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TITLE:

Determination of Chemical Inhibitor Efficiency against Intracellular *Toxoplasma gondii* Growth Using a Luciferase-Based Growth Assay

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KEYWORDS:

Toxoplasma gondii, CRISPR-Cas9, gene manipulation, luciferase, growth assay, inhibitor efficacy, parasite

SUMMARY:

Presented here is a protocol to evaluate the inhibition efficacy of chemical compounds against in vitro intracellular growth of *Toxoplasma gondii* using a luciferase-based growth assay. The technique is used to confirm inhibition specificity by genetic deletion of the corresponding target gene. The inhibition of LHVS against TgCPL protease is evaluated as an example.

ABSTRACT:

Toxoplasma gondii is a protozoan pathogen that widely affects the human population. The current antibiotics used for treating clinical toxoplasmosis are limited. In addition, they exhibit adverse side effects in certain groups of people. Therefore, discovery of novel therapeutics for clinical toxoplasmosis is imperative. The first step of novel antibiotic development is to identify chemical compounds showing high efficacy in inhibition of parasite growth using a high throughput screening strategy. As an obligate intracellular pathogen, *Toxoplasma* can only replicate within host cells, which prohibits the use of optical absorbance measurements as a quick indicator of growth. Presented here is a detailed protocol for a luciferase-based growth assay. As an example, this method is used to calculate the doubling time of wild-type *Toxoplasma* parasites and measure the efficacy of morpholinurea-leucyl-homophenyl-vinyl

sulfone phenyl (LHVS, a cysteine protease-targeting compound) regarding inhibition of parasite intracellular growth. Also described, is a CRISPR-Cas9-based gene deletion protocol in *Toxoplasma* using 50 bp homologous regions for homology-dependent recombination (HDR). By quantifying the inhibition efficacies of LHVS in wild-type and *TgCPL (Toxoplasma* cathepsin L-like protease)-deficient parasites, it is shown that LHVS inhibits wild-type parasite growth more efficiently than *Δcpl* growth, suggesting that TgCPL is a target that LHVS binds to in *Toxoplasma*. The high sensitivity and easy operation of this luciferase-based growth assay make it suitable for monitoring *Toxoplasma* proliferation and evaluating drug efficacy in a high throughput manner.

INTRODUCTION:

Toxoplasma gondii is a highly successful obligate intracellular parasite that infects approximately one-third of the human population. Its high transmission rate is predominantly due to its diverse routes of transmission, including consumption of undercooked meat, exposure to mammalian reservoirs, and congenital transmission during birth. *T. gondii* mainly causes opportunistic infections that can lead to severe morbidity and mortality in immunocompromised individuals[1-6]. The antibiotics currently used for treating acute toxoplasmosis are particularly inefficient in treating congenital and latent infections and cause severe reactions in some individuals[3,7,8]. Thus, an urgent need to identify novel therapeutics exists. Understanding the differences in subcellular processes within *Toxoplasma* and its host will help to identify potential drug targets. Therefore, efficient and convenient genome manipulation techniques are required to study the roles of individual genes within *Toxoplasma*. Additionally, *Toxoplasma* belongs to the phylum Apicomplexa, which includes several other significant human pathogens, such as *Plasmodium spp.* and *Cryptosporidium spp.* Hence, *Toxoplasma* can be used as a model organism to help study basic biology in other apicomplexan parasites.

To identify novel antibiotics against microbial pathogens, high throughput screening of a library of chemical compounds is initially performed to determine their efficacy in the repression of microbial growth. So far, several microplate-based growth assays have been developed for measuring intracellular growth *of T. gondii* (i.e., radioactive ^{3}H-uracil incorporation-based quantification[9], quantitative ELISA-based parasite detection using *T. gondii*-specific antibodies[10,11], reporter protein-based measurement using β-galactosidase or YFP-expressing *Toxoplasma* strains[12,13], and a recently developed high-content imaging assay[14]).

These individual strategies all have unique advantages; however, certain limitations also restrict their applications. For example, since *Toxoplasma* can only replicate within nucleated animal cells, autofluorescence and non-specific binding of anti-*T. gondii* antibodies to host cells cause interference in fluorescence-based measurements. Furthermore, usage of radioactive isotopes requires special safety compliance and potential safety issues. Some of these assays are more suitable for assessing growth at a single timepoint rather than continuous monitoring of growth.

Presented here is a luciferase-based protocol for the quantification of intracellular *Toxoplasma* growth. In a previous study, the NanoLuc luciferase gene was cloned under the *Toxoplasma* tubulin promoter, and this luciferase expression construct was transfected into wild-type (RHΔ*ku80*Δ*hxg* strain) parasites to create an RHΔ*ku80*Δ*hxg*::*NLuc* strain (referred to as RHΔ*ku80*::*NLuc* hereafter)[15]. This strain served as the parental strain for intracellular growth determination and gene deletion in this study. Using the RHΔ*ku80*::*NLuc* strain, parasite growth in human foreskin fibroblasts (HFFs) was monitored over a 96 h period post-infection to calculate parasite doubling time.

In addition, the inhibition efficacy of LHVS against parasite growth can be determined by plotting *Toxoplasma* growth rates against serial LHVS concentrations to identify the IC_{50} value. Previous literature has reported that TgCPL is a major target of LHVS in parasites and that treatment with LHVS decreases the development of acute and chronic *Toxoplasma* infections[16–19]. Additionally, RHΔ*ku80*::*NLuc* were used as the parental strain for genome modification to generate a *TgCPL*-deficient strain (RHΔ*ku80*Δ*cpl*::*NLuc*), and the inhibition of LHVS was measured against this mutant. By observing an upshift of IC_{50} values for LHVS in the *TgCPL*-deficient parasites compared to the WT strain, it was validated that TgCPL is targeted by LHVS in vivo.

In this protocol, RHΔ*ku80*::*NLuc* is used as the parental strain, which lacks an efficient non-homologous end-joining pathway (NHEJ), thereby facilitating double crossover homology-dependent recombination (HDR)[20,21]. Additionally, 50 bp homologous regions are flanked at both ends of a drug resistance cassette by PCR. The PCR product serves as a repair template to remove the entire gene locus via HDR using CRISPR-Cas9-based genome editing tools. Such short homologous regions can be easily incorporated into primers, providing a convenient strategy for production of the repair template. This protocol can be modified to perform universal gene deletion and endogenous gene tagging.

For instance, in our most recent publication, three protease genes, *TgCPL*, *TgCPB* (*Toxoplasma* cathepsin B-like protease), and *TgSUB1* (*Toxoplasma* subtilisin-like protease 1), were genetically ablated in *TgCRT* (*Toxoplasma* chloroquine-resistance transporter)-deficient parasites using this method[15]. Additionally, *TgAMN* (a putative aminopeptidase N [TgAMN, TGGT1_221310]) was endogenously tagged[15]. The Lourido lab also reported using short homologous regions in the range of 40–43 bp for the introduction of site-directed gene mutation and endogenous gene tagging in the *Toxoplasma* genome using a similar method[22]. These successful genome modifications suggest that a 40–50 bp homologous region is sufficient for efficient DNA recombination in the *TgKU80*-deficient strain, which greatly simplifies genome manipulation in *Toxoplasma gondii*.

PROTOCOL:

Toxoplasma gondii is categorized in Risk Group 2 and must be handled at a Biosafety Level 2 (BSL-2). The protocol has been reviewed and approved by the Institutional Biosafety Committee at Clemson University.

1. Luciferase-based *Toxoplasma* growth assay

1.1. Seed human foreskin fibroblasts (HFFs) 1 week before parasite inoculation to ensure that host cells are fully confluent. Perform a mock assay in a transparent plate to ensure that parasites remain intracellular throughout the evaluation period.

NOTE: Here, the assay is conducted in 96 well microplates. According to experimental needs, it can be scaled up to 384 or 1536 well microplates.

1.2. Pass *Toxoplasma* parasites into confluent HFFs 2 days prior to use by transferring ~0.3–0.4 mL of fully lysed parasites into a T25 flask. Incubate infected host cells at 37 °C with 5% CO_2 for 2 days.

1.3. Syringe 5 mL of freshly lysed parasites through a 21 G safety needle 5x to liberate intracellular parasites, then pass through a 3 µm filter to remove host cell debris. Rinse residual parasites out of the flask using 7 mL of phenol red-free D10 medium, then pass through the filter again.

1.4. Centrifuge parasites at 1000 *x g* for 10 min at room temperature (RT). Pour off the supernatant and resuspend in 10 mL of phenol red-free D10 media.

1.5. Count parasites using a hemocytometer to determine the concentration.

1.6. Dilute parasites to 1 x 10^4 parasites/mL for the wild-type (WT) strain. For growth-deficient parasite strains, increase the concentration accordingly to observe a significant increase in luciferase signals.

1.7. Aspirate media carefully from 96 well microplates pre-seeded with HFFs and inoculate 150 µL of parasite resuspension into wells in a format of three columns and five rows, which represents three technical replicates and five timepoints.

1.8. Incubate the microplate at 37 °C and 5% CO_2 for 4 h.

1.9. Aspirate media carefully from the wells to remove non-invaded parasites, then fill the wells with RT phenol red-free media in each row (except for the first row).

1.10. Mix equal volumes of PBS and 2x luciferase assay buffer and dilute the luciferase substrate to 12.5 µM.

1.11. Add 100 µL of dilute luciferase substrate into each well of the top row. Incubate the microplates at RT for 10 min to allow the cells to fully lyse.

1.12. Measure the luciferase activity using a microplate reader. The plate reader settings are listed in **Table 1**. Each reading represents the initial number of invaded parasites at 4 h post-infection.

1.13. Repeat steps 1.9–1.12 for each row every 24 h for 4 days without changing the medium. These readings reflect the total number of replicated parasites at 24 h, 48 h, 72 h, and 96 h post-infection.

1.14 Calculate the average readings at each timepoint and divide them by the average readings at 4 h to determine the fold changes in parasite growth over time.

1.15 Plot the data using graphing software. A representative growth reading table and plots of RHΔ*ku80*::*NLuc* parasites are shown in **Figure 1A,B**.

1.16 To calculate doubling time, plot the log2 values of fold changes at the individual timepoints over the incubation time. Use a linear regression function to calculate slope, which represents the doubling time of each strain (**Figure 1A,C**).

2. Evaluation of chemical compound inhibition efficacy against *Toxoplasma* growth

NOTE: Here, evaluation of the inhibition of LHVS in *Toxoplasma* growth is presented as an example. Eight different concentrations of LHVS are tested, and three technical replicates are performed for each of the three biological replicates for both RHΔ*ku80::NLuc* and RHΔ*ku80*Δ*cpl::NLuc* strains.

2.1. Prior to the parasite infection, seed HFFs to 96 well microplates in the format of three rows and nine columns for one biological replicate per compound per strain. Host cells will be allowed to grow for at least 7 days before use.

2.2. Pass RHΔ*ku80*::*NLuc* and RHΔ*ku80Δcpl*::*NLuc* parasites for 2 days prior to use. Follow steps 1.2–1.6 for parasite purification and quantification. Resuspend parasites in phenol red-free media at 1 x 10^4 parasites/mL.

2.3. Aspirate media from a plate of confluent HFFs and inoculate each well with 150 µL of parasite resuspension. Incubate the microplate at 37 °C and 5% CO_2 for 4 h.

2.4. Prepare LHVS at eight different concentrations in a 12 well reservoir by serial dilution. Generally, the concentrations are decreased by three-fold in a serial dilution manner.

NOTE: The lowest concentration is reduced by 6,561-fold relative to the highest concentration. The fold change of the dilution can be adjusted accordingly based on different properties of individual compounds.

2.5. At 4 h post-infection, aspirate media to remove non-invaded parasites and fill each well from columns 2–9 with 150 µL of media supplemented with LHVS at different concentrations. Leave the first column filled with regular medium to serve as a nontreated control.

2.6. Incubate the microplate at 37 °C and 5% CO_2 for an additional 96 h.

2.7. Perform steps 1.9–1.11 and measure luciferase activity of individual wells.

2.8. Average the luciferase activities of three technical replicates from wells of each individual LHVS concentration.

2.9. Divide the average luciferase activity for each LHVS concentration by the average luciferase activity derived from nontreated parasites to calculate the normalized luciferase activity as a percentage.

2.10. Plot the normalized luciferase activities against the individual LHVS concentrations using graphing software (**Figure 2**). Inhibition of pyrimethamine against parasite growth is also measured as a control. Pyrimethamine is a clinical antibiotic used to treat acute toxoplasmosis by inhibiting folic acid metabolism in *Toxoplasma*.

2.11. Calculate the IC_{50} values for individual compounds using the embedded method in the graphing software, normalized response vs. [inhibitor], under the "dose-response-inhibition" regression program. The IC_{50} is calculated using the following formula:

$$Y = 100/(1 + X/IC_{50})$$

Where: Y represents normalized luciferase activities of infected cells under different concentrations of inhibitor, and X represents individual concentrations of inhibitor.

3. CRISPR-Cas9-based gene deletion in *Toxoplasma* parasites

3.1. Generation of a plasmid construct expressing guide RNA (sgRNA) and Cas9 for deleting a gene of interest

3.1.1. Go to <www.ToxoDB.org> and retrieve the entire gene coding sequence, including introns and exons, along with 1.5 kb 5'-UTRs and 3'-UTRs (untranslated regions).

NOTE: Here, *TgCPL* (TGGT1_321530) is targeted as a representative example.

3.1.2. Copy the retrieved *TgCPL* sequence into the sequence analysis software (refer to **Table of Materials** for the name and version) and label the 5'- and 3'-UTR regions.

3.1.3. Select the **Tools** icon in the top menu bar, then select **Cloning** | **Find CRISPR Sites**.

3.1.4. Choose **3'(Cas9)'** for the PAM site location and select the folder containing the *Toxoplasma* genome sequence in the specificity scoring section. Leave the rest of the settings as defaults.

3.1.5. Choose a sgRNA with the following two criteria: 1) showing a high specificity score, generally >98%, and 2) lacking a G following the NGG, a protospacer adjacent motif (PAM) sequence. The selected sgRNA is usually located at sites close to the start and stop codons of the gene of interest.

3.1.6. Copy the sequence of the selected sgRNA and paste it into the following primer template.

sgRNA.TgCPL.F: GTCGTCCTCGCCGTTGAGGAGTTTTAGAGCTAGAAATAGC
sgRNA.R: AACTTGACATCCCCATTTACCAGAAGGCAAACACC

The portion in red represents the selected *TgCPL* sgRNA sequence. It can be replaced with different sgRNAs for various genes of interest.

3.1.6.1. If the selected sgRNA does not start with G, add G at the beginning of the sgRNA to help enhance its expression.

3.1.7 Perform a PCR reaction to modify the pre-existing plasmid expressing sgRNA (**Figure 3A**) that targets *Toxoplasma* uracil phosphoribosyltransferase (*TgUPRT*) gene[23] using a PCR premix with the settings provided in **Table 2**.

3.1.8 Run the PCR product on an agarose gel to confirm successful amplification. A 10 kb PCR product is expected to be amplified (**Figure 3B**).

3.1.9 Extract the PCR product using a DNA gel extraction kit and circularize it using a site-directed mutagenesis kit. Refer to **Table 3** for the recipe. Incubate the reaction for 10–20 min at RT.

3.1.10 Transform the circularized PCR product into *E. coli* and pick 10 clones for further verification of incorporation of designed sgRNA.

3.1.11 Grow two clones and extract plasmids. Cut the purified plasmids with BamHI and EcoRV. The candidate plasmids will yield two bands at 2.4 kb and 7.2 kb (**Figure 3C**).

3.1.12 Send the plasmids for Sanger sequencing using M13 reverse primers to confirm successful replacement of *TgUPRT* sgRNA with the designed sgRNA (**Figure 3D**).

3.2 Generation of repair template for gene deletion via HDR mechanism

3.2.1 According to the targeting sites of the selected sgRNA, locate 50 bp of 5'-UTRs or 3'-UTRs of the target gene for homology-dependent recombination (HDR, see discussion section). The

selection of regions follows the criteria listed below, depending on the location of the sgRNA targets.

3.2.1.1. If the cleavage site by Cas9 is located upstream from the start codon, select the following: a 50 bp DNA sequence upstream from the cleavage site as the left HDR region, and a 50 bp DNA sequence downstream from the stop codon as the right HDR region.

3.2.1.2. If the cleavage site by Cas9 is between the start and stop codons, select the following: a 50 bp DNA sequence upstream from the start codon as the left HDR region, and a 50 bp DNA sequence downstream from the stop codon as the right HDR region.

3.2.1.3. If the cleavage site by Cas9 is located downstream from the stop codon, select the following: a 50 bp DNA sequence upstream from the start codon as the left HDR region, and a 50 bp DNA sequence downstream from the cleavage site as the right HDR region.

NOTE: For the *TgCPL* gene, the cleavage site is located between the start and stop codons. Thus, the following primers are designed for amplifying the repair template using pMDC64 as the template, which encodes a pyrimethamine resistance cassette. The sequences in black anneal to the pMDC64 plasmid for PCR amplification. The regions labeled in red are *TgCPL*-specific sequences for homologous recombination.

TgCPLKO.DHFR.F:
GAGTTTTGACCGTTTGTCCCTCTTACCCCGCGGCCTCGCTCTCGAACACCGTCCGCGTTCGTGAAATTCTC
TgCPLKO.DHFR.R:
CGTCTTCCTTCTCACTTCCTTCCACGAACTTCCCGAAAGCCACGGTCGCGCGCTCTAGAACTAGTGGATC

3.2.2. Perform PCR using a PCR premix under the PCR conditions described in **Table 4**.

3.2.3. Run the PCR product on an agarose gel (**Figure 3E**), followed by gel extraction and standard nucleic acid quantification procedures.

NOTE: If the expected band cannot be successfully amplified, optimize PCR conditions and/or switch primer pairs.

3.3. *Toxoplasma* transfection

3.3.1. Pass RHΔ*ku80*::*NLuc* parasites for 2 days in a T25 flask containing confluent HFFs. A T25 flask of fully lysed parasites is sufficient for two to three transfections.

3.3.2. Syringe and filter-purify parasites as described in step 1.2. Resuspend parasites in cytomix buffer and spin down at 1,000 *x g* for 10 min at RT.

3.3.3. Wash pelleted parasites with 10 mL of the buffer and spin down the parasites at 1,000 *x g* for 10 min at RT.

3.3.4. Carefully pour off the supernatant and resuspend the parasites in the same buffer at a concentration of 1 x 10^8 parasites/mL.

3.3.5. Mix 2 µg of repair template DNA with 20 µg of the sgRNA/Cas9 expression plasmids (mass ratio = 1:5, equivalent to a 1:3 molar ratio). If the amplification yield of repair template is low, reduce the input of both DNA pieces accordingly. A minimum of 0.5 µg of repair template can be used.

3.3.6. Mix 400 µL of parasite resuspension, DNA, and 5 µL of 200 mM ATP/500 mM reduced glutathione (GSH) in a 1.5 mL centrifuge tube. Bring the total volume to 500 µL with cytomix buffer, if needed.

3.3.7. Transfer the mixture of parasites and DNA to an electroporation cuvette (4 mm gap width) and perform electroporation (2 kV voltage, 50 Ω resistance) using an electroporation apparatus.

3.3.8. Transfer electroporated parasites to a T25 flask containing confluent HFFs in fresh D10 medium. Apply appropriate antibiotic for drug selection after 24 h.

3.3.9. Keep under drug selective pressure until the growth of the transgenic parasites is stable.

3.3.10. Purify genomic DNA from the knockout population and check for integration of the pyrimethamine resistance cassette into the *TgCPL* locus by PCR. If verified, proceed to section 3.4. If not, perform another round of parasite transfection and drug selection. Inability to detect the correct integration of the drug resistance cassette usually suggests that the target gene is essential or that the gene locus is not accessible.

3.4. Cloning of knockout parasites

3.4.1. Seed two 96 well microplates with HFF cells and incubate at 37 ˚C and 5% CO_2 for 1 week prior to cloning parasites.

3.4.2. Pass ~0.3–0.4 mL of the population of transgenic parasites in a T25 flask containing confluent HFFs and grow them for 2 days. Consider passing more parasites if the mutant shows growth defects.

NOTE: To achieve the best yield and viability, the host cells are heavily infected by the parasites, and most of the parasites are kept in the intracellular stage.

3.4.3. Syringe infected host cells and filter-purify freshly lysed parasites as mentioned in step 1.3. Resuspend the parasites in D10 medium and spin them down at 1,000 x *g* for 10 min at RT.

3.4.4. Resuspend the pelleted parasites in 10 mL of D10 medium.

3.4.5. Count parasites using a hemocytometer to determine the parasite concentration.

3.4.6. Conduct a two-step dilution to bring the concentration to 10 parasites/mL in D10 medium supplemented with the appropriate antibiotic. Usually, the initial parasite resuspension is diluted by 1,000-fold, followed by a second dilution to 10 parasites/mL.

3.4.7. Aspirate media from 96 well microplates containing confluent HFFs and inoculate 150 µL of diluted parasites into each well.

3.4.8. Incubate plates at 37 ˚C with 5% CO_2 for 7 days without disturbance to allow plaque formation. The incubation period can be longer if transgenic parasites exhibit growth defects.

3.4.9. Screen the plates using a phase-contrast microscope and mark only the wells containing a single plaque.

3.4.10. Perform colony PCR to identify correct clones.

3.4.10.1. Use pipette tips to scrape the bottom of each well to lift infected HFF monolayers.

3.4.10.2. Pipet 75 µL of the cell resuspension from each marked well into 1.5 mL microcentrifuge tubes.

3.4.10.3. Centrifuge tubes for 10 min at maximum speed at RT. Carefully aspirate the supernatant and resuspend the pellet in 10.25 µL of lysis buffer containing dilution buffer and DNA release additive provided in the kit (**Table of Materials**).

3.4.10.4. Incubate the samples for 4 min at RT, then 2 min at 98 ˚C. Afterward, samples can be used for PCR or stored at -20 ˚C until use. Three sets of PCR reactions are used to test for the integration of the drug resistance cassette and loss of the gene of interest (**Figure 4A**). Refer to **Table 5** for PCR reaction setup and **Table 6** for thermocycler settings.

3.4.11. Identify the correct clones and transfer four clones into T25 flasks containing confluent HFFs.

3.4.12. After individual clones lyse host cells, purify genomic DNA for further PCR verification.

3.4.13. If an antibody recognizing the protein of interest is available, follow a standard immunoblotting procedure to verify loss of the target protein in the correct *Toxoplasma* knockouts. Representative images for screening a *TgCPL*-deletion mutant are shown in **Figure 4B,C**.

REPRESENTATIVE RESULTS:

Figure 1 represents an example of a growth curve for the RHΔ*ku80*::*NLuc* strain and the derived calculation for its doubling time. Generally, the assay is performed in three technical replicates for each of the three biological replicates to account for variations of luciferase activity readings. In order to calculate the normalized fold change of parasite growth, each reading at 24–96 h post-infection was divided by the initial reading at 4 h post-infection, which reflects the starting amount of live parasites in the assay (**Figure 1A,B**). In terms of determining parasite doubling time, the log2 of the normalized fold change of parasite growth was plotted against each timepoint. Next, the plot was subjected to a linear regression function to obtain the slope, which represents doubling time (**Figure 1C**).

The inhibition efficacies of LHVS in wild-type and Δ*cpl* strains were determined by plotting luciferase activities against eight inhibitor concentrations in **Figure 2**. It is essential to include infected cells without inhibitor treatment for normalization of raw luciferase activities in the assay. In addition, a mock experiment performed in a clear microplate is required for the assay to ensure that parasites are still in the intracellular stage at the end of the assay period.

In **Figure 3**, the generation and validation of a sgRNA expression construct targeting *TgCPL* and the production of a repair template for *TgCPL* deletion are shown. The 20 bp sgRNA matching to the *TgUPRT* gene encoded in the original plasmid was mutated to the DNA sequence targeting the *TgCPL* gene via PCR-based site-directed mutagenesis. To achieve this, the DNA sequences coding for the sgRNAs that recognize different genes were engineered to the forward primer, while the reverse primer was kept unchanged to simplify primer design.

Figure 3A shows a zoomed-in region of the sgRNA DNA sequences targeting the *TgUPRT* gene in the original template plasmid as well as the primer set used for the generation of the linearized sgRNA expression vector. **Figure 3B** shows a representative gel picture of the linearized *TgCPL*-targeting sgRNA expression plasmid. **Figure 3C** shows the restriction endonuclease digestion of the circularized *TgCPL*-targeting sgRNA expression plasmid. A M13 reverse primer was used to sequence the incorporated guide RNA within the sgRNA expression vector generated for the specific gene. In **Figure 3D**, the sequenced DNA region was aligned to the parental plasmid for the confirmation of successful mutagenesis. **Figure 3E** illustrates the start and end regions of the pyrimethamine resistance cassette, showing where the primers can anneal for production of the repair template for *TgCPL* gene deletion. The repair template was PCR-amplified and loaded into a 1% agarose gel for size verification and gel extraction.

The overall strategy for *TgCPL* knockout generation and screening is shown in **Figure 4**. Three sets of primers shown in **Figure 4A** were used to screen *TgCPL*-deletion parasites for the correct integration of 5'- and 3'-ARMs and deletion of the *TgCPL*-coding sequence. As shown in **Figure 4B**, generally, seven to eight clones were selected for screening initially. The screening usually starts with checking for deletion of the coding sequence for the gene of interest. This is followed by detection of 5' and 3'-ARMs, which helps minimize the total number of clones to be screened. Further verification by immunoblotting displayed in **Figure 4C** can be completed if an antibody recognizing the target protein is available.

FIGURE AND TABLE LEGENDS:

Figure 1: Intracellular growth quantification for *Toxoplasma* parasites using a luciferase-based method. (**A**) Raw luciferase activity readings in a spreadsheet software. The readings at 24 h, 48 h, 72 h, and 96 h post-infection were normalized against the initial readings at 4 h post-infection for calculating the fold changes in parasite growth. (**B**) The normalized data were averaged and plotted. (**C**) The log2 values of the fold changes were also plotted and subjected to linear regression for determination of the parasite's doubling time.

Figure 2: Inhibition efficacy assessment of LHVS and pyrimethamine using the luciferase-based growth assay. Parasites were inoculated into a 96 well microplate for 4 h to allow for invasion of host cells. Non-invaded parasites were washed away, and the plate was filled with media containing different concentrations of LHVS or pyrimethamine and incubated for an additional 96 h before determination of luciferase activity. The measured luciferase readings for parasites treated with individual inhibitor concentrations were normalized against the signal detected from untreated parasites. The data were plotted in a graphing program, and a regression analysis for IC_{50} determination was performed. The assay was repeated in three biological replicates with three technical replicates each. Data represent mean ± SEM, n = 3 biological replicates.

Figure 3: Generation of the plasmid construct expressing sgRNA targeting *TgCPL* and production of a repair template for *TgCPL* deletion. (**A**) The original pSAG1-Cas9-sgRNA-UPRT plasmid[23] was modified via a site-directed mutagenesis kit for replacement of the sgRNA targeting the *TgUPRT* gene to *TgCPL*. The sgRNA coding region is enlarged to show areas to which the primers anneal. After PCR, the mutated plasmid was linearized and loaded into a 1% agarose gel for verification of successful amplification, followed by gel extraction. (**B**) The gel image of the PCR-amplified linearized sgRNA expression construct. (**C**) After gel-extraction, the PCR product was circularized and subsequently transformed into *E. coli.* The clones containing the expected plasmids were screened by restriction endonuclease digestion and DNA sequencing. The band sizes after DNA digestion were 7.2 bp and 2.4 kb. (**D**) The M13 reverse primer labeled in the figure was used to sequence the mutated guide RNA region within the generated *TgCPL*-targeting sgRNA expression vector. The sequenced DNA region was aligned to the parental plasmid to confirm successful mutagenesis. (**E**) In this study, 50 bp homologous regions matching to the 5'- and 3'-UTRs of *TgCPL* were engineered into the primers for amplification of the repair template and flanked at the 5'- and 3'-ends of the pyrimethamine resistance cassette by PCR, respectively. Agarose gel electrophoresis was used to verify the correct size of the PCR product before gel extraction. The expected size of the repair template is ~2.7 kb. Usually, 5–6 µg of repair template can be obtained from 200 µL of PCR reaction.

Figure 4: PCR and immunoblotting confirmation of *TgCPL*-deficient parasites. (**A**) A schematic diagram depicting the general strategies of *TgCPL*-deletion in *Toxoplasma* and PCR-based screening of the correct *TgCPL* knockout clones. The primers used for the screening are labeled. (**B**) PCR and agarose gel electrophoresis were used to select clones containing the correct integration of the pyrimethamine resistance cassette into the *TgCPL* locus and loss of the *TgCPL*

gene. The genomic DNA of the Δ*cpl* population served as a positive control for 5'- and 3'-ARM detection, while the WT genomic DNA was used for the detection of the *TgCPL* gene as a positive control. Water was used instead of DNA template in the PCR reactions to serve as negative controls. The expected bands are denoted by arrows, whereas nonspecific PCR amplifications are labeled by asterisks. (**C**) Clone 1 identified by PCR screening was grown in tissue culture for cell lysate preparation and further immunoblotting analysis to confirm the loss of *TgCPL* expression in the knockout. TgActin was used as a loading control.

Table 1: Microplate reader settings for luciferase activity measurement during luciferase-based *Toxoplasma* growth assay.

Table 2: Thermocycler settings for generation of sgRNA expression vector.

Table 3: Reaction recipe for circularization of sgRNA expression vector.

Table 4: Thermocycler setting for generation of repair template.

Table 5: Colony PCR reaction recipe for screening single *Toxoplasma* clones.

Table 6: Thermocycler setting for screening single *Toxoplasma* clones.

Supplementary Table 1: Recipes for buffers.

DISCUSSION:

This protocol describes a luciferase-based protocol to assess intracellular *Toxoplasma* growth and evaluate the inhibition efficacy of chemical compounds against parasite growth. Compared to the existing strategies available for measuring intracellular *Toxoplasma* growth, this method exhibits high sensitivity and specificity. While monitoring parasite growth, a mock assay in a clear 96 well microplate is recommended to confirm that the tested strain does not prematurely lyse host cells before the end of the evaluation period. Otherwise, the luminescence readings will not accurately reflect parasite growth, since *Toxoplasma* only replicates within host cells.

It has been observed that phenol red dye quickly quenches luciferase activity, which can result in significant differences in the luciferase readings among technical replicates due to a delay in individual well measurements by the plate reader. Therefore, it is optimal to prepare HFFs in phenol red-free medium prior to seeding in the 96 well microplates. Also, in the case of high luciferase activity, cross-well interference may lead to significant variation among neighboring wells exhibiting strong luciferase activity. Hence, it is recommended to place an empty column between each strain.

Specifically, for the RHΔ*ku80*::*NLuc* strain, 1,500 parasites are inoculated into each well for the growth assay. Since the doubling time for WT *Toxoplasma* parasites is ~6–8 h[24], it is expected to

see an increase in luciferase activity by 8- to 16-fold at 24 h post-infection. However, certain strains with significant growth defects will only yield a slight increase in luciferase activity. Therefore, if the initial parasite inoculum is low, the inherent variation in luciferase activity will mask the observation of an increase in luminescence over the growth period. Hence, it is recommended to inoculate a higher number of parasites to achieve an accurate fold change for strains with growth deficiencies.

In the protocol, the guide RNA design follows the general rules used for CRISPR-Cas9-based genome modification in mammalian cells[25]. Currently, many types of software and online platforms provide services for guide RNA design in various organisms, such as CHOPCHOP[26], E-CRISP[27], and EuPaGDT[28]. Here, commercial software (**Table of Materials**) is used to design sgRNA. Compared to the previously mentioned online programs, this software provides a local environment for sgRNA design. It calculates activity and specificity scores for each candidate sgRNA using a previously published algorithm[25,29].

Technically, any sgRNA located within a gene of interest that contains a high specificity score can efficiently mediate the cleavage of genomic DNA for downstream homology-dependent recombination. In practice, sgRNA targeting a region close to the start or stop codon is preferred. Genes of interest can be endogenously epitope-tagged using the sgRNA, which generates a double-stranded gap at the end of the gene. Endogenously tagging a gene prior to its deletion will help confirm gene loss via immunoblotting detection in the case that an antibody against the protein of interest is not available. Epitope-tagging of a gene can also help determine the subcellular location of the protein of interest via immunofluorescence microscopy. Furthermore, if the target gene is essential, sgRNA recognizing the start region of the gene can be used for the replacement of its cognate promoter to a tetracycline-responsive promoter to generate a conditional knockout.

Additionally, the protocol describes a technique for gene deletion in *Toxoplasma* by replacing the *TgCPL* gene with a pyrimethamine resistance cassette. By using different plasmid templates encoding other drug resistance cassettes, investigators can modify primer sequences to incorporate other antibiotic resistance genes into the repair template via PCR. Furthermore, this protocol can be modified to perform other genome modifications, such as endogenous gene tagging, promoter replacement, and site-directed mutagenesis. It is noteworthy that the homologous regions used in this protocol are only 50 bp in length. A separate study successfully used 40–43 bp homologous DNA sequences to introduce single-nucleotide mutations and gene epitope tagging in *Toxoplasma* parasites[22]. Homologous DNA sequences this short can be easily incorporated into primers. Although we did not quantitatively evaluate the HDR efficiency for this particular length of homologous region, it seems that a 40–50 bp region is sufficient for efficient DNA recombination in the *TgKu80*-deficient *Toxoplasma* strain, as evidenced by the successful genetic manipulation of several genes recently achieved[15,22].

During the efficacy determination of chemical compounds, if the prospective drug target gene is essential, a comparison of shifts in IC_{50} values between the wild-type and knockout strains is not practical. In this scenario, an assay directly measuring the ability of chemical compounds to

inhibit recombinant protein activity is required to evaluate efficacy and specificity of the drugs. Recent literature reported the fitness scores of individual genes in *Toxoplasma* by performing a genome wide CRISPR screen[30], which can serve as a guide to help assess the difficulty of generating a straight knockout mutant.

Taken together, the protocol describes successful completion of a luciferase-based intracellular *Toxoplasma* growth assay and an evaluation strategy for chemical inhibitors against *Toxoplasma* growth. Also detailed is a CRISPR-Cas9-based genome editing protocol for gene deletion in *Toxoplasma* parasites, which has been widely used in the field. Individual labs can modify the described protocol according to experimental needs, such as endogenous gene tagging, switching drug selection markers, and altering the evaluation period for intracellular parasite growth.

DISCLOSURES:

The authors have nothing to disclose.

ACKNOWLEDGEMENTS:

The authors would like to thank Drs. Sibley and Carruthers for sharing pSAG1-Cas9-sgRNA-TgUPRT plasmid and anti-TgCPL and TgActin antibodies. This work was supported by the Clemson Startup fund (to Z.D.), Knights Templar Eye Foundation Pediatric Ophthalmology Career-Starter Research Grant (to Z.D.), a pilot grant of an NIH COBRE grant P20GM109094 (to Z.D.), and NIH R01AI143707 (to Z.D.). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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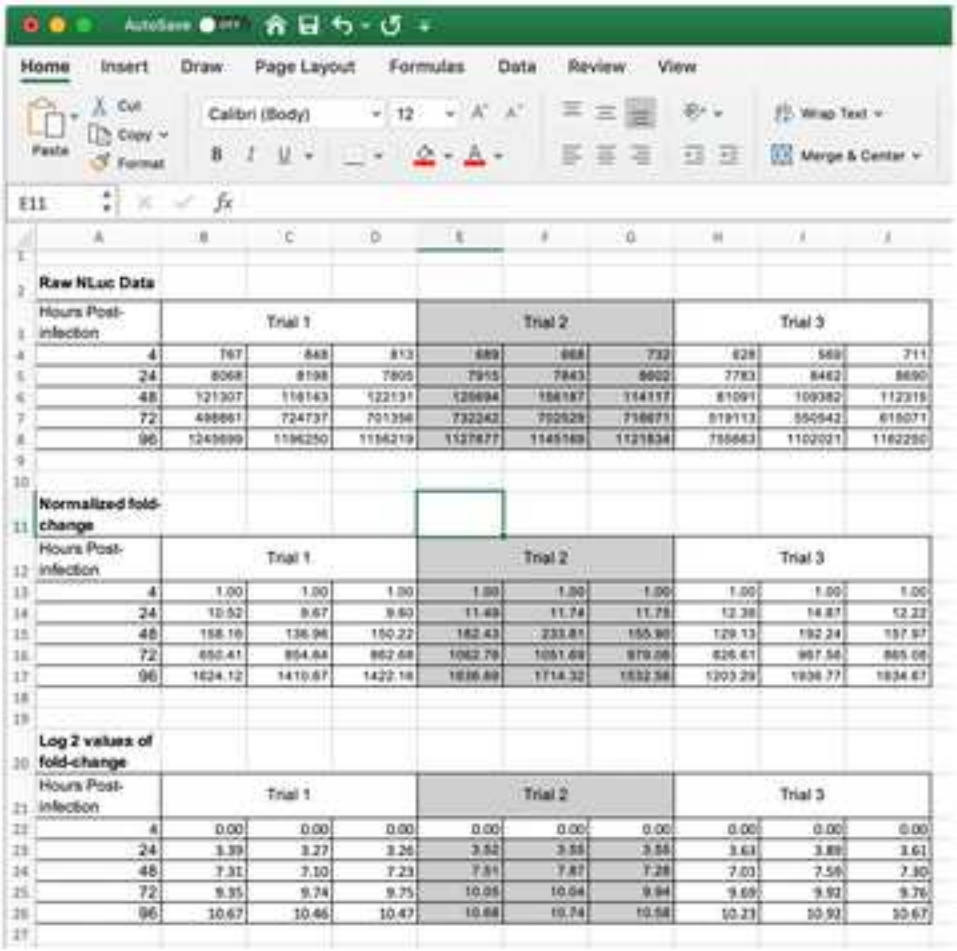
Figure 1

A



Raw NLuc Data

Hours Post-infection	Trial 1			Trial 2			Trial 3		
4	767	848	813	689	668	732	629	569	711
24	8068	8198	7805	7915	7843	8602	7783	8462	8690
48	121307	116143	122131	126694	156187	114157	81091	109382	112315
72	498661	724737	701356	732242	702529	718671	519113	550542	616071
96	1243699	1196250	1156219	1127877	1145169	1121834	755663	1102021	1162250

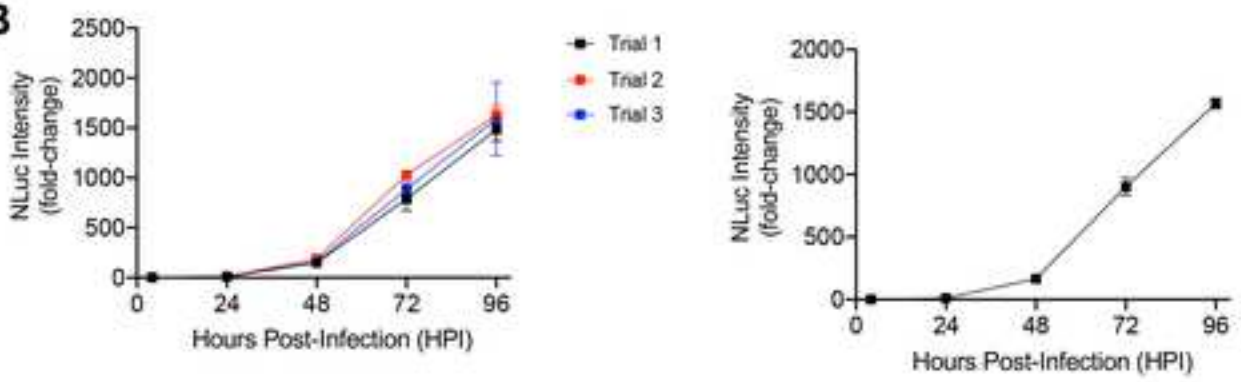
Normalized fold-change

Hours Post-infection	Trial 1			Trial 2			Trial 3		
4	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
24	10.52	9.67	9.60	11.49	11.74	11.75	12.38	14.87	12.22
48	158.16	136.96	150.22	182.43	233.81	155.90	129.13	192.24	157.97
72	650.41	854.64	862.68	1062.79	1051.69	979.08	826.61	967.56	865.08
96	1624.12	1410.67	1422.18	1636.69	1714.32	1532.58	1203.29	1936.77	1634.67

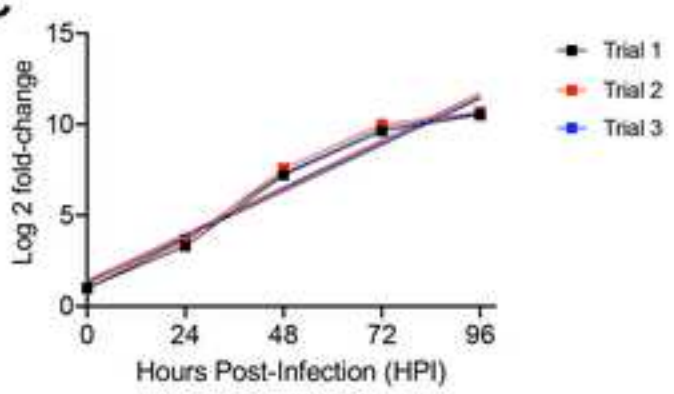
Log 2 values of fold-change

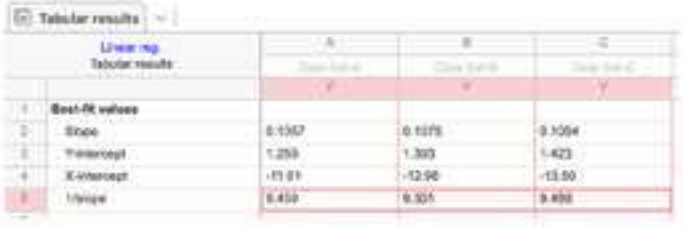
Hours Post-infection	Trial 1			Trial 2			Trial 3		
4	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
24	3.39	3.27	3.26	3.52	3.55	3.55	3.63	3.89	3.61
48	7.31	7.10	7.23	7.51	7.87	7.28	7.01	7.59	7.30
72	9.35	9.74	9.75	10.05	10.04	9.94	9.69	9.92	9.76
96	10.67	10.46	10.47	10.68	10.74	10.58	10.23	10.92	10.67

B



C



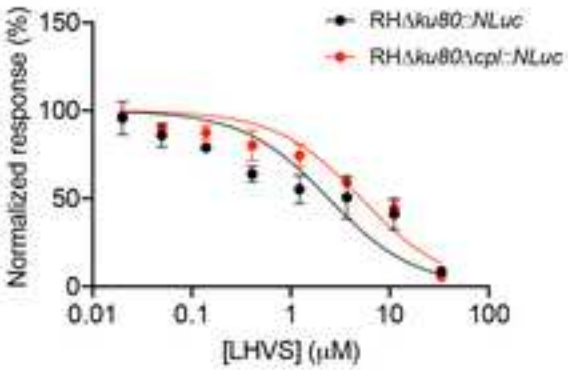


Doubling time

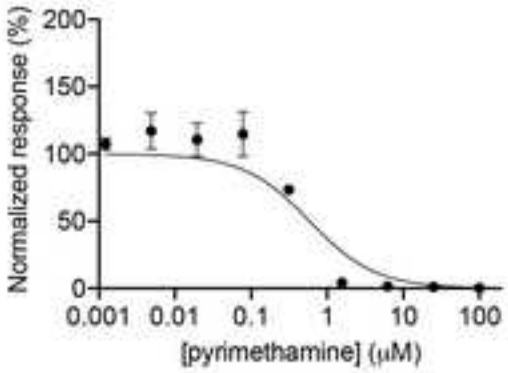
Trial 1: 9.5 h

Trial 2: 9.3 h

Trial 3: 9.5 h



IC_{50} (LHVS) for RHΔ*ku80*::*NLuc* = 2.89 ± 1.22 μM
IC_{50} (LHVS) for RHΔ*ku80*Δ*cpl*::*NLuc* = 5.17 ± 0.55 μM



IC_{50} (pyrimethamine) = 0.59 ± 0.09 μM

Fig 3

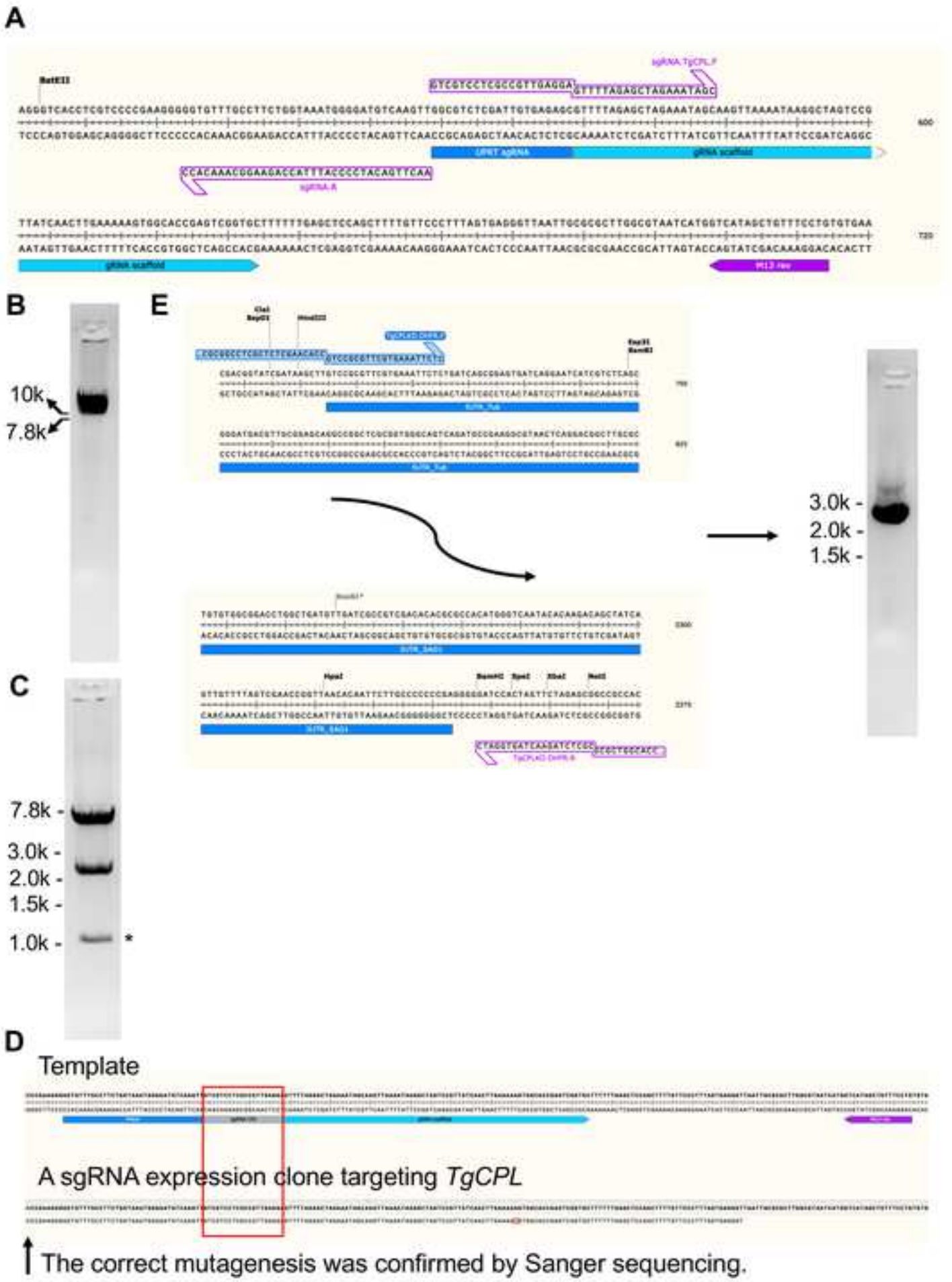
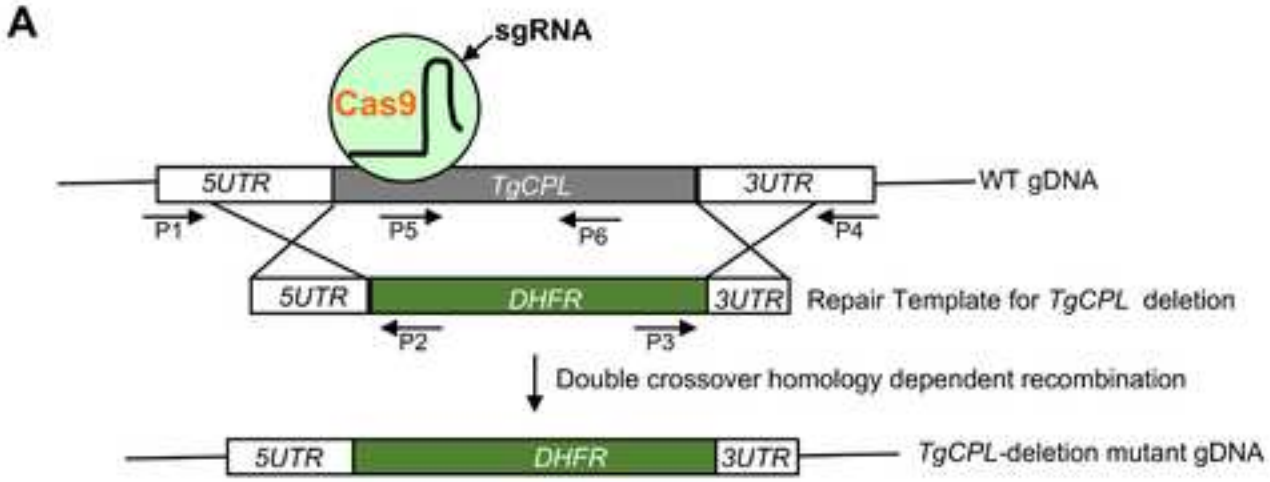
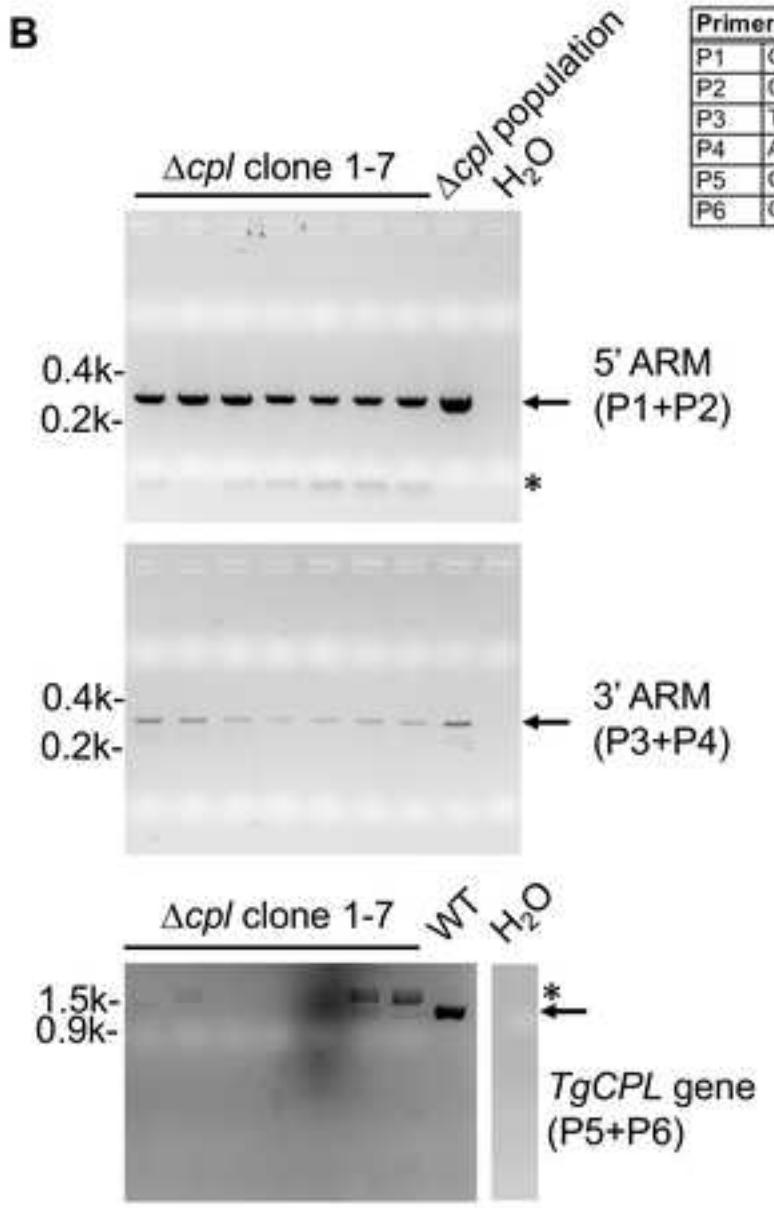


Fig 4



Primers used in Fig. 4A	
P1	GCAAGCGTCATTCGCCGC
P2	CGCTGAGACGATGATTCCTG
P3	TTAGTCGAACCGGTTAACAC
P4	ATACAGTGGACTGACTCG
P5	GAAGTCGCACCACCTTGG
P6	GTCCGATACCTGTGTTCC



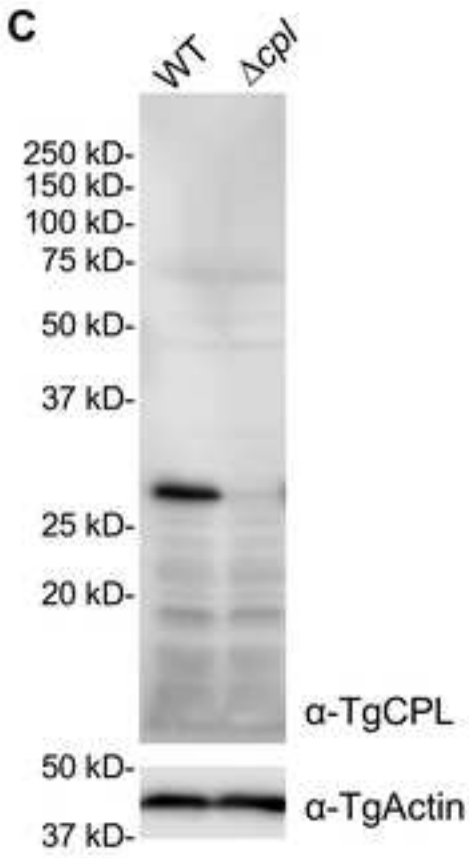


Table 1. Microplate Reader Settings	
Luciferase:	Endpoint
Integration time:	1 s
Filter Set - Emission:	Full light
Optics:	Top
Gain:	135
Read speed:	Normal
Delay:	100 ms
Read height:	4.5 mm

Table 2. Thermocycler setting for the generation of sgRNA expression vector	
Initial denaturation:	98°C for 5 min
25 cycles of	
Denaturing:	98°C for 5 s
Annealing:	60°C for 15 s
Extension:	72°C for 1 min
Final extension:	72°C for 10 min

Table 3. Reaction recipe for circularization of sgRNA expression vector	
Sample	Volume (µl)
PCR product (10-50 ng)	1
2X KLD (kinase, ligase, DpnI) Reaction Buffer	5
10X KLD Enzyme Mix	1
Nuclease-free water	3
Total	10

Table 4. Thermocycler setting for the generation of repair template	
Initial denaturation:	98°C for 5 min
35 cycles of	
Denaturing:	98°C for 15 s
Annealing:	58°C for 15 s
Extension:	72°C for 30 s per kb
Final extension:	72°C for 10 min

Table 5. Colony PCR reaction recipe for screening single *Toxoplasma* clones	
Sample	Volume (µl)
total *Toxoplasma* genomic DNA	1
Forward primer (25 µM)	0.2
Reverse primer (25 µM)	0.2
2x PCR master premix	5
Nuclease-free water	3.6
Total	10

Table 6. Thermocycler setting for screening single *Toxoplasma* clones	
Initial denaturation:	98°C for 5 min
35 cycles of	
Denaturing:	98°C for 5 s
Annealing:	55 - 62°C for 5 s
Extension:	72°C for 20 s per kb
Final extension:	72°C for 1 min

Name of Material/Equipment	Company	Catalog Number
Agarose gel extraction kit	New England BioLabs	T1020L
BamHI	New England BioLabs	R0316S
Biotek Synergy H1 Hybrid Multi-Mode Microplate Reader	BioTek Instuments	
BTX Gemini Twin Waveform Electroporation System	Harvard Apparatus	
Chemically competent *E. coli* cells	New England BioLabs	C29871
CloneAmp HiFi PCR premix	Takara Bio	639298
Coelenterazine h	Prolume	301-10 hCTZ
EcoRV	New England BioLabs	R3195S
Phire Tissue Direct PCR Master Mix	Thermo Scientific	F170L
Plasmid miniprep kit	Zymo Research	D4054
Q5 Site-Directed Mutagenesis kit	New England BioLabs	E0554S
Software		
Geneious software for sgRNA design (version: R11)		
GraphPad Prism software (8^{th} version)		
SnapGene for molecular cloning (version: 4.2.11)		

RESPONSE TO REVIEWERS (JoVE60985; Key, Bergmann, Micchelli, Thornton, Millard, Dou)

We thank the reviewers for taking the time to review our manuscript and for their constructive comments. We were very happy that the reviewers recognized that the protocols reported in the manuscript can be adopted in the field to help measure the efficacy of potential anti-*Toxoplasma* antibiotics and verify the corresponding drug targets by genetically deleting the corresponding genes. We were also pleased to read that both reviewers thought that the procedures documented in the manuscript were logically written. We have made changes in response to the editorial and reviewers' comments. Below we have included the comments and provided our responses (see italics below). We hope that the reviewers and editor find our work suitable for publication in the *Journal of Visualized Experiments.*

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.
We carefully revised and proof-read the manuscript.

2. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points
We changed font, font size, spacing, margins as requested.

3. We can only show first co-authors in the journal website.
Yes, Melanie Key and Amy Bergmann both shared the first authorship in this manuscript.

4. Please provide an email address for each author.
We included the author's emails in the revised version.

5. Please rephrase the Short Abstract/Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: "Here, we present a protocol to …". Presently it is more than the required word limit
We shortened the abstract as requested.

6. Please ensure that the long Abstract is within 150-300-word limit and clearly states the goal of the protocol.
We also shortened the abstract as requested.

7. Please define all abbreviations during the first-time use.
We did as requested.

8. JoVE cannot publish manuscripts containing commercial language. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.

9. For example: NanoLuc, BioTek Synergy H1 Hybrid Multi-Mode Microplate Reader, GraphPad Prism software, Geneious software (version R11), CloneAmp HiFi PCR premix, NEB Monarch DNA Gel Extraction kit, NEB Q5 site-directed mutagenesis kit, Cytomix, BTX Gemini Twin Waveform Electroporation System, Thermo Scientific Phire Tissue Direct PCR Master Mix,
For Item 8 and 9, as requested we replaced these commercial terms with general names. We

still kept NanoLuc and Cytomix in the manuscript, since we feel that the use of luciferase probably will confuse the audience due to the existence of different kinds of luciferases and Cytomix is not a commercial name. In addition, we found that NanoLuc was widely used in published literature. For example, please refer to this research article from this link. https://www.frontiersin.org/articles/10.3389/fphar.2016.00027/full

10. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please ensure that individual steps of the protocol should only contain 2-3 actions per step.
We removed most of large paragraphs of text between sections, but we do feel that a general brief introduction describing the following procedures is required.

11. Please do not include any materials, reagents etc in the protocol section. Please include all these in the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials in separate columns in an xls/xlsx file.
As requested, we expanded the table of materials, including the essential supplies, reagents, and equipment with the name, company, and catalog number in separate columns.

12. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.”
We switched to imperative tense in the revised protocol section.

13. In the JoVE Protocol format, “Notes” should be concise and used sparingly. They should only be used to provide extraneous details, optional steps, or recommendations that are not critical to a step. Any text that provides details about how to perform a particular step should either be included in the step itself or added as a sub-step.
We changed this as requested.

14. Please revise the protocol text to avoid the use of any personal pronouns in the protocol (e.g., "we", "you", "our" etc.).
We changed this as requested.

15. Please ensure you answer the “how” question, i.e., how is the step performed?
We changed this as requested.

16. 1.1: How is this done?
We revised it as requested.

17. 1.2: How do you grow the parasite? How do you lyse these? What is the volume/number used for passing through the syringe?
We changed this as requested. We made sure to specify if the parasites were naturally lysing out of the host cells or if lysis was done manually with a syringe.

18. 2.10: How is this done?
We changed this as requested.

19. 3.1.7: Please include reaction set up for the PCR reaction as a separate table.
We changed this as requested.

20. 3.1.9-3.1.12: Please briefly describe how is this done?
We changed this as requested.

21. 3.2.1: How?
We changed this as requested.

22. Lines 349-375: Please move this to the table of materials.
We changed this as requested.

23. Please consider making a table for the plate readings, reaction set up, thermocycler programs etc. All tables should be uploaded separately to your Editorial Manager account in the form of an .xls or .xlsx file. Each table must be accompanied by a title and a description after the Representative Results of the manuscript text and can be referenced in the protocol text wherever applicable.
A separate table was created for plate readings, reaction set up, thermocycler programs, and the titles of the individual tables were included in the text.

24. There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.
We marked the sections to be filmed in yellow.

25. Please place the representative result after the protocol section. Please expand the result in the context of the technique you have described, e.g., how do these results show the technique, suggestions about how to analyze the outcome, etc. The paragraph text should refer to all of the figures. Data from both successful and sub-optimal experiments can be included.
We combined all of the representative results into a section that followed the protocol section.

26. Please include all the Figure Legends together at the end of the Representative Results in the manuscript text. Each Figure Legend should include a title and a short description of the data presented in the Figure and relevant symbols.
We did this as requested.

27. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."
We did not use any copyrighted pictures in this manuscript.

28. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique

We revised the Discussion section as requested.

Reviewers' comments:
Reviewer #1:

Manuscript Summary:
This manuscript describes three protocols 1) a luciferase-based intracellular Toxoplasma growth assay; 2) an evaluation strategy for chemical inhibitors against Toxoplasma growth; 3) CRISPR-Cas9-based gene deletion in Toxoplasma parasites. These efficient protocols will enrich laboratory techniques in the investigation of Toxoplasma, and especially aid those who need to evaluate Toxoplasma chemical inhibitors.
We greatly appreciated the comments from the reviewer that our protocol is useful for Toxoplasma research and provides aid for the investigators who work on drug screening.

Major Concerns:
The manuscript is well-written and organized. According to my experience in Toxoplasma research, these protocols should be very efficient. I have no major suggestions for improvement.

Minor Concerns:
1. Is it more accurate to use "parental strain" instead of "WT" throughout the manuscript (at least some places)?
According to the suggestions, we changed "WT" to "parental strain" in some places.

2. Line 92: "(HDR)" should be added after "homology-dependent recombination".
We changed this.

Reviewer #2:

Manuscript Summary:
The manuscript by Key, Bergmann and colleagues describes a protocol for screening compounds against Toxoplasma gondii using a luciferase-expressing strain in a 96-plate well format. The authors then generated a TgCPL knockout cell line to demonstrate the efficiency of their approach and show that a 50-bp homology region is sufficient for DNA recombination in the parasite.

Overall, this protocol and the video associated with it will be a useful tool for anyone trying to assess drug efficiency by measuring the growth rates of T. gondii.
Again, we greatly appreciated the comments from this reviewer as well.

Major Concerns:
None

Minor Concerns:

1) The authors demonstrate the feasibility of their approach in a 96-well plate format. I was wondering if they have tried this approach in a larger format (ie. 384 or 1536 well plates) or if they could mention if the approach could be scaled up in the discussion section.
In this protocol, we only used a 96-well plate as an example. We feel that the method can be scaled up to 384- or 1536-well plate. We added these comments in the section titled "Protocols".

2) The authors state that using 50bp of homology for DNA recombination is an "improved protocol". However, the same type of approach has been already performed by Sidik et al. 2014, where they provide a repair template with 40 to 43 bp homology regions to achieve homologous recombination in the CDPK3 locus of T. gondii. If I understand correctly, the improvement stems from an older version of the author's protocol, so they should state so.
Thanks for pointing out this important information. Accordingly, we incorporated the findings from the paper published by Sidik et al. and cited it in the manuscript.

3) The authors do not cite the Larson et al. 2009 paper which formally demonstrated that TgCPL is the target of LHVS.
As suggested, we cited this paper.

4) Figure 1: there is no explanation for figure 1C nor it is mentioned in the figure legends.
Thanks for recognizing this error. We have addressed this in the figure legend of our revised version.

5) Figures 3 and 4: Authors should provide the plasmid maps required to express the sgRNA targeting TgCPL and to generate the proper repair template. However, I find that the plasmid maps are unnecessary. The authors should just provide a schematic representation of the region of interest (the region where the sgRNA is located). The two figures can also be merged into one as both are required for the same experiment.
Thanks for the suggestions. We combined Figure 3 and 4 into a single figure. In Figure 3A, we only showed the sgRNA region targeting TgUPRT gene in the template plasmid, which is replaced to different sgRNA depending on individual genes by site-directed mutagenesis. In Figure 3E, we incorporated the start and end areas of the pyrimethamine resistance cassette to show the regions where the primers can anneal to for the production of repair template.

6) Figure 5: For the PCRs of the clones carrying the correct integration, a negative control (ie. the parental strain) should be provided to further demonstrate the success of the approach.
I suggest to add a legend to each control the authors use for their PCRs (Δ*cpl* or WT genomic DNA) instead or writing "positive control".
PCR with P5+P6: what are those bands in the gel? Can you speculate why do we see them in some clones but not in others?
For the Western Blot, which one of the clones was picked up for further analysis? The authors do not mention this.
As suggested, we incorporated the negative controls in Figure 4B. We also updated the names of the positive controls in the figure and figure legend. In terms of detection of the loss of TgCPL coding sequence by using P5 and P6, the primer set generated non-specific amplification. We marked and annotated these non-specific bands in the figure. We also included negative controls in Figure 4B. Since all of clones are correct, we picked the first clone for further validation by immunoblotting. We included this information in the figure legend as well.

Supplementary Table 1

Cytomix buffer	25 mM HEPES, pH 7.6, 120 mM KCl, 10 mM K2HPO4/KH2PO4, 5 mM MgCl2, 0.0
D10 medium	DMEM 1X (Corning, Cat #: 10-013-CV), 10 mM HEPES, 10% (v/v) Cosmic Calf Ser
Phenol red-free mediun	DMEM/ Highly Modified (Hyclone, Cat #: SH30284.02), 10 mM HEPES, 10% (v/v)
2X NLuc Buffer	100 mM MES, pH 6.0, 1mM CDTA, 0.5% Tergitol, 0.05% Mazu DF 204, 150 mM K

15 mM CaCl2, and 2 mM EGTA.
um (Hyclone, Cat #: SH30087.03), 1 mM sodium pyruvate, 4 mM L-glutamine, 100 units/mL of penicillin,
Cosmic Calf Serum (Hyclone, Cat #: SH30087.03), 1 mM sodium pyruvate, 4 mM L-glutamine, 100 units/
Cl, 1 mM DTT, 35 mM Thiourea.

, and 100 µg/mL of Streptomycin
/mL of penicillin, and 100 µg/mL of streptomycin.