

Journal of Visualized Experiments

QTL mapping and CRISPR/Cas9 editing to identify a drug resistance gene in *Toxoplasma gondii* --Manuscript Draft--

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Full Title:	QTL mapping and CRISPR/Cas9 editing to identify a drug resistance gene in <i>Toxoplasma gondii</i>
Article Type:	Invited Methods Article - JoVE Produced Video
Keywords:	Quantitative Trait Locus (QTL) mapping; Clustered regularly interspaced short palindromic repeats (CRISPR); guide RNA (gRNA); sinefungin; single nucleotide polymorphism (SNP); genetic cross; whole genome sequencing (WGS)
Manuscript Classifications:	3.3: Parasitic Diseases; 3.3.752: Protozoan Infections; 5.5.393: Genetic Techniques; 5.5.393.731: Reverse Genetics; 5.5.393.760: Sequence Analysis; 7.5.360.340: Genome; 7.5.360.340.24.380.937: Quantitative Trait Loci; 8.1.158.273.343.330: Genetics, Microbial
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Abstract:	<p>Scientific knowledge is intrinsically linked to available technologies and methods. This article will present two methods that allowed for the identification and verification of a drug resistance gene in the Apicomplexan parasite <i>Toxoplasma gondii</i>; the method of quantitative trait locus (QTL) mapping using a whole genome sequence (WGS) based genetic map and the method of clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 based gene editing. The approach of QTL mapping allows one to test if there is a correlation between a genomic region(s) and a phenotype. Two datasets are required to run a QTL scan, a genetic map based on the progeny of a recombinant cross and a quantifiable phenotype assessed in each of the progeny of that cross. These datasets are then formatted to be compatible with R/qtl software that generates a QTL scan to identify significant loci correlated with the phenotype. Although this can greatly narrow the search window of possible candidates, QTLs span regions containing a number of genes from which the causal gene needs to be identified. Having WGS of the progeny was critical to identify the causal drug resistance mutation at the gene level. Once identified, the candidate mutation can be verified by genetic manipulation of drug sensitive parasites. The most facile and efficient method to genetically modify <i>T. gondii</i> is the CRISPR/Cas9 system. This system comprised just two components both encoded on a single plasmid, a single guide RNA (gRNA) containing a 20 bp sequence complementary to the genomic target and the Cas9 endonuclease that generates a double-strand DNA break (DSB) at the target, repair of which allows for insertion or deletion of sequences around the break site. This article provides detailed protocols to use CRISPR/Cas9 based genome editing tools to verify the gene responsible for sinefungin resistance and to construct</p>

	transgenic parasites.
Author Comments:	
Additional Information:	
Question	Response
If this article needs to be "in-press" by a certain date, please indicate the date below and explain in your cover letter.	



June 30, 2016
Dr. Teena Mehta
Scientific Editor
Journal of Visualized Experiments

Dear Dr. Mehta,

Please see our manuscript entitled "QTL mapping and CRISPR/Cas9 editing to identify a drug resistance gene in *Toxoplasma gondii*" by Shen B. *et al.* that we would like to submit for publication in the Journal of Visualized Experiments. This manuscript provides detailed protocols to map, identify and confirm a gene responsible for sinefungin resistance in *T. gondii*. Specifically, two protocols are of interest as indicated in the title, QTL mapping and CRISPR/Cas9 editing. QTL mapping is a robust method to map genetic loci correlated with phenotypes, and is one for which JoVE has a limited number of publications utilizing the technique. To properly describe this approach we provide detailed command line syntax and step by step usage of freely available software that should complement your journal. CRISPR/Cas9 is a new and powerful tool to efficiently edit genomes that promises to continue to be effective. We give details on how CRISPR/Cas9 works, how to create a new CRISPR/Cas9 plasmid using site-directed mutagenesis, and how to use that plasmid to generate targeted mutations or insert constructs in *T. gondii*. The contributions of the authors are as follows; Shen and Behnke designed the protocols, Shen, Powell and Behnke performed the experiments/analyzed the data, and Shen and Behnke wrote the manuscript. During the preparation and submission of this manuscript, we have been kindly assisted by Teena Mehta, as Scientific Editor of Journal of Visualized Experiments, who we thank for the invited submission of this manuscript.

Please, see the list of recommended reviewers that follows.

Thank you for receiving our manuscript and considering it for review. We appreciate your time and look forward to your response.

Yours sincerely,

A handwritten signature in black ink, appearing to read "M. Behnke". The signature is fluid and cursive, with the first name "Michael" and last name "Behnke" clearly distinguishable.

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August 8, 2016
Dr. Mala Mani
Science Editor
Journal of Visualized Experiments

Dear Dr. Mani,

We appreciate the post-review Editorial comments for our manuscript entitled "QTL mapping and CRISPR/Cas9 editing to identify a drug resistance gene in *Toxoplasma gondii*". We have made the recommended changes which make the article more consistent and clear. Please see the document "55185_R0_072516-edited" which has tracked changes for all edits. Also, we have included detailed responses (red) to the Editorial comments below. Lastly, please see the Rightslink document which has a statement from ASM, the publisher of Eukaryotic Cell, granting permission to re-publish figures.

Sincerely,

A handwritten signature in black ink, appearing to read "M. Behnke". The signature is fluid and cursive, with the first and last names being clearly legible.

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Editorial Comments

- JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent. Please remove all commercial sounding language from your manuscript (text and figures). Examples of commercial sounding language in your manuscript are: REDHORSE (if REDHORSE is an open source software, please ignore this), Illumina, Addgene, NEB, nanodrop, BTX ECM-830, Q5 High-Fidelity, Gibson Assembly, Excel, etc. All commercial products should be sufficiently referenced in the table of materials/reagents. Please replace all commercial sounding language in your manuscript with generic names that are not company-specific.

We have changed all commercial related language to generic terms, occasionally pointing the reader to the Materials list for further information.

- Please ensure that all items mentioned have been included in the Materials/Equipment list, and are accompanied by a catalog number.

The Materials list has been completed.

- Please define all abbreviations before use. For e.g., DMEM, LB, GSH, PBS, etc.

Abbreviated terms have been defined at their first instance in the article.

- Your Long Abstract exceeds our 300 word limit. Please revise the Long Abstract so that it clearly states the goal of the protocol within 300 words.

The Long Abstract is now 300 words.

- Please ensure that all text in the protocol section is written in the imperative tense as if you are telling someone how to do the technique (i.e. “Do this”, “Measure that” etc.). 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”, however, notes should be used sparingly and actions should be described in the imperative tense wherever possible.

- Please rewrite the following steps in the imperative tense: 3.1.1, 3.1.2, 3.2.1, 4.1.2 (last sentence), 4.1.3.1, 4.1.4.1.1, 4.3.2.1, 4.4.2, and 4.4.4.1.

Tense for the listed sections has been modified.

- 1.1: Please provide additional details on how to maintain and culture the parasite. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

Additional details and reference have been added.

- 1.5: Is it a hemocytometer?

Instances of hemacytometer has been changed to hemocytometer.

- 1.6: Please add stepwise detail on how to pass the parasite to a HFF culture. Please mention the sinefungin concentration used in your studies.

Details have been added.

- 1.7: Please add details on how to monitor the parasite growth.

Wording has been changed to indicate growth of the parasite.

- 1.8: Please add additional details on how to score the progeny. How is growth/no growth and lysis observed?

Details have been added.

- Hyperlinks: Please move all hyperlinks to the Reference section and provide the superscript reference number in the relevant protocol steps.

Hyperlinks have been removed from the Protocols sections and added as references.

- Please note that the highlight was removed from sections 2 and 3 as these sections cannot be filmed.

We would like to work with the Editor and script writing team to determine what will be filmed.

- 3.4: The protocol section should be made up almost entirely of discrete steps, without large paragraphs of text between sections. Please break up the paragraphs into simple steps (with 2-3 actions per step) as much as possible throughout the protocol. The Protocol should contain only action items that direct the reader to do something. Alternatively, please consider moving long paragraph style text to the Discussion.

Discrete steps have been added to section 3.4.

- 4.1.1: If this step is to be filmed, please add stepwise detail on how to obtain the genomic sequence. Alternatively, remove the highlight and provide a citation.

The proper citation was added.

- 4.1.3.1: If this step is to be filmed, please add stepwise detail on how to synthesize the two primers. Alternatively, remove the highlight and provide a citation.

The proper citation was added.

- 4.1.4.1: If this step is to be filmed, please add stepwise detail on how to perform Q5 site-directed mutagenesis.

The proper citation was added.

- 4.1.5: Please add details on how to transform the mutagenesis product. Please mention the mutagenesis products used in your experiments.

Additional details have been added.

- 4.1.5.1: If this step is to be filmed, please add details on how to extract the plasmids, analyze DNA and sequence the plasmid. Please note that steps referring to manufacturer's instructions and cite a reference on how to perform a method cannot be filmed.

Highlighting has been removed.

- 4.2.1: Please add stepwise detail on how to inoculate HFF with the parasite. In addition, please mention the culture conditions.

Additional details have been added.

- 4.2.2: How are lysed cells observed?

Additional details have been added.

- 4.2.9: Please provide a reference.

Reference has been added.

- 4.3.1: Please add details on how to seed HFF cells on the coverslip. How many cell? When are the cells seeded on the coverslips? Please mention the culture conditions, medium, volume, etc.

Additional steps have been added describing these methods.

- 4.3.2.1: What volume of 4% formaldehyde? Please mention the antibody dilutions. Please add stepwise detail on how to perform IFA or provide a reference.

The appropriate changes have been made.

- 4.3.3.2: Please define "regular medium". Please mention the media volumes used.

Media volumes and details have been added.

- 4.3.3.3: Please mention the cell culture conditions. What volume of ethanol and crystal violet? Please add more details on how to stain and how to observe the plaques.

Details have been added.

- 4.4.1: Please mention the media, volume and culture conditions. Please add details on how to determine if the resistant pool becomes stable.

The appropriate details have been included.

- 4.4.2: Please add details on how to subclone the resistant pool. How many HFF cells? What medium?

Details for the media have been added. HFFs grown in 96 wells indicates how many cells.

- 4.4.3: Please mention what “contents”. How are the contents transferred?

“Contents” changed to “cells”, and pipetting details have been added.

- 4.4.5: Please mention the PCR amplification conditions. For e.g., reagents for the PCR reaction, cycles, temperature, etc.

PCR conditions have been added.

- 4.4.7: If this step is to be filmed, please add stepwise detail on how to compare the sequences. Alternatively, please provide a reference.

Reference has been added.

- Section 5 (including sub-steps): Please simplify steps of your protocol section so that individual steps contain only 2-3 related actions per step and a maximum of 4 sentences per step. We recommend that you split your longer steps into sub-steps numbered accordingly, i.e. step 1 is followed by sub-step 1.1 then 1.1.1 as necessary. The Protocol should contain only action items that direct the reader to do something. Please move any discussion about the protocol to the Representative Results or Discussion section.

Section 5 has been modified to discrete steps.

- Section 5.6 and 5.7: Please provide references for Western blotting, RT-PCR, and microscopy. Please add details on how to freeze the stocks (medium, etc.).

Appropriate references have been added.

- After you have made all of the recommended changes to your protocol (listed above), please re-evaluate the length of your protocol section. There is a 10-page limit for the protocol text, and a 3- page limit for filmable content. If your protocol is longer than 3 pages, please highlight (in yellow) 2.75 pages or less of text (which includes headings and spaces) to identify which steps should be visualized to tell the most cohesive story of your protocol steps. Please see JoVE's instructions for authors for more clarification. Remember that the non-highlighted protocol steps will remain in the manuscript and therefore will still be available to the reader.

- Please disregard the comment below if all of your figures are original. If you are re-using figures from a previous publication, please obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]." *We do not require the permission to re-use the figures until after internal and peer review. You can however, upload the permission to re-use email/file any time during the review process via your editorial manager account.*

Please see the Rightslink document granting permission for re-publishing of figures from ASM journals.

- Figure 1: Please provide a short description of each panel and/or a general description in the legend.

The legend has been added.

- Please expand your discussion to cover the following in detail and in paragraph form: 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.

The Discussion has been extended by three paragraphs providing further details about protocol limitations, critical steps, and significance and future applications.

- Please revise the protocol text to avoid the use of any pronouns (i.e. "we", "you", "your", "our" etc.).

Language using pronouns has been changed.



August 9, 2016
Dr. Mala Mani
Science Editor
Journal of Visualized Experiments

Dear Dr. Mani,

Thank you for accepting the edits we recently provided, and for the additional recommendations. We have made the changes, as well as have reread the manuscript making a few grammatical changes along the way. The 55185_R1_080916-edit.doc file has all the edits tracked. Please see the responses to the Editorial Comments below.

Sincerely,

A handwritten signature in black ink, appearing to read "M. Behnke". The signature is fluid and cursive, with the first letter of the last name being a large, stylized "B".

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Editorial Comments

- 4.1.5: Please provide a reference for chemical transformation.

Reference to the supplier's protocol has been added.

- 4.1.5.1.1: Please mention the storage conditions.

Conditions have been added.

- 4.3.2.2: How are successful transfectants identified?

Details have been added.

- 4.3.2.3: How are GFP positive and total parasites observed?

Use of a microscope has been indicated.

- Please note that files uploaded to the “Supplemental Files (as requested by JoVE)” section of the JoVE submission site are only for JoVE’s internal use and will NOT be published with your article. If you would like your files to be available for download with your article, then please move them to the “Supplemental Code Files” section of the JoVE submission site.

Supplemental files have been uploaded as Code Files

- Data files 1, 2 and 3 are missing. Please upload these files to the “Supplemental Code Files” section. A description of the files should be included with the Figure legends.

Files have been renamed, although when I try to upload Data File 2.zip the file is unzipped and each .txt file is uploaded individually. The six .txt files (chrid.txt, genotype.txt, markerpos.txt, mname.txt, phenonames.txt, phenos.txt) are intended to be provided as a zipped file named Data File 2.

- If the supplemental files (included in the current revision, e.g. chrid.txt, genotype txt, markerpos txt, etc.) are to be published along with your article, please move them to the “Supplemental Code Files” section and provide a title and description for each file in the Figure legend section.

Figure legends for each Data File have been added.



August 19, 2016
Dr. Mala Mani
Science Editor
Journal of Visualized Experiments

Dear Dr. Mani,

Thank you for accepting the edits we recently provided, and for the additional recommendations. We have made the required changes to the length of the Protocol section by moving various Notes to Supplementary File 1 and Protocol 5 to Supplementary File 2. The Protocol should now be 10 pages in length. The 55185_R2_081016-edit.doc file has all the edits tracked.

Sincerely,

A handwritten signature in black ink, appearing to read "M. Behnke". The signature is fluid and cursive, with the first letters of the first and last names being capitalized and prominent.

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October 14, 2016
Dr. Mala Mani
Science Editor
Journal of Visualized Experiments

Dear Dr. Mani,

We appreciate the encouraging peer reviews and comments. Please, see our response to the Editorial and Reviewers' comments following this letter. All edits have been tracked in the new versions of the article files uploaded with this resubmission. Also, the invoice for publication has been paid. We hope that the current state of the paper is sufficient to begin working with the JoVE Production Team. Again, we would like to include a video of Dr. Shen introducing the CRISPR section, a video he will have to shoot as he lives in China. Additionally, we would like to include a couple animations in the video, one describing crosses/QTL and the other describing CRISPR. Once the Production Team is included, we can work with them to figure out how to accomplish these goals.

Thanks again for your Editorial oversight of this article.

Sincerely,

A handwritten signature in black ink, appearing to read "M. Behnke". The signature is fluid and cursive, with the first name "Michael" and last name "Behnke" clearly distinguishable.

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Response to Editorial and Reviewers' Comments

Editorial Comments:

- Figure 1: Please modify the figure to remove the reference to Protocol section 5.

"5" has been removed from Figure 1.

- Scattered grammar issues should be corrected:

-1.3. "Scrape the monolayer with a cell scrapper" scraper

Fixed

-4.1.4.1.1. "Use the following cycling conditions for PCR: 98 °C for 1 min, followed by 25 cycles of 98 °C for 10 s, 55 °C for 30 s and 72 °C for 5 min, followed by final extension for 2 min at 72 °C." – this has no real associated filmable action, and should be converted to a note. "Refer to the mutagenesis kit manual to finish the rest of the mutagenesis reactions." can then be removed.

Changed to a Note and highlighting has been removed.

-SI units should be used throughout (mL, µL, L).

Units have been modified to reflect SI standards.

-In general, where primer sequences appear, or thermocycler settings, these do not need to be included in full within a step and can instead be placed as a note immediately following the action (and not highlighted). They can also just be included in the supplemental materials and referenced there.

Two such sections have been converted to Notes and de-highlighted.

-4.4.8 should be converted to a note.

Converted to a Note.

- Additional detail is required:

-1.3: "...pass the parasite solution through a 10 ml syringe/22 gauge blunt needle 2 - 3 times." What is the purpose this step? You are sucking up the scraped solution and passing it back out of the syringe?

"to lyse the host cells releasing parasites. Solution can be pulled back up into the syringe for multiple needle passages." Has been added to Protocol 1.3

-4.1.5 “Grow the transformants on lysogeny broth (LB) plates containing ampicillin.”
What temperature condition? 37 °C?

“at 37 °C” has been indicated

•Branding – 4.4.7: We don’t need to reference Clustal W, “sequence alignment tool” is fine.

Reference to ClustalW has been removed

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The manuscript describes QTL mapping in *T. gondii* using progeny from a cross to map a drug resistance gene. A detailed protocol of the bioinformatic analysis is included. The authors then describe the use of the CRISPR/Cas9 system to create disruptions of the candidate gene to validate its role in drug resistance. The paper is dense but that is likely necessary for the detailed bioinformatic analysis

Major Concerns:

None

Minor Concerns:

1. Include the type of input data that you are going to analyze for the QTL scan. Consider including any requirements in terms of estimating required depth of sequencing and replicates.

The input data format is already described in protocol 2.3, either gary's format or csv. We have added additional description to Protocol 2.3 in the main text to alert the reader to the supplement where this information can be found.

The read depth has already been addressed in Protocol 3.2.2 with the VarScan options, i.e. minimum read depth of 5 to call SNPs.

2. Consider including a more detailed map of the Cas9GFP plasmid and targeting plasmids with guide RNAs. Otherwise the figures are very good.

The Addgene plasmid page for the CRISPR plasmids have detailed maps. We have pointed the reader to Addgene in Protocol 4.1.

3. Consider including a recipe for cytomix.

The working concentrations for cytomix were provided in Protocol 4.2.2.

4. Check spelling on Caution using pyrimethamine. Explain the risk and IBC justification for its use as a selectable marker.

Additional language as to the risks and IBC guidelines have been added to the Note after Protocol 5.3.2.

Additional Comments to Authors:
N/A

Reviewer #2:

Manuscript Summary:

This manuscript provides a detailed description on the identification of candidate gene(s) potentially responsible for drug resistance to sinefungin (SNF) based on whole genome-based QTL and the verification of the candidate gene by CRISPR/Cas9-based genetic tool. This protocol will be a great resource not only for the Toxoplasma research community, but the approaches are also applicable to the identification and verification of desired phenotypes in other organisms such as other protozoan parasites.

Major Concerns:

No major concerns. The protocols are well and clearly presented.

Minor Concerns:

I only have three minor concerns that can be easily clarified by the authors without being further reviewed:

1) In the beginning of the protocol, it may be necessary to briefly describe the progenies that are used in this protocol, or have a short paragraph to describe materials and methods.

Much of this was provided in Protocol 2 in Supp File 1. We have included specific reference to details about the progeny in this section, and have indicated that there are 24 progeny in Protocols 1.8 & 2 and in the Introduction.

2) At the beginning of individual sections of the protocol, one or two sentences to tell the purpose of the subsequent procedures may be helpful.

In keeping with the JoVE formatting guidelines for the Protocol section, we feel the titles sufficiently translate the intent of each section. Also, many of the Notes which contain further detailed description of the Protocols were moved to Supp File 1 due to length restrictions. Reference to each of these Supp Notes has been provided in the main Protocol text and we hope the reader will make the effort to access the Supp files to obtain additional details.

3) Somewhere in the section 1 (such as in section 1.5), clarify that the "parasites" are merozoites.

We have added "and the tachyzoite stage readily grows in tissue culture." to the first Note in Protocol 1.

TITLE:

QTL mapping and CRISPR/Cas9 editing to identify a drug resistance gene in *Toxoplasma gondii*

AUTHORS:

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KEYWORDS:

Quantitative Trait Locus (QTL) mapping; Clustered regularly interspaced short palindromic repeats (CRISPR); guide RNA (gRNA); sinefungin; single nucleotide polymorphism (SNP); genetic cross; whole genome sequencing (WGS)

SHORT ABSTRACT:

Details are presented on how QTL mapping with a whole genome sequence based genetic map can be used to identify a drug resistance gene in *Toxoplasma gondii* and how this can be verified with the CRISPR/Cas9 system that efficiently edits a genomic target, in this case the drug resistance gene.

LONG ABSTRACT:

Scientific knowledge is intrinsically linked to available technologies and methods. This article will present two methods that allowed for the identification and verification of a drug resistance gene in the Apicomplexan parasite *Toxoplasma gondii*, the method of quantitative trait locus (QTL) mapping using a whole genome sequence (WGS) based genetic map and the method of clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 based gene editing. The approach of QTL mapping allows one to test if there is a correlation between a genomic region(s) and a phenotype. Two datasets are required to run a QTL scan, a genetic map based on the progeny of a

recombinant cross and a quantifiable phenotype assessed in each of the progeny of that cross. These datasets are then formatted to be compatible with R/qtl software that generates a QTL scan to identify significant loci correlated with the phenotype. Although this can greatly narrow the search window of possible candidates, QTLs span regions containing a number of genes from which the causal gene needs to be identified. Having WGS of the progeny was critical to identify the causal drug resistance mutation at the gene level. Once identified, the candidate mutation can be verified by genetic manipulation of drug sensitive parasites. The most facile and efficient method to genetically modify *T. gondii* is the CRISPR/Cas9 system. This system comprised just two components both encoded on a single plasmid, a single guide RNA (gRNA) containing a 20 bp sequence complementary to the genomic target and the Cas9 endonuclease that generates a double-strand DNA break (DSB) at the target, repair of which allows for insertion or deletion of sequences around the break site. This article provides detailed protocols to use CRISPR/Cas9 based genome editing tools to verify the gene responsible for sinefungin resistance and to construct transgenic parasites.

INTRODUCTION:

Host range determines the extent of a parasites' prevalence. Some parasites have very specific host requirements that limit the area from which they are found, others are generalists. One such generalist is *Toxoplasma gondii*. This parasite is found worldwide as it can infect all mammals and many birds. Humans are also susceptible and it is estimated that approximately one-third of the global population has been infected. Fortunately, a robust immune response normally controls the growth of the parasite, but in situations where the immune system is compromised the parasite can grow unchecked and cause diseases, often encephalic. Also, this parasite can cause congenital diseases if previously uninfected women are infected during pregnancy as they lack immune memory to quickly limit the spread of the parasite. Additionally, there is a burden of ocular toxoplasmosis that can result in vision loss ¹. For these reasons *T. gondii* has become a focus of study, and due to the many molecular methods developed for its study, a model for Apicomplexan parasites. Two methods that will be discussed here are quantitative trait locus (QTL) mapping and clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 gene editing. QTL mapping and CRISPR/Cas9 editing are, respectively, forward and reverse genetic approaches that have been used in previous studies to identify and/or characterize *T. gondii* virulence genes. Here, these methods are combined to identify and confirm the function of a sinefungin resistance (SNF^r) gene, TgME49_290860, and its orthologs (annotated as *SNR1*) ².

Although *T. gondii* can infect a wide variety of intermediate hosts, it must pass through and infect the intestinal epithelial cells of a felid to complete the life cycle. Cats are the definitive hosts of the parasite where the sexual stages are produced and genetic recombination via meiosis occurs. To conduct a QTL study one must create a genetic cross, and in the case of *Toxoplasma* this means passing two different parasite strains that differ in a phenotypic trait, the parental stains, through a cat to produce recombinant progeny ³. Before being fed to cats, the parental strains are made resistant to separate drugs to allow for more efficient identification of recombinants by double

drug selection of the progeny ⁴. Three drugs have been used in *T. gondii* for this purpose; fluorodeoxyribose (FUDR) for which uracil phosphoribosyl transferase (*UPRT*) is the resistance gene ⁵, adenosine arabinoside (ARA) for which adenosine kinase (*AK*) is the resistance gene ⁶, and sinefungin (SNF) for which the resistance gene was unknown ⁷. Several genetic crosses have been created for *T. gondii*, but only the 24 progeny of the ME49-FUDR^r X VAND-SNF^r cross were genotyped using whole genome sequencing (WGS) ⁸. This opened the possibility of mapping and identifying the SNF resistance gene using this cross as the VAND parent was made sinefungin resistant by chemical mutagenesis of the drug sensitive VAND (VAND-SNF^s) strain, and the VAND reference genome was sequenced using the VAND-SNF^s strain thus allowing for the identification of all polymorphisms between the progeny WGS and the VAND-SNF^s reference genome, including the inherited parental VAND-SNF^r mutation that rendered some of the progeny sinefungin resistant.

In order to identify the causal single nucleotide polymorphism (SNP) in the SNF^r progeny, several computational based open source resources can be used to analyze the data. To create the genetic map for the ME49-FUDR^r X VAND-SNF^r cross the REDHORSE software suite ⁹ was developed that uses WGS alignments of the parents and progeny to accurately detect the genomic positions of genetic crossovers. This mapping information can then be combined with the phenotypic data (SNF^r in the progeny) to format a dataset compatible with the 'qtl' package ¹⁰ in the R statistical programming software where a QTL scan can be run to reveal significant loci correlated with the phenotype. To identify the causal SNP located within the QTL locus, the WGS reads of the progeny can be individually aligned to the sinefungin sensitive VAND genome using the Bowtie 2 alignment program ¹¹ from which SNPs can be called using the VarScan mpileup2snp variant caller program ¹². Using these SNPs, the QTL locus can then be scanned for polymorphisms that are present in the SNF^r but not in the SNF^s progeny. With the causal SNP identified in the coding region of a gene, genetic modification of the candidate SNF^r gene can be performed in a SNF^s strain to verify the drug resistance function.

The CRISPR/Cas9 genome editing system was recently established in *Toxoplasma* ¹³, which added important tools for the exploration of the complex biology of this parasite, particularly for genetic studies in non-laboratory adapted strains. Because of the highly active non-homologous end joining (NHEJ) activity in wildtype (WT) *Toxoplasma* cells, targeted genome modification is difficult to achieve since exogenously introduced DNA is randomly integrated to the genome at extremely high frequency ¹⁴. To increase the success rate of locus specific modification, different approaches have been employed to increase the efficiency of homologous recombination and/or decrease the NHEJ activity ^{15,16}. One of these approaches is the CRISPR/Cas9 system. Compared to other methods, the CRISPR/Cas9 system is efficient at introducing site-specific modifications and is easy to design ^{13,17,18}. In addition, it can be used in any *Toxoplasma* strain without extra modification to the parasite ^{13,19}.

The CRISPR/Cas9 system originated from the adaptive immune system of *Streptococcus pyogenes*, which uses it to defend the invasion of mobile genetic

elements such as phages²⁰⁻²². This system utilizes the RNA-guided DNA endonuclease enzyme Cas9 to introduce double-strand DNA break (DSB) into the target, which is subsequently repaired either by the error-prone NHEJ to inactivate target genes through short indel mutations, or by homology directed recombination to alter the target locus exactly as designed^{23,24}. The target specificity is determined by a small RNA molecule named single guide RNA (gRNA), which contains an individually designed 20-nt sequence that has 100% homology to the target DNA²². The gRNA molecule also contains signatures recognized by Cas9 that guide the nuclease to the target site, which includes a special Protospacer Adjacent Motif (PAM, sequence is 'NGG')^{25,26}. Therefore, the gRNA molecule and the PAM sequence work together to determine the Cas9 cleavage site in the genome. One can easily change the gRNA sequence to target different sites for cleavage.

When the CRISPR/Cas9 system was first developed in *Toxoplasma*, a single plasmid expressing the Cas9 nuclease and the gRNA molecule was used to introduce DSB at the targeting site^{13,17}. It has been shown that the CRISPR/Cas9 system drastically increases the efficiency of site-specific genome modification, not only by homologous recombination, but also non-homologous integration of exogenous DNA¹³. It does this in wildtype strains that contain NHEJ activity. Therefore, this system can be used in essentially any *Toxoplasma* strain for efficient genome editing. In a typical experiment, the target specific CRISPR plasmid and the DNA fragment used to modify the target are co-transfected into parasites. If the DNA fragment used to modify the target contains homologous sequences to the target locus, homologous recombination can be used to repair the DSB introduced by CRISPR/Cas9 to allow precise modification of the target. On the other hand, if the introduced DNA fragment does not contain homologous sequence, it can still be integrated into the CRISPR/Cas9 targeting site. The latter is often used to disrupt genes by insertion of selectable markers or to complement mutants at loci that allow negative selection¹³. Here the *SNR1* locus serves as an example to show how CRISPR/Cas9 can be used for gene disruption and the generation of transgenic parasites.

PROTOCOL:

1. Assess *SNF*^r in the progeny of the ME49-FUDR^r X VAND-*SNF*^r cross

Note: *T. gondii* is an obligate intracellular parasite and the tachyzoite stage readily grows in tissue culture.

1.1. To grow the *T. gondii* parasite, maintain them in confluent human foreskin fibroblast (HFF) monolayer culture seeded to T25 flasks with Dulbecco's modified Eagle's medium (DMEM) media supplemented with 10% fetal bovine serum (FBS) (D10 media) at 37 °C and 5% CO₂.

Note: See Protocol 1.1 Note in Supplementary File 1.

1.2. To test individual progeny clones for sinefungin resistance, grow each clone to high parasite density in a T25 HFF culture flask for 2-3 days, until majority of the parasites begin to lyse the host cells.

1.3. Scrape the monolayer with a cell scraper and pass the parasite solution through a 10-mL syringe/22-gauge blunt needle 2-3 times to lyse the host cells releasing parasites. The solution can be pulled back up into the syringe for multiple needle passages.

1.4. Filter the parasite solution into a new conical tube through a membrane with the pore size of 3 μm to remove HFF cells and cellular debris.

1.5. Count the parasites on a hemocytometer and determine the number of parasites per mL.

1.6. Using a pipettor, pass 2.5×10^5 parasites to a new T25 HFF culture flask containing D10 media with 0.3 μM working concentration of the sinefungin drug.

1.7. Grow the parasites at 37 °C and 5% CO₂ for 7-10 days.

1.8. Score the progeny for the growth phenotype in sinefungin drug. Observe the monolayer under an inverted phase-contrast microscope and score 0 for no growth (sinefungin sensitive), score 1 for growth and lysis of monolayer (sinefungin resistant).

Note: See Protocol 1.8 Note in Supplementary File 1.

2. Run a QTL scan of the SNF^r phenotype in R/qtl

Note: See Protocol 2 Note in Supplementary File 1.

2.1. Install the R programming language software on a local computer. See ²⁷.

2.2. Run R and install the package 'qtl'. Either do this from the R GUI interface option 'Packages->Install package(s)', or run the following command from the R command line (the first ">" symbol in each example denotes the beginning of a command, not to be copied):

```
>install.packages("qtl")
```

Note: Once R and the 'qtl' package have been installed, there are two ways to run R/qtl, from the R command line or using a graphical user interface (GUI) program called J/qtl ²⁸ : see ²⁹ to download J/qtl. This protocol will provide the R/qtl command line syntax with occasional reference to the equivalent function in J/qtl.

2.3. Load the 'qtl' package:

```
> library(qtl)
```

Note: See Protocol 2.3 Note in Supplementary File 1 for dataset format and downloads.

2.4. Load the dataset file into R/qtl.

2.4.1. For “csv” format (See: Data File 1):

```
>SNFR <- read.cross(format="csv", file="$PATH/Rqtl-SNFR.csv", genotypes=c("0", "1"),  
na.strings=c("-"), convertXdata=FALSE)
```

2.4.2. Or for “gary” format (See: Data File 2):

```
>SNFR <- read.cross(format="gary", dir="$PATH", chridfile="chrid.txt",  
mnamesfile="mname.txt", mapfile="markerpos.txt", genfile="genotype.txt",  
phefile="phenos.txt", pnamesfile="phenonames.txt", convertXdata=FALSE)
```

Where \$PATH is the directory path to the file(s) containing the qtl dataset. For example: /Users/DiggityDog/QTLfiles

Note: The files can also be loaded into J/qtl with the previous commands using the ‘Insert Comment or Command’ option, or load the data with the GUI ‘File->Load Cross Data’ option.

2.5. Calculate probabilities for the map:

```
> SNFR <- calc.genoprob(SNFR, step=2.0, off.end=0.0, error.prob=1.0E-4,  
map.function="haldane", stepwidth="fixed")
```

2.6. Run a single scan using a binary distribution of the sinefungin resistance phenotype across all chromosomes:

```
> SNFR.scan <- scanone(cross=SNFR, chr=c("Ia", "Ib", "II", "III", "IV", "V", "VI", "VIIa",  
"VIIb", "VIII", "IX", "X", "XI", "XII"), pheno.col=c(1), model="binary", method="em")
```

Note: If running the VIR phenotype use the ‘model=”normal”’ distribution option.

2.7. Run 1000 permutations to calculate significance thresholds and then attribute the permutations to the SNFR.scan variable:

```
> SNFR.scan.permutations <- scanone(cross=SNFR, chr=c("Ia", "Ib", "II", "III", "IV", "V",  
"VI", "VIIa", "VIIb", "VIII", "IX", "X", "XI", "XII"), pheno.col=c(1), model="binary",  
method="em", n.perm=1000, perm.Xsp=FALSE, verbose=FALSE)
```

```
>attr(SNFR.scan, "pheno.col") <- c(1)
```

Note: Steps 2.5 through 2.7 can be run in J/qtl with the ‘Analysis->Main Scan-> Run One QTL Genome Scan’ option.

2.8. Plot the scan results:

```
>plot(SNFR.scan, gap=0, bandcol="grey")
```

Note: The plot produced in J/qtl is interactive.

2.8.1. Plot the scan results for just chromosome IX, the chromosome with the highest peak:

```
>plot(SNFR.scan, chr=c("IX"), gap=0, bandcol="grey", show.marker.names=TRUE)
```

2.9. Show all the marker positions and LOD scores from the single scan:

```
>SNFR.scan
```

2.10. Show the threshold results of the permutation test:

```
>summary(SNFR.scan.permutations)
```

2.11. Show only those markers greater than the $\alpha=.05$ threshold value (taken from the previous output):

```
>SNFR.scan[SNFR.scan$lod > 2.68,]
```

2.12. Show the markers with the highest LOD score on each chromosome:

```
>summary(SNFR.scan)
```

2.13. Show those markers that have the max LOD score value (taken from the previous output):

```
>SNFR.scan[SNFR.scan$lod >= 6.57,]
```

Note: See Protocol 2.13 Note in Supplementary File 1.

3. Identify the causal SNF^r mutation using WGS reads from the progeny

Note: See Protocol 3 Note in Supplementary File 1.

3.1. Align WGS reads of individual progeny to the sinefungin sensitive VAND (VAND-SNF^s) parental genome and call SNPs

Note: Download the VAND reference genome (SNF^s) from NCBI Assembly AEYJ000000000.2 and the WGS reads (.sra files) for the 24 progeny from NCBI SRA PRJNA258152. Also, download the following programs: Bowtie2 ¹¹, NCBI SRA Toolkit, SAMtools ³⁰, VarScan ¹², and MUMmer ³¹.

3.1.1. Create a Bowtie 2 compatible index from the VAND reference genome FASTA file using the bowtie2-build program, here naming the index "VAND":

```
>bowtie2-build GCA_000224845.2_TGVAND_v2_genomic.fna VAND
```


3.1.2. Convert the SRA formatted WGS read files to FASTQ files using fastq-dump with the --split-files option for paired reads (the SRR1555372.sra file for progeny P1_14VB will be used as an example):

```
>fastq-dump --split-files SRR1555372.sra
```

Note: Run this and all the following commands in Protocol 3.1 for all 24 progeny.

3.1.3. Align the WGS reads to the reference genome using bowtie2 with output to a SAM (.sam) file and the --end-to-end option:

```
>bowtie2 -x VAND -1 SRR155372_1.fastq -2 SRR1555372_2.fastq -S  
SRR155372.sam --end-to-end
```

3.1.4. Convert the .sam file to a BAM (.bam) file:

```
>samtools view -bS SRR155372.sam > SRR155372.bam
```

3.1.5. Sort the .bam file:

```
>samtools sort SRR155372.bam SRR155372.sort
```

3.1.6. Index the sorted .bam file:

```
>samtools index SRR155372.sort.bam
```

3.2. Call SNPs for the progeny using VarScan

Note: See Protocol 3.2 Note in Supplementary File 1.

3.2.1. Convert all indexed BAM (.sort.bam) files into one pileup formatted file using samtools mpileup with the VAND genome reference file, all progeny .sort.bam files, and output to a file named AllProgeny-mpileup.txt:

```
>samtools mpileup -f GCA_000224845.2_TGVAND_v2_genomic.fna  
SRR1555599.sort.bam SRR1555372.sort.bam SRR1555660.sort.bam  
SRR1555672.sort.bam SRR1556052.sort.bam SRR1556122.sort.bam  
SRR1556192.sort.bam SRR1556194.sort.bam SRR1556195.sort.bam  
SRR1556199.sort.bam SRR1556200.sort.bam SRR1556202.sort.bam  
SRR1556203.sort.bam SRR1556274.sort.bam SRR1556276.sort.bam  
SRR1556278.sort.bam SRR1556395.sort.bam SRR1556396.sort.bam  
SRR1556397.sort.bam SRR1556398.sort.bam SRR1556399.sort.bam  
SRR1556400.sort.bam SRR1556401.sort.bam SRR1556402.sort.bam > AllProgeny-  
mpileup.txt
```

3.2.2. Call all SNPs using the VarScan mpileup2snp program using the AllProgeny-mpileup.txt file, minimum read coverage of 5, minimum variant frequency across reads of .8, a p-value of .01, and output to a file names AllProgeny-SNPs.txt:

```
>java -jar VarScan.jar mpileup2snp AllProgeny-mpileup.txt --min-coverage 5 --min-var-freq .8 --p-value .01 > AllProgeny-SNPs.txt
```

Note: The AllProgeny-SNPs.txt file is a tab delimited text file that will be used in Protocol 3.4 to locate the causal SNP.

3.3. Find VAND genome coordinates that correspond to coordinates of the ME49 based QTL locus

Note: See Protocol 3.3 Note in Supplementary File 1.

3.3.1. Use the MUMmer nucmer program to align the ME49 genome (available for download from ToxoDB.org ³²) and the VAND reference genome file (output goes to the out.delta file):

```
>nucmer ToxoDB-28_TgondiiME49_Genome.fasta  
GCA_000224845.2_TGVAND_v2_genomic.fna
```

3.3.2. Use the MUMmer show-coords program to obtain the coordinates of the two genome alignments, output to a file named ME49vsVAND-coords.txt:

```
>show-coords out.delta > ME49vsVAND-coords.txt
```

Note: The ME49vsVAND-coords.txt file can be used to find the VAND contigs and positions that correspond to the QTL locus; markers MV359 to MV366 located on ME49 chromosome IX between 3,187,537 to 4,202,258 bp. There are two VAND contigs that span the corresponding QTL locus; KN044604.1:657,441-1 bp and KN042501.1:430,910-41,870 bp (both contigs align reverse to the ME49 chromosome).

3.4. Assess progeny SNPs located within the QTL locus for SNPs inherited only in the SNF^r progeny

Note: See Protocol 3.4 Note in Supplementary File 1.

3.4.1. Review Data File 3 to identify the causal mutation located within the QTL locus. Scan the data for a pattern where the SNF^r progeny have a SNP and the SNF^s do not (feasible because progeny SNPs were obtained by comparison to the VAND-SNF^s reference genome). This should only result in one position with this pattern, position 348130 on VAND contig KN042501.1.

3.4.2. See row 6604 in Data File 3 (Figure 3).

Note: See Protocol 3.4.2 Note in Supplementary File 1.

4. Verification of identified hits by CRISPR/Cas9 mediated gene inactivation.

Note: To confirm the causal SNP identified by QTL mapping and WGS SNP analysis, the corresponding genetic changes need to be made in a wild-type SNF^s background

and the resulting phenotype examined. In the case of sinefungin resistance, the responsible mutation results in inactivation of the *SNR1* gene by early termination ². Therefore, disruption of *SNR1* can be used for confirmation. Here a detailed protocol is provided for using CRISPR/Cas9 induced indel mutations to disrupt *SNR1*, to demonstrate its involvement in sinefungin resistance (Figure 4).

4.1. *SNR1* specific CRISPR plasmid construction

Note: See Protocol 4.1 Note in Supplementary File 1 to obtain plasmids and maps.

4.1.1. Obtain the genomic sequence for the target gene (*SNR1*, TGME49_290860) from ToxoDB ³².

4.1.2. Use online tools such as E-CRISP ³³ to design target specific gRNAs. Do not include the PAM sequence (NGG) in the gRNA.

Note: See Protocol 4.1.2 Note in Supplementary File 1.

4.1.3. Synthesize primers to construct the target specific CRISPR plasmid.

4.1.3.1. According to the design from step 4.1.2, synthesize two primers to change the *UPRT* targeting gRNA in the original CRISPR plasmid to the selected gRNA sequence by site-directed mutagenesis.

Note: The sequences of these two primers are as follow: gRNA-Fw: NNNNNNNNNNNNNNNNNNNNGTTTGTAGAGCTAGAAATAGC (N20 is the target specific gRNA sequence) and gRNA-Rv: AACTTGACATCCCCATTTAC ².

4.1.4. Perform the site-directed mutagenesis reactions.

4.1.4.1. Using the *UPRT* targeting CRISPR plasmid (pSAG1::CAS9-U6::sgUPRT) as template and the two primers listed above, perform site-directed mutagenesis reactions according to the manufacturer's instructions.

Note: Use the following cycling conditions for PCR: 98 °C for 1 min, followed by 25 cycles of 98 °C for 10 s, 55 °C for 30 s and 72 °C for 5 min, followed by final extension for 2 min at 72 °C.

4.1.5. Transform the mutagenesis products containing the GOI-specific CRISPR plasmids to *E. coli* competent cells by chemical transformation according to the supplier's protocol (see Materials). Grow the transformants on lysogeny broth (LB) plates containing ampicillin (100 µg/mL) at 37 °C. Subsequently, pick 2 - 4 clones and individually grow them in 5 mL LB medium supplemented with 100 µg/mL ampicillin for 12 – 16 h.

4.1.5.1. Extract plasmids from the cultures using a DNA isolation kit and analyze them by DNA gel electrophoresis to check the size of the plasmids (expected plasmid size is 9674 bp, use the original CRISPR plasmid as control). Use the M13-Rev primer (5'-

CAGGAAACAGCTATGACC) to sequence the plasmids to confirm the target specific gRNA sequence.

4.1.5.1.1. Once positive clones are identified, store both plasmid DNA at -20 °C and corresponding bacterial culture at 4 °C for future use.

Note: The CRISPR plasmid to be used for transfection should be dissolved in deionized water and the concentration determined by spectrophotometry or an alternative method.

4.2. Parasite transfection.

Note: General methods for culturing *T. gondii* in human foreskin fibroblast (HFF) cells in D10 medium have been described previously ³⁴.

4.2.1. Two to three days before transfection, add enough parasites to a T25 flask containing a confluent HFF cell monolayer ($0.5 - 1 \times 10^6$ for type 1 strains, $1 - 2 \times 10^6$ for other strains) to achieve 70 – 80% HFF cell lysis within 2 – 3 days.

4.2.2. Examine the culture under an inverted phase contrast microscope, when the HFF monolayer is 70 – 80% lysed by the parasites. Gently remove the medium with a pipette and wash the cells off the flask surface with 5 mL cytomix buffer (120 mM KCl, 0.15 mM CaCl_2 , 10 mM $\text{K}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$ pH=7.6, 25 mM HEPES, 2 mM EDTA, 5 mM MgCl_2 , pH=7.6). Subsequently, transfer the parasite solution to a 15-mL conical tube.

Note: See Protocol 4.2.2 Note in Supplementary File 1.

4.2.3. Pass the parasite solution through a 10-mL syringe/22-gauge blunt needle 2 - 3 times.

4.2.4. Filter the parasite solution into a new conical tube through a membrane with the pore size of 3 μm to remove HFF cells and cellular debris.

4.2.5. Pellet the filtered parasites by centrifugation at 400× g for 10 min.

4.2.6. Pour off the supernatant and resuspend the pelleted parasites with 10 mL cytomix buffer, take a 10 μL aliquot to determine the parasite concentration with a hemocytometer and pellet the rest by centrifugation at 400× g for 10 min.

4.2.7. Remove supernatant and resuspend the pellet in cytomix buffer to obtain a density of 4×10^7 parasites/mL.

4.2.8. In a 4 mm gap cuvette, mix 250 - 300 μL parasite solution ($1 - 1.3 \times 10^7$ parasites) with 7.5 μg CRISPR plasmid, 6 μL ATP (100mM), and 6 μL glutathione (GSH) (250mM).

4.2.9. Electroporate the parasites ³⁵. Include a separate electroporation as a negative control in which a CRISPR plasmid targets elsewhere, such as the *UPRT* targeting CRISPR plasmid.

Note: The electroporation settings depend on the device. For the electroporator listed in the Materials use the 4 mm gap cuvettes. The following protocol is recommended: 1700 V, 176 μ s of pulse length, 2 pulses with 100 ms interval.

4.3. Determine the frequency of sinefungin resistance after CRISPR targeting.

4.3.1. Seed HFF cells to coverslips placed in 24-well plates 3 - 4 days before electroporation so that they are confluent by the time to perform transfection in 4.2.

4.3.1.1. Trypsinize the HFF cells from one confluent T25 flask and subsequently add 12 mL D10 medium and mix well.

4.3.1.2. Add a coverslip to each well of a 24-well plate and aliquot 500 μ L HFF cell solution to each well. Incubate the plate at 37 °C for 3-4 days to let the cells grow until confluent.

Note: Cells from one T25 flask are enough to seed one 24-well plate.

4.3.1.3. Add 50 μ L of electroporated parasite solution from step 4.2.9 into one well of 24-well plate containing a coverslip seeded with confluent HFF cells. Transfer the rest of electroporated parasite solution into a T25 flask seeded with confluent HFF cells.

4.3.2. Assess the efficiency of transfection.

4.3.2.1. Grow the cells in the 24-well plate for 24 h and subsequently fix the cells (host cells and parasites) with 500 μ L 4% formaldehyde to check the expression of Cas9-GFP by immunofluorescent assay (IFA).

4.3.2.2. Probe the cells with an anti-GFP antibody and a *Toxoplasma* specific antibody (such as anti-TgALD) to label total parasites. See the antibody product sheet for the recommended dilution (usually, 1:1000 is sufficient for IFAs). If the antibodies are unconjugated, use two different secondary antibodies conjugated with fluorescent dyes to label the primary antibodies.

4.3.2.3. Observe the labeled cells on a fluorescent microscope with filters appropriate for the secondary fluorescent dyes. Obtain transfection efficiency by dividing the number of GFP positive parasites by the number of total parasites.

Note: The two antibodies used for IFA should come from two different host species, for example using the combination of mouse anti-GFP and rabbit anti-TgALD.

4.3.3. Determine the frequency of sinefungin resistance in transfected parasites.

4.3.3.1. For the transfected parasites cultured in T25 flasks, grow them for 2 – 3 days until natural egress. Then collect the parasites, blunt needle lyse host cells to release intracellular parasites, and purify them by filtration through membranes with pore size of 3 μ m. Count the parasites with a hemocytometer to estimate the density.

4.3.3.2. Add purified parasites into 6-well plates seeded with confluent HFF cells: for the first row (3 wells), add 200 parasites/well and grow them in regular D10 medium (2 mL/well); for the second row, add 5000 parasites/well and grow them in D10 medium containing 0.3 μ M sinefungin.

4.3.3.3. Put the plates in a 5% CO₂ incubator and grow the parasites for at 37 °C 8 - 10 days without disturbance to allow plaques to form. Fix the samples with 70% ethanol (2 mL/well) and stain the monolayer with 0.1% crystal violet (2 mL/well). Wash the wells with water to visualize the plaques (clear zones) formed by parasite growth.

Note: The negative control should be processed the same way side by side.

4.3.4. Calculate the rate of CRISPR induced sinefungin resistance.

4.3.4.1. Obtain the average number (X) of plaques from wells containing no drug and calculate the parasite viability as X/200. Obtain the average number (Y) of plaques from wells containing sinefungin to calculate the rate of CRISPR induced sinefungin resistance as follow:

Rate of CRISPR induced resistance = $200 \times Y / (5000 \times X \times \text{transfection efficiency})$

Note: Transfection efficiency is obtained from 4.3.2.

4.4. Examine the indel mutations induced by CRISPR/Cas9

4.4.1. Grow the electroporated parasites from section 4.2.9 in a T25 flask seeded with HFF cells for 2 days at 37 °C. Then, replace the medium with 5 mL D10 medium containing 0.3 μ M sinefungin (selection medium).

4.4.1.1. Keep the parasites in selection medium for at least 3 passages until the resistant pool becomes stable (indicated by the absence of parasite growth in the negative control group but robust parasite growth in the experimental group).

4.4.2. Subclone the sinefungin resistant pool to obtain clonal strains.

4.4.2.1. Collect freshly egressed parasites, purify by 3 μ m membrane filtration and subclone into 96-well plates seeded with confluent HFF cells in 150 μ L D10 medium. Grow the subcloning cultures in a CO₂ incubator at 37 °C for 7 - 10 days without disturbing the plates.

Note: See Protocol 4.4.2.1 Note in Supplementary File 1.

4.4.3. Check the 96-well plates under an inverted phase-contrast microscope to look for wells that contain only one plaque. Mark such wells and subsequently transfer the cells from each well to 24-well plates seeded with HFF cells using a pipette.

4.4.4. When 80 – 90% of the HFF cells in a well are lysed, harvest the parasites (about 500 μ L) and pass 50 μ L parasite solution to a new well to maintain the strain. Use the rest (\approx 450 μ L) to extract genomic DNA for PCR amplification.

4.4.4.1. For genomic DNA isolation from the SNF^r clone, pellet the parasites (small amounts of HFF cell contamination is tolerable) at 1000 \times g for 10 min. Wash the pelleted parasites with phosphate-buffered saline (PBS) once and pellet them again. Isolate genomic DNA from pelleted cells using a commercial kit or by boiling. Resuspend parasites in 50 - 100 μ L PBS.

Note: Perform PCR to obtain a fragment of the *SNR1* gene for sequencing. Use the following primers to amplify the *SNR1* locus²: SNR1-Amp-Fw: 5' CCGACCACAA CAATTTTC and SNR1-Amp-Rv: 5'GACGTGATTCACTTTTTTACAGACAGAC. Use high fidelity DNA polymerases. Amplify the WT locus for control purposes.

4.4.5. Perform the PCR with 20 μ L reaction volume and run it with the following program: 98 °C for 1 min, followed by 30 cycles of 98 °C for 10 s, 55 °C for 30 s and 72 °C for 2.5 min, followed by final extension for 2 min at 72 °C.

Note: Sequence the PCR products using the following primers: SNR1-Seq1: 5' GCC ACA TGC TTT AGC GTG, SNR1-Seq2: 5' TCC TCT CCA TCA CGG GTT GG, SNR1-Seq3: 5' GCA AGA GCC GCG TGA CG, SNR1-Seq4: 5' CTC TCC CGC GGT CGA G, SNR1-Seq5: 5' GCA CCG TCC GCA AGC, and SNR1-Seq6: 5' CCG GAA GGT GAA TCG TTC TTC.

4.4.6. Compare the sequences of *SNR1* gene from sinefungin resistant mutants to that of the WT strain using a sequence alignment tool.

Note: See Supplementary File 2 Protocol 5 for details on the utilization of negative selection at the *SNR1* locus for genetic complementation or transgenic strain construction.

REPRESENTATIVE RESULTS

This article outlines in detail several methods that can be used in succession to identify a gene responsible for drug resistance (Figure 1). The 24 progenies of the ME49-FUDR^r X VAND-SNF^r cross were assessed for resistance to the drug sinefungin as described in Protocol 1. Using the genetic map and the SNF^r phenotype of the progeny, a QTL scan was run in R/qtl - Protocol 2 (Figure 2). This resulted in one significant peak on chromosome IX spanning approximately 1 Mbp. It is in this region that the causal mutation is located.

To identify the causal mutation, WGS reads from the progeny were aligned to the VAND-SNF^s reference genome using Bowtie2 - Protocol 3.1, SNPs were called using VarScan mpileup2snp - Protocol 3.2, and the QTL locus in the VAND genome was identified using MUMmer - Protocol 3.3. Progeny SNPs within the QTL locus were extracted and scanned for a pattern where the SNF^r progeny have a SNP and the SNF^s do not, which is feasible because progeny SNPs were obtained by comparison to the

VAND-SNF^s reference genome (Figure 3). Only one SNP matched this pattern that results in an early stop codon in a putative amino acid transporter gene named *SNR1*.

Confirmation that *SNR1* is the SNF^r resistance gene was performed using the CRISPR/Cas9 system. A new CRISPR/Cas9 plasmid engineered for *T. gondii* gene editing was made containing a gRNA targeting the *SNR1* gene near the SNF^r mutation identified in the progeny – Protocol 4 (Figure 4A). The *SNR1* targeting CRISPR/Cas9 plasmid was electroporated into a SNF^s WT parasite strain and resistant mutants were obtained when cultured in 0.3 μ M sinefungin. No SNF^r parasites were obtained when electroporated with the UPRT targeting CRISPR/Cas9 plasmid. Several SNF^r CRISPR mutants were cloned and the region around the *SNR1* gRNA target was sequenced. Each mutant had an indel that disrupted the coding sequence of the *SNR1* gene (Figure 4B). This method can also be used to insert a targeting construct into the *SNR1* locus via NHEJ (Figure 5), or by HR (Figure 6) – Supplemental File 2.

Figure 1. Schematic workflow for experiments outlined in the protocols. The main steps in identifying and confirming the SNF^r gene using the ME49-FUDR^r X VAND-SNF^r cross are shown with reference to the appropriate Protocols outlined in this article.

Figure 2. R/qtl commands to run QTL scan on the SNF^r phenotype. The commands as outlined in Protocol 2 were run in R using the qtl package. Representative commands and plot are shown.

Figure 3. Location of the causal SNP. Progeny WGS reads were aligned to the VAND-SNF^s reference genome with Bowtie 2, SNPs were called with VarScan, and the corresponding VAND QTL locus identified with MUMmer. SNPs located within the QTL locus were imported into a spreadsheet and the causal SNP was identified. SNF^r progeny (yellow), SNF^s progeny (green), SNF^r SNP (red) (see Data File 3).

Figure 4. Confirming *SNR1* as SNF^r gene using CRISPR/Cas9. A) CRISPR/Cas9 induced indel mutations (green) in the *SNR1* locus. B) Representative indels in *SNR1* caused by two different *SNR1* specific CRISPR plasmids (C5 and C6 respectively). RH is the WT strain and C5 and C6 are SNF^r mutants. (Figure 4B is taken from ²).

Figure 5. Insertional mutagenesis using CRISPR/Cas9. A) Insertion of complementing or transgenic genes into the *SNR1* locus by CRISPR/Cas9 mediated site-specific integration. Two possible orientations of the inserted *DHFR*^{*} mini gene and the primers used for identification, F1/2 and R1/2 represent the priming sites of oligos used in diagnostic PCRs. B) Diagnostic PCRs of one *snr1::DHFR*^{*} clone, RH is used as a wild-type control. PCR of the GRA1p is included as a control checking the quality of genomic DNA as templates. Asterisks indicate lanes with unspecific bands (Figure 5B is taken from ²).

Figure 6. Gene knockout using CRISPR/Cas9. A classic design for CRISPR/Cas9 mediated homologous gene replacement in *T. gondii*. The orientation of inserted

transgene is fixed in this case. F1/R1, F2/R2 and F3/R3 represent the priming sites of oligos used in diagnostic PCRs.

Data File 1. ME49-FUDR^r X VAND-SNF^r cross file for use in R/qtl, “csv” format. One .csv file that can be loaded into R/qtl with the format=“csv” option.

Data File 2 (chrid.txt, genotype.txt, markerpos.txt, mname.txt, phenonames.txt, and phenos.txt). ME49-FUDR^r X VAND-SNF^r cross files for use in R/qtl, “gary” format. Six .txt files that can be loaded in R/qtl with the format=“gary” option.

Data File 3. Spreadsheet with progeny SNPs across the SNF^r QTL locus. Contains one worksheet with the progeny SNPs, and a second worksheet with the phenotypic data of the parents and progeny of the cross.

Supplemental File 1: Additional protocol details.

Supplemental File 2: Protocol 5 - Utilization of negative selection at the *SNR1* locus for genetic complementation or transgenic strain construction.

DISCUSSION

These protocols present several methods that when combined allow for the identification of a drug resistance gene in *T. gondii*. Two in particular were integral to the project, the relatively seasoned method of QTL mapping and the recently developed method of CRISPR/Cas9 gene editing. Lander and Botstein published their influential paper in 1989 demonstrating QTL mapping which correlates genetic loci with phenotypes³⁶. More recently in 2012, Dounda and Charpentier described the CRISPR/Cas9 editing system in *Streptococcus pyogenes*²² that was quickly adapted as a genetic tool in many different models, including *T. gondii*^{13,17}. Both methods were useful here, where QTL mapping defined the locus containing the drug resistance mutation that was ultimately identified using WGS based SNP detection, and CRISPR/Cas9 editing provided the means to confirm *SNR1* is the sinefungin drug resistance gene.

The ME49-FUDR^r X VAND-SNF^r cross⁸ was originally developed to interrogate a virulence phenotype¹⁹, but the parental strains also happened to have an additional phenotypic difference for which the causal gene was not known, sinefungin resistance in the VAND parent. This highlights one benefit of crosses in that they can be repurposed when additional phenotypic differences in the parents are observed. This was the case for another *Toxoplasma* cross, type 2 X type 3, where several genes involved in virulence were found by mapping multiple phenotypes³⁷⁻⁴⁰. To date, four different *T. gondii* crosses have been described and used to map genes responsible for phenotypes^{8,37,41,42}, all of which have the potential be reused to map new phenotypes for which the genetic basis is unknown. Along these lines, the genomes for 62 *T. gondii* strains representing the known global genetic diversity have been sequenced⁴³. New crosses could be made from this pool for strains that differ in interesting phenotypes. Having extolled the advantages of QTL mapping, it needs to be said that generating a

cross isn't a minor undertaking. There are other methods that can be used to identify causal genes. One powerful technique uses chemical mutagenesis to create mutants that can be screened for phenotypes. To find the causal gene, mutants can either be complemented with cosmid libraries⁴⁴ or genome resequencing methods can be used to find the causal mutation⁴⁵. For more on this, see two JoVE articles by Coleman *et al.* and Walwyn *et al.* that outline these approaches^{46,47}.

Many of the steps leading to the identification of the causal SNF^r mutation (Protocols 2 and 3) rely on computational methods conducted with software that is freely available for academic use. Detailed commands for each step are provided and when run with the proper files will allow the user to recreate the datasets necessary to find the causal SNF^r SNP. Keep in mind that some of the syntax in the commands refer to filenames or directory structure (\$PATH) that can be modified to the user preference. Although the commands given here certainly do not exhaust the ways one can analyze a cross with QTL analysis and WGS based SNP identification, they are comprehensive enough to repeat the experiment described in this article and should allow the user to become more familiar with how these approaches are utilized in a step by step fashion.

Although QTL mapping and WGS sequence based SNP detection was sufficient to identify a candidate SNF^r gene, additional experiments are needed to confirm its role in drug resistance. This can be convincingly shown through gene disruption or knockout techniques, both of which can be achieved using CRISPR/Cas9 gene editing. Detailed methods for using CRISPR/Cas9 to either generate indel mutations or insert transgenic constructs into a target gene in *T. gondii* are given. The targeting specificity which CRISPR/Cas9 provides increases the efficiency of gene editing over traditional methods. Also, this increased efficiency has made it possible to use non-laboratory adapted strains for genetic studies which were previously difficult to modify¹³. Even though in its infancy, CRISPR/Cas9 has already been used to make gene disruptions^{2,13,17,18,48,49}, tag genes¹⁷, and make gene knockouts^{16,19,50-54} in *T. gondii*, promising to be a useful tool for many other studies in the future.

The discovery that *SNR1* inactivation leads to sinefungin resistance makes the *SNR1* locus a promising site for transgene insertion or genetic complementation. To maximize the success of using CRISPR/Cas9 mediated gene targeting to direct the integration of transgene into the *SNR1* locus, the following aspects should be considered during the experimental design. First, a selection marker is recommended to be included in the transgenic construct to increase the efficiency of strain construction. If a selection marker is included, both positive and negative selection can be used, resulting in almost 100% of the doubly selected parasites being transgenic with the GOI integrated at the *SNR1* locus. In contrast, if the transgenic construct does not contain additional selectable traits and relies on negative selection at the *SNR1* locus, the efficiency of successful transgenesis largely depends on the efficiency of co-transfection of the CRISPR plasmid and the transgenic construct.

Second, although CRISPR/Cas9 mediated site-specific integration of a non-homologous DNA fragment is frequently used for complementation and transgenesis, it should be

noted that the orientation of insertion cannot be guaranteed in such cases as either direction is possible. This may pose problems to some applications. For example, to complement a mutant with different gene alleles, it is difficult to ensure that all alleles are inserted in the same orientation, and discrepancies in orientation may cause expression differences. For this type of application, DNA constructs with sequences homologous to the *SNR1* locus are recommended, which will drive the proper integration orientation (Figure 6).

Third, during transfection, the ratio between the CRISPR plasmid and the transgenic DNA molecule is critical for successful transgenic strain construction. This ratio needs to be adjusted according to the selection strategies. The following guidelines are recommended: 1) If the transgenic construct contains a drug resistant marker and the corresponding drug is the only selection used to generate transgenic parasites, i.e. sinefungin is not used, the suggested molar ratio between the transgenic construct and the CRISPR plasmid is 1:5. Using more CRISPR plasmid in this case maximizes the likelihood parasites that receive the transgenic construct will also receive the CRISPR plasmid, therefore the drug resistant parasites are more likely to have the marker inserted at the CRISPR targeting site. If the ratio is reversed, most parasites that receive the transgenic construct won't get the CRISPR plasmid. As a consequence, the vast majority of drug resistant parasites obtain the transgenic construct through random integration not associated with CRISPR/CAS9 mediated site-specific insertion. 2) If the transgenic construct contains a drug resistant marker and the corresponding drug is used along with sinefungin to select transgenic parasites, the suggested molar ratio between the transgenic construct and the CRISPR plasmid is 1:1. This strategy provides the highest efficiency of transgenic strain construction. 3) If the transgenic construct does not contain a selectable marker, relying on negative selection by sinefungin alone to obtain transgenic parasites, the suggested molar ratio between the transgenic construct and the CRISPR plasmid is 5:1. The rationale for this design is the same as in the first guideline above. Since both pyrimethamine and sinefungin were used for selection in Protocol 5, the ratio between *DHFR** mini gene and the *SNR1* targeting CRISPR plasmid was set as 1:1.

Taken together, the methods outlined here have a level of detail that was not possible to convey in the original publication that identified *SNR1*². These protocols; specifically, the command line syntax, sequential layout of the programs utilized, and the use of CRISPR/Cas9 should aid future endeavors to identify new genes responsible for phenotypes.

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DISCLOSURES

The authors declare that they have no competing financial interests.

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Figure 1

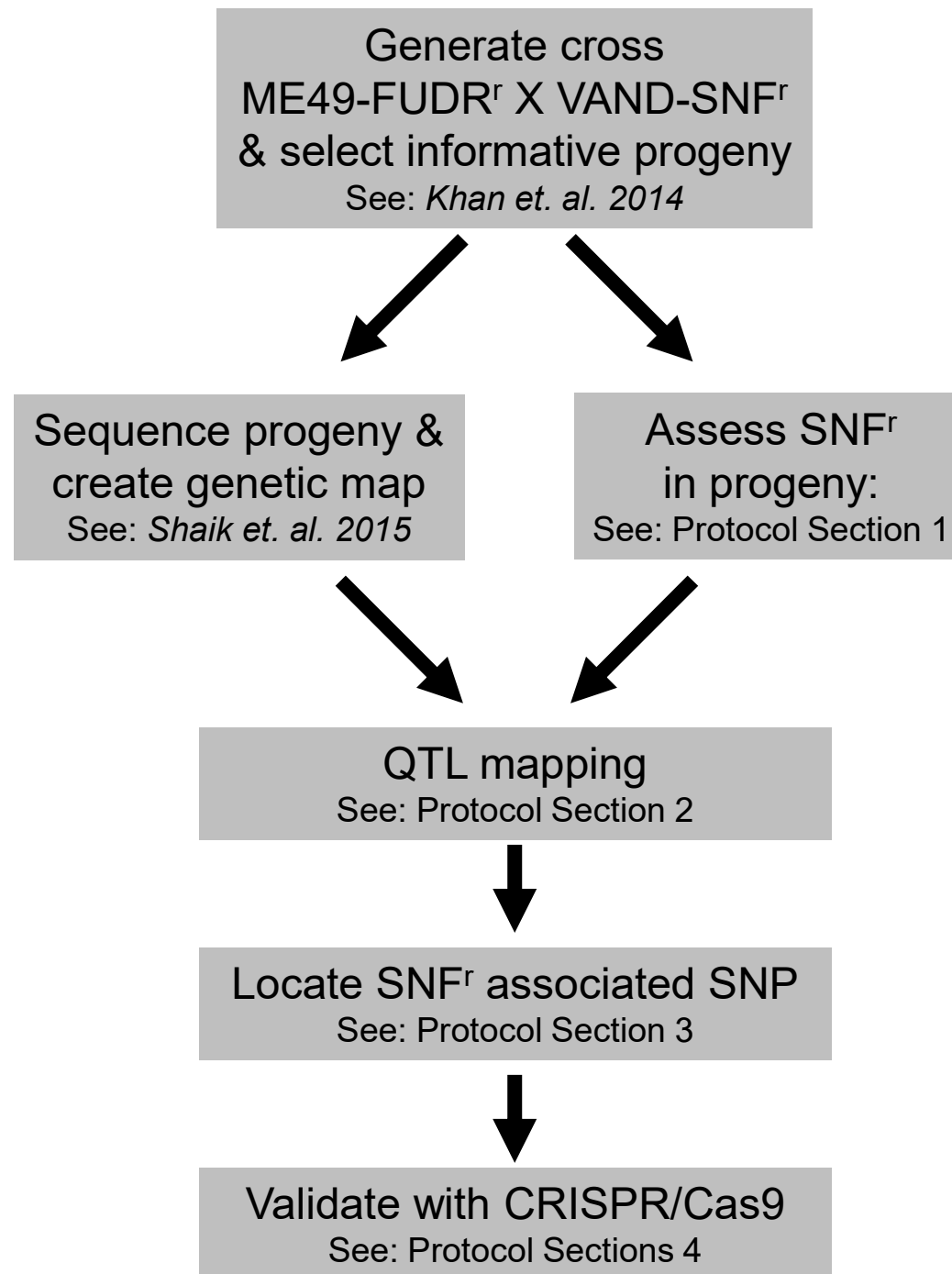


Figure 2

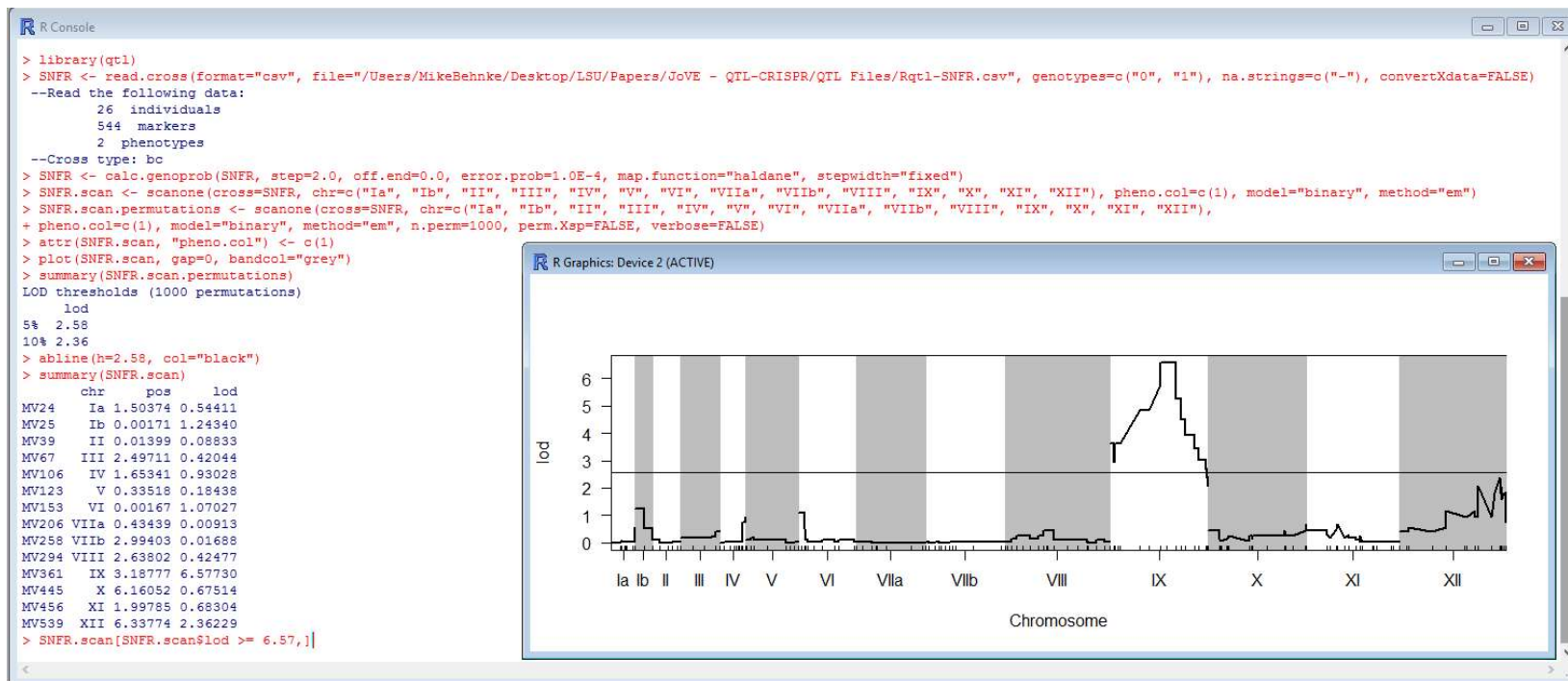
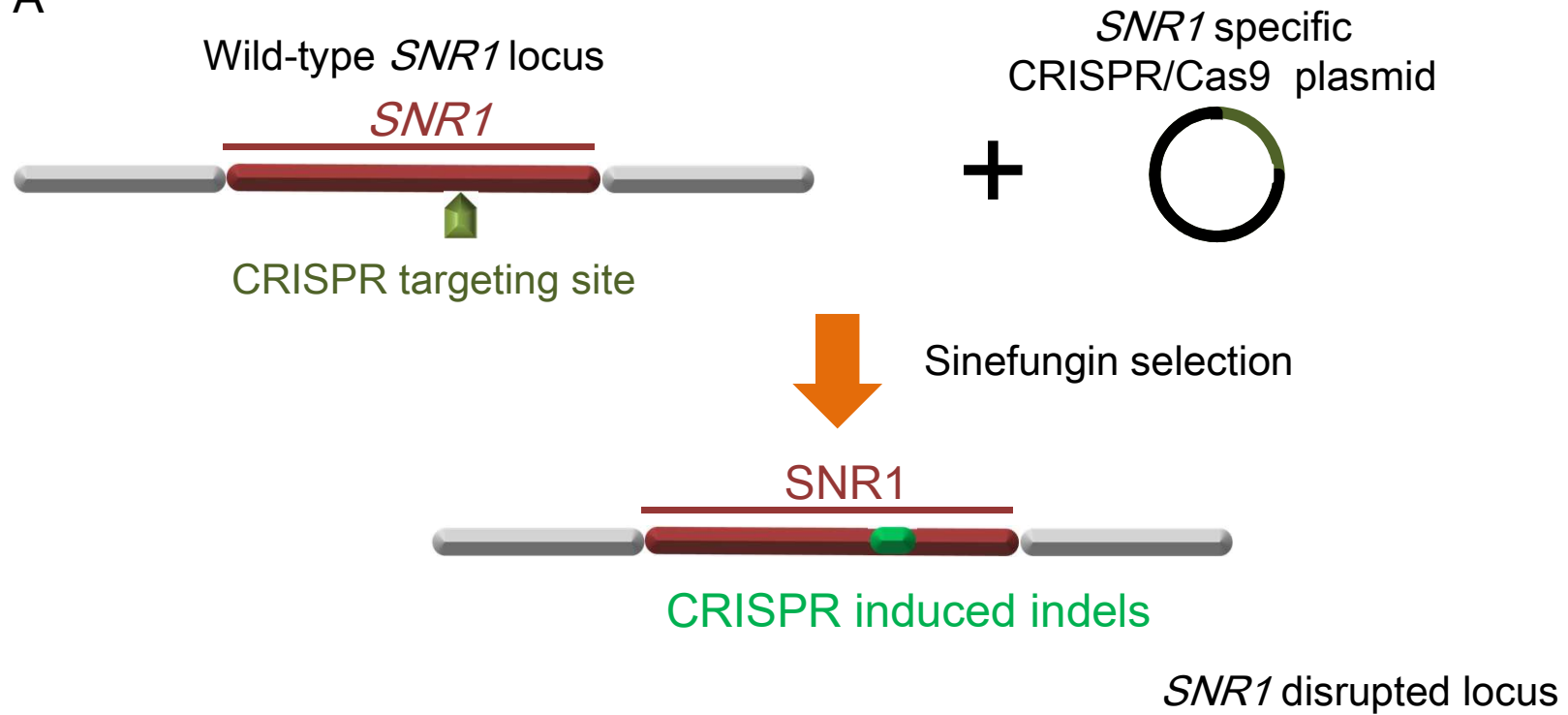


Figure 3

Contig	Pos	Ref	Var	P1 15VB	P1 14VB	P1 24VB	P2 29VBSF	P1 18VBSF	P1 39VBSF	P1 45VB	P1 49VBSF	P1 65VBSF	P1 80VBSF	P1 43VB	P1 3VBSF	P1 25VB	P1 28VB	P1 74VBSF	P1 86VBSF	P1 94VBSF	P2 16VBSF	P2 63VB	P1 18VB	P1 16VB	P1 39VB	P2 39VB	P1 44VB
KN042501.1	352665	G	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	A	A	A	A	A
KN042501.1	352600	T	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C	C	C	C	C	C
KN042501.1	352364	A	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	T	T	T	T	T
KN042501.1	352246	C	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	A	A	A	A	A
KN042501.1	352235	G	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	N	N	N	N	N	N
KN042501.1	352217	T	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	G	G	G	G	G
KN042501.1	352194	C	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	G	G	G	G	G
KN042501.1	352177	A	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	G	G	G	G	G
KN042501.1	351399	A	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	N	-	-	G	G	G	G	G	G
KN042501.1	351377	A	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	N	N	N	N	N
KN042501.1	351375	G	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C	N	N	C	N	N
KN042501.1	351358	T	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	G	G	G	G	G
KN042501.1	351326	C	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	G	G	G	G	G
KN042501.1	351105	A	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C	C	C	C	C	C
KN042501.1	350892	T	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C	N	C	C	N	N
KN042501.1	350870	A	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	G	G	G	G	N
KN042501.1	350524	G	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	A	A	A	A	A
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KN042501.1	346529	A	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	G	G	G	G	G
KN042501.1	346319	C	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	A	A	A	A	A
KN042501.1	346316	A	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C	C	C	C	C	C
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KN042501.1	345137	A	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	G	G	G	G	G
KN042501.1	344942	A	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	G	G	G	G	G
KN042501.1	344889	T	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C	C	C	C	C	C

Figure 4

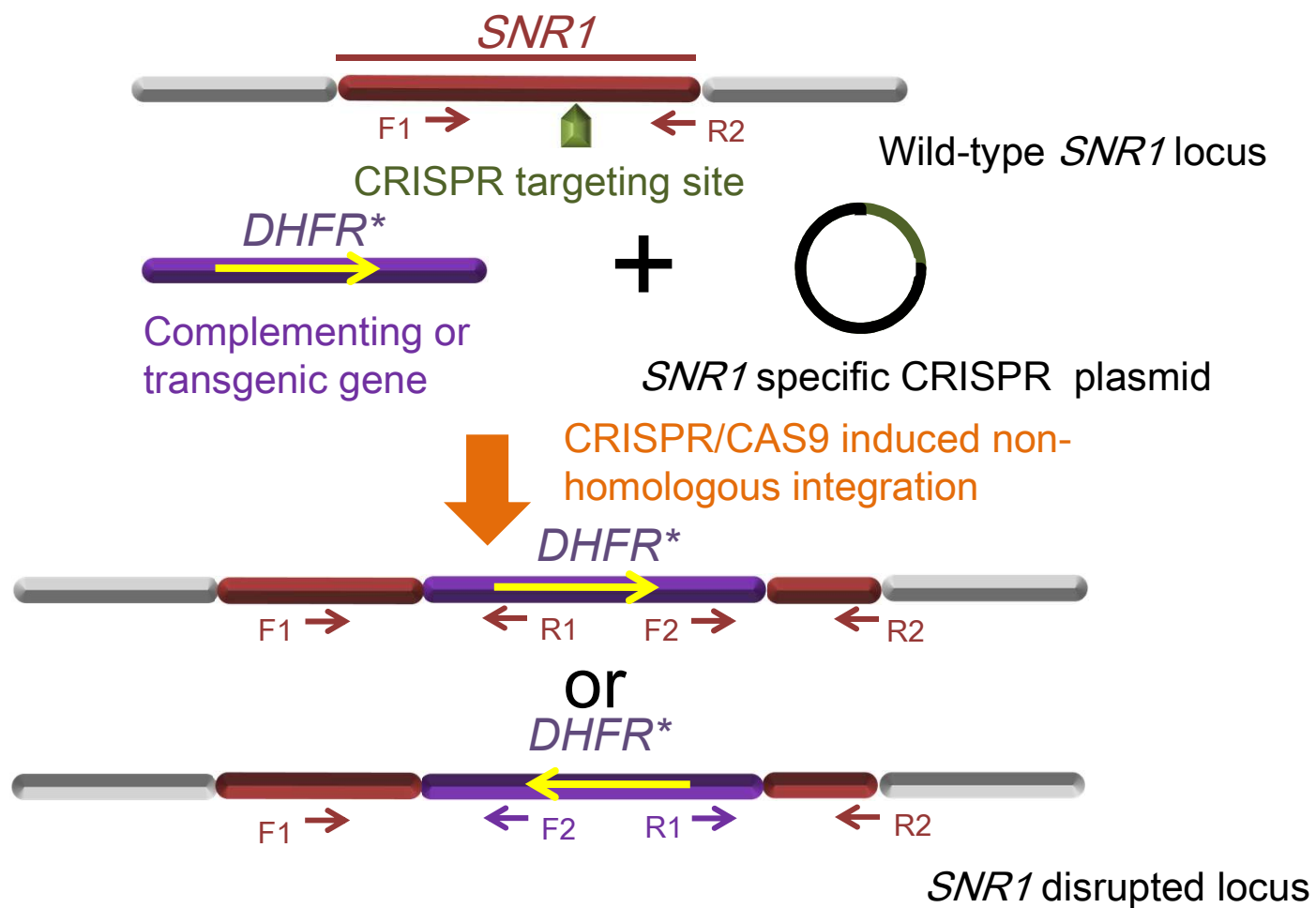
A



B

			PAM				PAM
RH	1722	AGCGAT-GTGTGG	1733	RH	1507	GCAGACGT--TTTTGG	1520
C6-1		AGCGATTGTGTGG		C5-1		GCAGACGTGGTTTTGG	
C6-2		AGCGATTGTGTGG		C5-2		GCAGAC----TTTTGG	
		*****	*****			*****	*****

Figure 5 A



B

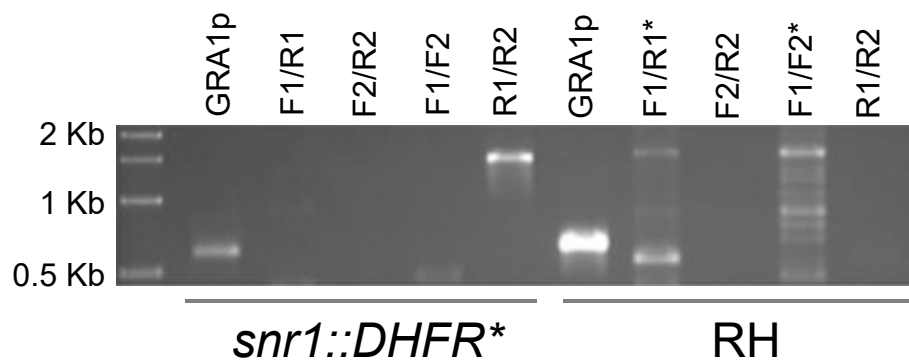
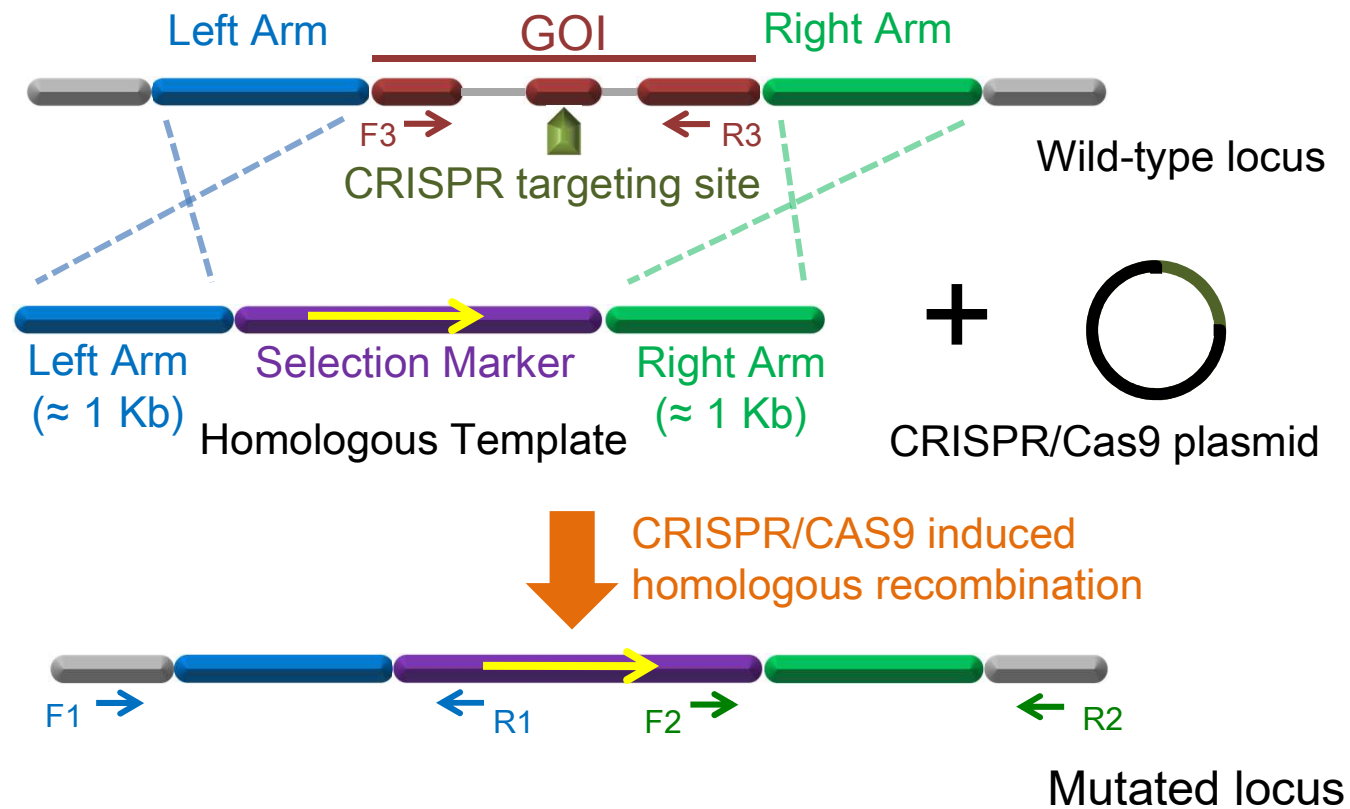


Figure 6



Name of Reagent/ Equipment	Company
T25 flasks	Corning
HFF	ATCC
<i>T. gondii</i> ME49 strain	ATCC
<i>T. gondii</i> VAND strain	ATCC
DMEM (No Sodium Bicarbonate)	Life Sciences
Sodium Bicarbonate	Sigma Aldrich
HEPES	Sigma Aldrich
Fetal Bovine Serum Premium Grade	VWR International
L-Glutamine 200mM	Sigma Aldrich
Gentamicin (10 mg/mL)	Life Technologies
Cell Scraper for Flasks	VWR International
Syringe 10ml	BD
Blunt needles 22g x 1"	BRICO Products
Nuclepore Filter 3.0 µm, 25mm	GE Healthcare
Swin-Lok Filter Holder 25mm	GE Healthcare
Hemocytometer	Propper MFG
Sinefungin	Enzo Life Sciences
R	The R Foundation
J/qtl	The Churchill Group
Bowtie2	John Hopkins University
NCBI SRA Toolkit	NCBI
SAMtools	Wellcome Trust Sanger Institute
VarScan	Washington University, St Louis
MUMmer	JCVI & Univ Hamburg
pSAG1::CAS9-U6::sgUPRT	Addgene
pSAG1::CAS9-U6::sg290860-6	Addgene
pUPRT::DHFR-D	Addgene
Q5 Site-Directed Mutagenesis Kit	New England Biolabs
QIAprep Spin Miniprep Kit	QIAGEN
NanoDrop 2000	Thermo Scientific
LB Broth	Fisher Scientific
Potassium chloride	Sigma Aldrich
Calcium chloride	Sigma Aldrich
Potassium phosphate monobasic	Sigma Aldrich
Potassium phosphate dibasic	Sigma Aldrich
EDTA	Sigma Aldrich
Magnesium chloride	Sigma Aldrich
Adenosine triphosphate (ATP)	Sigma Aldrich
L-glutathione (GSH)	Sigma Aldrich
ECM 830 Electroporation System	BTX
Electroporation Cuvette 4 mm	Harvard Apparatus
Crytal Violet	Alfa Aesar
6-well TC plate	Corning
24-well TC plate	Corning
Cover glass 12mm round	VWR International
96-well TC plate	Corning
Gibson Assembly Cloning Kit (Multi-fragment)	New England Biolabs
Q5 High-Fidelity Polymerase	New England Biolabs
Pyrimethamine	TCI AMERICA

Catalog Number

430639

SCRC-1041

50840

PRA-344

12800017

S5761

H3375

97068-085

G7513

15710064

10062-904

309604

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110612

420200

90001

380-070-M001

<https://www.r-project.org/>

<http://churchill.jax.org/software/jqtl.shtml>

<http://bowtie-bio.sourceforge.net/bowtie2/index.shtml>

http://www.ncbi.nlm.nih.gov/Traces/sra/?view=toolkit_doc

<http://www.htslib.org/>

<http://varscan.sourceforge.net/>

<http://mummer.sourceforge.net/>

Plasmid #54467

Plasmid #59855

Plasmid #58528

E0552S

27106

ND-2000

DF0446-07-5

P9541

746495

P9791

P5504

E5134

M1028

A6419

G4251

45-0002

45-0126

B21932-14

353046

353935

89015-724

353075

E5510S

M0491S

P2037-1G

Comments/Description

50611

T. gondii CRISPR plasmid to cut the UPRT gene
T. gondii CRISPR plasmid to cut the TG*_290860 SNR1 gene
Template for *DHFR** mini gene

Use cuation when using pyrimethamine resistant parasites - see Protocol



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Author: Michael S. Behnke, Asis Khan, L. David Sibley et al.

Publication: Eukaryotic Cell

Publisher: American Society for Microbiology

Date: Feb 1, 2015

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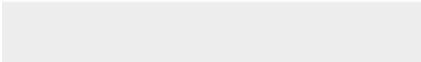
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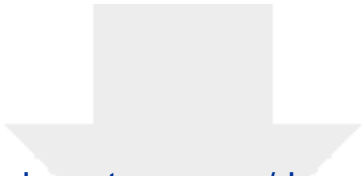
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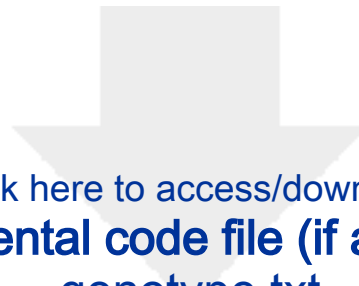




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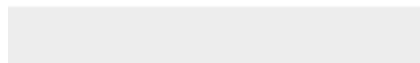
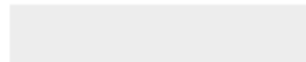
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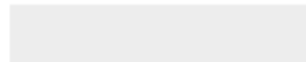
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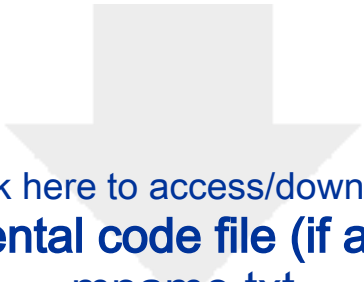




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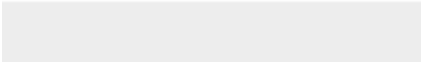
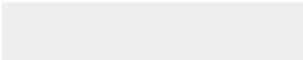
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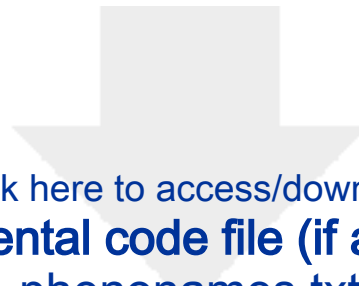




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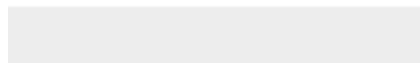
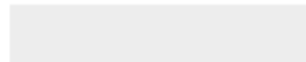
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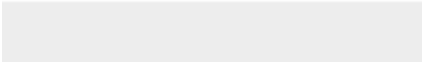

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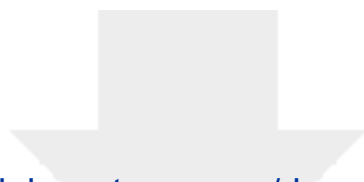




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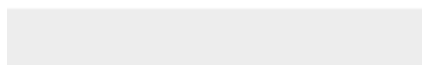
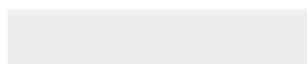
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