describe the actions being performed. If there are no specific actions I suggest unhighlighting these. **I thought that this could be somewhere that the students could speak about how to design the different components templates. We could potentially also show a computer screen of how to make the different components.**

11. 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. Some examples:

1.2.1-1.2.4: Please describe how this is done. **We have done this.**

1.3.2: Please specify centrifugation force (in x g). What happens after centrifugation? **We have done this.**

1.4.2: Since this step is highlighted for filming, please make sure that step here can stand alone. Please add more details here. **We have unhighlighted this as PCR purification is something that many people are familiar with and can do without our guidance.**

1.5.2: Please specify the type of water used in this step. **We have done this.**
1.8, 2.4.3, 2.4.4: Please describe how this is done. **We have done this.**
1.9: What are considered to be correct plasmids? **We clarified this.**
4.1: How large is the petri dish? **We have clarified this.**
7.1: Please specify culture temperature. **We have done this.**
Please ensure that conditions and primers are listed all PCR procedures.
12. 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.
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14. 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.
15. 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. **These are discussed in the discussion already. If we were to discuss significant trouble shooting we would get into different types of protocols and this could get confusing.**
16. References: Please do not abbreviate journal titles. **We have changed these.**

Reviewers' comments:

Reviewer #1:
Manuscript Summary:
Protocol on CRISPR for C. albicans. Because C. albicans is diploid, CRISPR is very useful because it typically modifies both alleles at the same time - this is a powerful improvement over classical gene modification techniques

Major Concerns:
None

Minor Concerns:
Would be very useful to add to Figure 1 an example of how to design a gRNA based on the negative strand. It's a little bit tricky and sometimes it's the only option. **The example that we gave was on the negative strand but we have provided a positive strand example as well now and changed figure 1 D appropriately.**

Reviewer #2:
Manuscript Summary:
In this manuscript, Evans and colleagues describe a protocol for CRISPR-Cas9 based genetic editing in the

fungal pathogen Candida albicans. They describe the design of the CRISPR editing system as well as the transformation and verification process. This is a timely protocol that will be of interest to many. Some points will help improve and enhance the impact of this work.

Major Concerns:
1. Are there any measures taken during design of guide RNAs to ensure specificity? It would be very helpful for readers to understand how to best design and chose an optimal guide RNA.

In line 1.1.2. we give rules for guide design.

1.1.2. The forward guide sequence must be the 20 bases directly upstream of a NGG PAM site and not contain more than 5 Ts in a row.

2. The authors mention plasmid pv1524, but this is not defined. Perhaps it should be given a more generic name to explain the use and importance of this plasmid. It could also be described in more detail (which resistance markers are present, how was Cas9 optimized for C. albicans, etc).

We have added below to 3.1

Note: pV1524 contains an ampicillin and nourseothricin markers. Cas9 has been codon optimized for *C. albicans*.

pV1524 is the name given the plasmid in the primary literature. If we gave it a generic name that could cause confusion as there are other Cas9 experssion plasmids from both our group and other groups for which it could be confused.

3. A little more detail could be provided for the statement: "Purify plasmids from transformants and sequence with Sequencing Primer (Table 1)." - plasmids are purified with miniprep kits? **This has been specified.** Is the entire plasmid sequenced **We have now specified that the insertion sequence is sequenced not the entire plasmid**?

4. For 2.4.2, what are the PCR cycling conditions? **The annealing temperatures will vary for different primer sets but an example of PCR conditions is now given. Example extension conditions: 2 min 95 °C, (30 s 95 °C, 1 min 50 °C, 1 min 68 °C)x34, 10 min 68 °C.**

5. In 3.5 - are then any details about the salmon sperm? Concentration**? It is has been provided.**
6. The authors should speak to the expected efficiency of various parts of this protocol (bacterial cloning, transformation in yeast, editing, etc). **Editing and tranformation efficiencies vary greatly depending up on the gene and particular sequence being inserted and we feel it would be in appropriate to give expected efficiency. We have stated that guide cloning into pv1524 is quite efficient and added the number of colonies that should be sequenced. Added Note: Most of the time, sequencing four transformants is sufficient to identify a correct clone.**

7. The discussion could be expanded to explain possible applications of this in the context of this pathogen - and what sort of important biological relevance can be gained from generating mutations in this pathogen (ie studying virulence, other pathogenic traits etc) Statement below has been added.

Many gene families such as the Aspartyl Proteases and Agglutinin-Like Sequence proteins are important for *C. albicans* virulence. CRISPR genome editing will facilitate investigation of these gene families.

8. The authors mention how this CRISPR editing "has greatly improved the efficiency of genome engineering in C. albicans". This could benefit from a description of previous methods that were used, and their limitations. Below is what we state in the intro which we feel explains this.

In addition, some *C. albicans* loci are heterozygous, further complicating genetic interrogation[5]. To genetically manipulate *C. albicans*, one performed multiple rounds of homologous recombination[6]. However, the diploid nature of the genome and laborious construct development made this a potentially tedious process, especially if multiple changes were required.

9. The authors should describe possible troubleshooting steps, and pitfalls/limitations of this technique.

Below are some of the common pitfalls that we mention in the discussion.

One must ensure that only a single copy of the guide sequence has been cloned into CaCas9 expression vectors as extra copies will impede genome editing. If multiple copies of the guide are introduced consistently one should lower the concentration of annealed guide used in ligation.

When editing single *C. albicans* alleles, one must examine adjacent DNA sequences to determine if your clone maintained a diploid SNP profile.

Minor Concerns:

1. Word missing between lines 44-45

The *C. albicans* CRISPR system described is flanked by FRT sites and encodes Flippase. Upon induction of Flippase, the antibiotic marker, CaCas9, and guide RNA are removed from the genome. This allows the investigator to perform subsequent edits to the genome.

2. If TPK2 is being used as an example, this could be clarified in the introduction, but indicated that this exemplifies who editing could be done for other genes.

We state in the intro

Below is described editing of TPK2 but all *C. albicans* open reading frames can be targeted multiple times by CRISPR.

We also list resources where guides for all Candida genes can be found.

3. Restriction enzymes are listed with arabic instead of roman numerals
We have fixed these errors.

Reviewer #3:
Manuscript Summary:
The manuscript describes a method for CRISPR-mediated gene editing in Candida. The author is well qualified to write this review as the first to publish this tool in Candida albicans.

Major Concerns:
None. The article is well-written and clear.

Minor Concerns:
-Line 34 Do you also need homology for the repair template to knock out multiple alleles?

Good catch, we have changed to

Multimember gene families can be edited in parallel if suitable conserved sequences exist in all family members.
-Line 199 Would it be helpful to add more detail on the treatment on salmon sperm DNA?

We have changed to 40 μl boiled and quick cooled salmon sperm (10mg/ml)
-Why YPD+Uri? The host strain should be described.

We supplement our media with uridine. It is not technically necessary as we are not using auxotrophic strains but we find that the yeast grow better under our conditions with the supplementation.

We have added SC5314 to below.

3.4. Grow overnight culture of *C. albicans* SC5314, wild type prototroph, at 25 °C in Yeast Peptone Dextrose supplemented with 0.27 mM Uridine (YPD+Uri), ideally to OD_{600} less than 6.
SC5314 is a prototrophic clinical isolate.

-Add a quick overview of what is being targeted by CRISPR in the protocol and what the strain background is would be helpful, as well as an overview of the major steps involved.

-In other systems, other off target errors introduced by CRISPR.thus, it might be helpful to suggest whole genome sequencing for key mutants or to complement.

We have found very few off target effects and the NHEJ pathway is very inefficient in C. albicans. We mention

Targeting specific alleles however must be done carefully as loss of heterozygosity (LOH) at an allele or of an entire chromosome has been observed. When editing single *C. albicans* alleles, one must examine adjacent DNA sequences to determine if your clone maintained a diploid SNP profile.

This discusses similar concerns that are more prevalent in C. albicans in our experience.

We have also added

In addition, off target effects are quite low for *C. albicans* CRISPR but whole genome sequencing can be considered for key strains.

-References to other Candida CRISPR methods would be helpful to readers (PMID: 29695626, 29062088; 28657072; 28657070; 28497115.)

We have added a number of these references where we believe they could be helpful in the manuscript.

Reviewer #4:

Manuscript Summary:

The authors described a protocol for CRISPR-mediated gene editing in C. albicans. The authors chose the TPK2 gene to be their target gene for editing. They used used a yeast codon optimized Cas9, guide sequence and a repair template with selection markers and restriction sites in pV1524 plasmid. First, they designed and cloned the guide DNA sequence into the plasmid. Then they prepared a repair DNA template with the desired modifications before transforming the C. albicans and performing colony PCR to identify the mutants.

Major Concerns:

1. Several steps in the methods section need more details to make it easier for other researchers to replicate the protocol.

We have added many details consistent with the editorial review and peer review which should satisfy this comment.

2. There is no description of the effects of genome editing of TPK2 on the phenotype of the fungus.

It is our understanding that the results section provides an example of how this technology could be used. A detailed characterization of the how the mutation effects Candida phenotype seems outside of what is appropriate for a JOVE publication. We have added a brief description of the function of TPK2 in the results.

3. In the steps that is performed to prepare a product that will be used for gene editing, e.g. the steps for the preparation of repair DNA template, there is no notes on troubleshooting. For example, what should be done to confirm the success of this step before proceeding to the next step? What are the causes not succeeding e.g. getting low yield ..... etc. Same for other steps.

Quantitate the primer extension products to ensure sufficient DNA by determining the absorbance at 260 nm. Note: Typical final concentration of the primer extension product is ~200-300 ng/µl.

Has been added to quantitate the repair template.

Note: A restriction digestion site will be included in the repair template sequence to facilitate efficient screening of clones **(Figure 1C)**.

Has been added to help identify correct clones.

Purify plasmids from four transformants by miniprep and sequence the insertion sequence with Sequencing Primer **(Table 1)**. **Note:** Most of the time sequencing four transformants is sufficient to identify at least one correct clone.

Has been added to give further details for guide cloning.

4. The results section is extremely short.
It is the authors understanding that the purpose of the results sections is provide an example of how a result would look and that is what we have done. Throughout the manuscript we have provided a number of citations that contain additional examples of results. As no specific recommendations have been made as to how to lengthen the results we feel our results are adequate for a JOVE publication as the technique is the focus.
Minor Concerns:
Abbreviations should be defined at the first time they are mentioned or there should be a list of abbreviation, depending on the journal style. There are several abbreviations that are not defined.

We have defined all abbreviations.
Line 61: the repair template DNA that is used to repair the cleavage site…..

We don't know what the reviewer means by this comment.
Line 85: definition of TPK2 and a brief description of its function may be helpful to the reader since this is the main gene that is being edited here. This may be added in the introduction.

This has been added to the results.
Line 107: Do you inactivate the restriction enzyme after the plasmid digestion is complete? Or continue to the next step without inactivation?

The enzyme need not be inactivated thus we have not included this step in the protocol.
Line 111: What is the speed that you spin at?

This has been added.
Line 119-120: Do you check if the digestion is complete? Or continue to the ligation step directly?

No we do not check if the digestion is complete thus this is not included in the protocol.
Line 135: there is no step 1.4.3

This is not needed as we go to 1.5 next.

Line 138: What is CIP? If it is calf intestinal phosphatase, the abbreviation should to be added to step 1.4.1

This is defined earlier in the paper.
Line 153: how do you identify the correct plasmid?

This has been detailed in the paper more thoroughly, described above.

Line 167-168: What's the concentration of dNTPs? Also, Taq polymerase should be expressed in units.

This has been added.
Line 170: What are the PCR conditions? It should be mentioned here so that other researchers can reproduce the protocol.

Example extension conditions: 2 min 95 °C, (30 s 95 °C, 1 min 50 °C, 1 min 68 °C)x34, 10 min 68 °C. Has been added.

Lines 174-175: How do you check if the primer extension was completed successfully and you have the double stranded template? Measuring the concentration may not indicate successful extension.

We have found that concentration of template indicates successful extension. Extension products could be run on a gel if you have reason to think extension did not work properly but we have not had this issue.
Lines 178, 180, 185: there should be consistency of using the full name or the chemical formula of lithium acetate.

We have made this consistent.
Lines 180-185: the concentrations mentioned here (for example, 10 mM Tris-Cl pH 7.5) are the final concentrations? If so, there is no need to mention the pH for each solution separately.

These are the final concentrations. We have added a note all stock solution pH 7.5.
Line 190: how do you inactivate the restriction enzyme after the digestion is complete? Also, how do you check if the digestion is complete before proceeding to the next step?

We do not inactivate the restriction enzyme or check for complete digestion, we find that the protocol described leads to sufficient digestion for genome editing.

Line 200, 203: shouldn't it be salmon sperm DNA? What's the concentration?

This has been added and changed.
Line 200: How much do you add of each of the digested plasmid and the repair template? Also, what's the range? More than 10 µg is not a range.

We have added specific amounts. Adding both a range and a specific amount will be confusing.

Lines 183, 201: what does PLATE stand for?

This has been added to the first use of the acronym.
Line 211: The PLATE mixture to discard is the supernatant?

Supernatant has been added.
Line 219: Which media do you use? Is it the same as step 3.11?

YPD+Uri which is stated. We believe all medias are defined.
Line 224: After streaking for single colonies, what do you do with the plates? Incubate them to allow single colonies to grow? At which conditions? And for how long?

4.1.2. Streak for single colonies using aseptic technique and allow colonies to grow at 30 °C for 2 days. Has been added.

Line 230, 231: Again, what is the concentration of dNTPs, and how many units of Taq? Same for DMSO.

This has been added.

Line 237: Again, what are the cycling conditions of the PCR?

We do not wish to added cycling conditions as the individual primers used will change the annealing temp the brand of Taq may affect the conditions necessary as well.
Step 6.1: which restriction enzyme do you use here? Is it EcoRI? How much do you add of it (in units)?

Number of units of enzyme and exact conditions will vary depend the amount of PCR product you get from the reaction and enzyme used.

We have added the following Note: The restriction enzyme used here is the site encoded in your repair template.

Line 251: For how long they can be stored at -80?

Note: Correct strains can be stored at -80 °C for many years. Has been added.
Line 259: How do you count or estimate this number of cells?

Lines 276-281 are methods not results.

We feel that for a JOVE article it is appropriate to provide some methods with the results to orient the reader as to what step of the protocol is being performed.

Line 318: how to ensure that a single copy of the guide has been cloned? If there are any recommendations to do so, it would be very helpful if mentioned in the methods section.

We have now mentioned this.

One must ensure that only a single copy of the guide sequence has been cloned into CaCas9 expression vectors by sequencing as extra copies will impede genome editing. If multiple copies of the guide are introduced consistently one should lower the concentration of annealed guide used in ligation.

Lines 416-421: check reference #12 and 13 as it appears that they are the same.

We have fixed this error.
Figure 1A, B and C: it would be better to label each DNA strand with 5' or 3'.

These have been labeled.

Review Rebuttal_1

Editorial comments:

The manuscript has been modified and the updated manuscript, **58764_R1.docx**, is attached and located in your Editorial Manager account. **Please use the updated version to make your revisions. We have made all of the revisions. If we have missed something please feel free to contact me.**

Best,

Doug

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

We have reviewed these factors.

2. Please do not highlight notes for filming.

We have unhighlighted all notes.

3. Please define all abbreviations before use, e.g., EDTA, PEG, TE, BSA, etc.

We have done this.

4. Please do not abbreviate journal titles for all references.

We have fixed these although the PeerJ Endnote format keeps trying to abbreviate them.

5. Please specify all primers.

We have done this.

6. Please use standard SI unit symbols and prefixes such as µL, mL, L, g, m, etc.

I think that all units are now the stand symbols.

7. Step 1.1.1-1.1.3: Please write each step in imperative tense. These steps cannot be filmed unless there is Graphical User Interface involved. For steps that are done using software, a step-wise description of software usage must be included in the step. Please mention what button is clicked on in the software, or which menu items need to be selected to perform the step.

We have written out what needs to be done on the graphical interface.

8. Step 1.2.1-1.2.4: These steps cannot be filmed unless there is Graphical User Interface involved. For steps that are done using software, a step-wise description of software usage must be included in the step. Please mention what button is clicked on in the software, or which menu items need to be selected to perform the step.

We have added this.

9. 1.3.1: Please specify the composition of the buffer.

This is cut smart buffer which you said I was not allowed to mention in the manuscript as it is trademark. It is proprietary so I do not know the exact components.

10. 1.6: Please do not highlight a step without highlighting any of the sub-steps.

We have fixed this.

11. 2.1, 2.2: How to design? Is a software being used?

Yes we use software. This is now detailed and the GUI can be filmed.

12. There are two Step 5.1.

We have fixed this.

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