Editorial comments:
Changes to be made by the Author(s) regarding the written manuscript:
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We have done this.
2. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]." **We have done this and attached the links from the online permission.**
3. Please provide an email address for each author. **We have done this.**
4. Please spell out each abbreviation the first time it is used. **We have done this.**
5. Please use SI abbreviations for all units: L, mL, µL, h, min, s, etc. **We have done this.**
6. Please include a space between all numbers and their corresponding units: 15 mL, 37 °C, 60 s; etc. **We have done this.**
7. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. For example: CutSmart, etc. **We have done this.**
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.
13. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.
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.