**Protocol Notes**

Protocol 1.1:

Note: General methods for culturing *T. gondii* in human foreskin fibroblast (HFF) cells in D10 medium have been described previously1. For 1 L of D10, mix 1 packet DMEM powder, 3.7 g sodium bicarbonate, 2.38 g HEPES, 100 mL FBS, 10 mL of L-Glutamine (200 mM), and 1 mL Gentamicin (10 mg/mL).

Protocol 1.8:

Note: Once all 24 progeny have been scored the data will be used as a binary phenotype for QTL mapping using R/qtl as discussed in the next section. One can use the parental strains as controls to test the effectiveness of the culture conditions, where the sinefungin sensitive Me49-FUDRr strain should not grow and the sinefungin resistant VAND-SNFr should grow when cultured with drug.

Protocol 2:

Note: The genetic map for the ME49-FUDRr X VAND-SNFr cross was created using the REDHORSE software suite that was designed and written for this purpose, please see J. S. Shaik *et al.* BMC Genomics 2015 2and A. Khan *et al.*, BMC Genomics 2014 3 for detailed information on both the map and the 24 progeny. REDHORSE uses the alignment of parental and progeny WGS to accurately determine the genomic locations of crossovers in the progeny that resulted from recombination of the parental strains during meiosis. These crossover locations are ideally suited for use as markers in a genetic map. The following steps combine the ME49-FUDRr X VAND-SNFr genetic mapand the SNFr phenotype assessed in the progeny (Protocol 1) to run a QTL scan using the qtl library in R.

Protocol 2.3:

Note: For more information on how to run R/qtl visit the website ([www.rqtl.org](http://www.rqtl.org)) for example datasets, manuals, and tutorials. There are several ways to format the genetic map/phenotype dataset for R/qtl, two are presented here. The “csv” format where all the data are in a single .csv file or the “gary” format where separate parts of the dataset are in individual tab delimited text files. Examples of both for this cross can be found in Data File 1 and Data File 2, respectively. Both sets contain data for two phenotypes, sinefungin resistance (SNFr) discussed here and virulence (VIR), which was assessed in another study 4. A full table of the phenotypes and progeny are in the second worksheet of Data File 3.

Protocol 2.13:

Note: The output from step 2.13 shows the SNF locus spans markers MV359 to MV366 located on chromosome IX between 3,187,537 to 4,202,258 bp. These markers define the boundary of the QTL locus for sinefungin resistance as they all have the same max LOD score, also shown on the plot from step 2.8 (Figure 2). This locus will serve as the region within which the inherited mutation that results in SNFr will be identified in Protocol 3. The VIR phenotype included in Data File 1 & 2 is provided as an additional example dataset that can be run in R/qtl. The virulence phenotype is the result of a different gene than *SNR1*, and thus maps to a different QTL 4.

Protocol 3:

Note: Although the SNFr locus has been identified by QTL mapping, the locus contains a number of open reading frames any of which could be the causal gene. The VAND strain was made resistant to SNF by chemical mutagenesis with N-ethyl-N-nitrosourea (ENU). Because ENU is a nucleobase alkylating agent which induces point mutations, the most likely explanation for the cause of resistance was the generation of a mutation at the nucleotide level such as a SNP in the drug resistance gene. This prompted the search for SNP(s) in the locus present only in the drug resistant progeny. To do this, WGS reads from all the progeny are individually aligned to the SNF sensitive VAND genome using Bowtie 2, SNPs are called from those alignments using VarScan, and the genomic region spanning the SNFr QTL locus is scanned for the expected SNP pattern.

Protocol 3.2:

Note: Protocol 3.1 generated individual indexed BAM files for all 24 progeny, each aligned to the VAND-SNFs reference genome. The next steps combine all 24 BAM files to call SNPs from those alignments. The variant caller program VarScan requires the alignment input to be in the SAMtools pileup format.

Protocol 3.3:

Note: Since the genetic map used for QTL mapping (Protocol 2) is based on the ME49 reference genome, the coordinates of the QTL markers correspond to the ME49 genome, not the VAND genome. Also, the VAND genome is not assembled into chromosome level supercontigs (*Toxoplasma* has 14 chromosomes), but into 2141 contigs; see the VAND reference GCA\_000224845.2\_TGVAND\_v2\_genomic.fna file. To determine the VAND contig(s) that correspond to the ME49 based QTL marker locations use the MUMmer nucmer program to align both ME49 and VAND genomes.

Protocol 3.4:

Note: The AllProgeny-SNPs.txt file from Protocol 3.2 contains progeny SNPs located across all 14 VADN-SNFs chromosomes. To find the mutation responsible for SNF resistance, only those SNPs within the QTL locus need to be analyzed. All SNPs falling within the QTL locus (VAND contig KN044604.1:657,441-1 bp and VAND contig KN042501.1:430,910-41,870 bp – Protocol 3.3) were extracted from the VarScan generated AllProgeny-SNPs.txt file. The data were then parsed to only include the contig name, position of SNP, reference base, variant base, and SNP calls for the 24 progeny. This data was then imported into a spreadsheet and SNPs were ordered as they occur along chromosome IX (Data File 3). To facilitate review of the SNP data, SNP Table 1 in Data File 3 was copied to SNP Table 2, and all positions with the reference base were changed to a “-“ and columns were color coded by the SNFr (yellow)or SNFs (green) phenotype (Data File 3). The data show that the SNFr progeny inherited the QTL locus from the VAND parent as for most positions they retain the reference base, *i.e.* a “-“.

Protocol 3.4.2:

Note: This position corresponds to chromosome IX:3901106 in the reference ME49 genome, which is located within a gene annotated as a putative amino acid transporter (TGME49\_290860), and results in a T → A substitution that creates an early stop codon in the coding sequence, see the ToxoDB.org gene page for TGME49\_290860. As this gene is the main SNFr candidate it has been designated *SNR1*, for sinefungin resistance gene 1.

Protocol 4.1:

Note: Six CRISPR plasmids that specifically target *SNR1* have been made previously 5 including pSAG1::CAS9-U6::sg290860-6 (see the Materials list for plasmid availability and map/sequence). Any one of these plasmids can be used for direct inactivation of *SNR1*. These plasmids were generated by replacing the gRNA sequence in the *UPRT* targeting CRISPR plasmid with *SNR1* specific gRNAs using the following generic protocol for locus specific CRISPR plasmid construction 6.

Protocol 4.1.2:

Note: Use genomic sequences, not coding sequences with introns spliced out, to design gRNAs. The default setting for many gRNA design programs (including E-CRISP) excludes non-exon hits. This setting should be preserved when designing gRNAs to inactivate genes by indel mutation or to direct the insertion of selectable markers by non-homologous integration. However, for other applications, this option may be adjusted. For example, to replace an intron containing gene with an epitope tagged or LoxP flanked version of the same gene without introns (coding sequence only), the CRISPR gRNAs need to target introns, otherwise the CRISPR system will not only target the endogenous gene of interest (GOI) but also the exon containing transfecting donor.

Protocol 4.2.2:

Note: Removal of the growth medium before parasite collection helps to get rid of parasites that are detached from the surface and floating in the medium because these parasites are of low viability.

Protocol 4.4.2.1:

Note: To achieve the best subcloning efficiency, infect each well with 2 - 3 parasites for type 1 strains or 3 - 5 parasites for other strain types. Such inoculation density often produces 30 - 40 single clones from each 96-well plate. Growth of single clones produces single plaques in the wells but the time required to produce visible plaques varies. Type 1 strains usually form visible plaques within 7 - 8 days whereas other strains need 8 - 10 days or even longer.

**References**

1 Roos, D. S. Molecular genetic tools for the identification and analysis of drug targets in Toxoplasma gondii. *Curr Top Microbiol Immunol.* **219** 247-259 (1996).

2 Shaik, J. S., Khan, A., Beverley, S. M. & Sibley, L. REDHORSE-REcombination and Double crossover detection in Haploid Organisms using next-geneRation SEquencing data. *BMC Genomics.* **16** (1), 133, doi:s12864-015-1309-7 [pii]

10.1186/s12864-015-1309-7, (2015).

3 Khan, A. *et al.* NextGen sequencing reveals short double crossovers contribute disproportionately to genetic diversity in Toxoplasma gondii. *BMC Genomics.* **15** (1), 1168, doi:1471-2164-15-1168 [pii]

10.1186/1471-2164-15-1168, (2014).

4 Behnke, M. S. *et al.* Rhoptry Proteins ROP5 and ROP18 Are Major Murine Virulence Factors in Genetically Divergent South American Strains of Toxoplasma gondii. *PLoS Genet.* **11** (8), e1005434, doi:10.1371/journal.pgen.1005434, (2015).

5 Behnke, M. S., Khan, A. & Sibley, L. D. Genetic Mapping Reveals that Sinefungin Resistance in Toxoplasma gondii Is Controlled by a Putative Amino Acid Transporter Locus That Can Be Used as a Negative Selectable Marker. *Eukaryot Cell.* **14** (2), 140-148, doi:EC.00229-14 [pii]

10.1128/EC.00229-14, (2015).

6 Shen, B., Brown, K. M., Lee, T. D. & Sibley, L. D. Efficient gene disruption in diverse strains of Toxoplasma gondii using CRISPR/CAS9. *MBio.* **5** (3), e01114-01114, doi:mBio.01114-14 [pii]

10.1128/mBio.01114-14, (2014).