

Video Article

Genetic Manipulation in $\Delta ku80$ Strains for Functional Genomic Analysis of *Toxoplasma gondii*

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Abstract

Targeted genetic manipulation using homologous recombination is the method of choice for functional genomic analysis to obtain a detailed view of gene function and phenotype(s). The development of mutant strains with targeted gene deletions, targeted mutations, complemented gene function, and/or tagged genes provides powerful strategies to address gene function, particularly if these genetic manipulations can be efficiently targeted to the gene locus of interest using integration mediated by double cross over homologous recombination.

Due to very high rates of nonhomologous recombination, functional genomic analysis of *Toxoplasma gondii* has been previously limited by the absence of efficient methods for targeting gene deletions and gene replacements to specific genetic loci. Recently, we abolished the major pathway of nonhomologous recombination in type I and type II strains of *T. gondii* by deleting the gene encoding the KU80 protein^{1,2}. The $\Delta ku80$ strains behave normally during tachyzoite (acute) and bradyzoite (chronic) stages *in vitro* and *in vivo* and exhibit essentially a 100% frequency of homologous recombination. The $\Delta ku80$ strains make functional genomic studies feasible on the single gene as well as on the genome scale¹⁻⁴.

Here, we report methods for using type I and type II $\Delta ku80\Delta hxgprt$ strains to advance gene targeting approaches in *T. gondii*. We outline efficient methods for generating gene deletions, gene replacements, and tagged genes by targeted insertion or deletion of the hypoxanthine-guanine phosphoribosyltransferase (*HXGPRT*) selectable marker. The described gene targeting protocol can be used in a variety of ways in $\Delta ku80$ strains to advance functional analysis of the parasite genome and to develop single strains that carry multiple targeted genetic manipulations. The application of this genetic method and subsequent phenotypic assays will reveal fundamental and unique aspects of the biology of *T. gondii* and related significant human pathogens that cause malaria (*Plasmodium* sp.) and cryptosporidiosis (*Cryptosporidium*).

Video Link

The video component of this article can be found at <https://www.jove.com/video/50598/>

Introduction

Toxoplasma gondii is a common obligate intracellular protozoan parasite that frequently and chronically infects a wide range of animals and humans⁵. It is estimated that more than 1 billion humans are currently and chronically infected by this pathogen. In addition to the importance of disease caused by *T. gondii* infection, the increasing availability of experimental tools, powerful genomics resources⁶, ease of *in vitro* growth and excellent mouse models have made *T. gondii* a leading model system for the broader study of intracellular eukaryotic pathogens and other significant apicomplexan parasites that cause devastating diseases such as malaria (*Plasmodium* sp.) and Cryptosporidiosis (*Cryptosporidium*)^{5,7}. A significant limitation of *Toxoplasma gondii* as a model organism has been the inefficient recovery of progeny that carry targeted genetic manipulations. This problem in gene targeting is due to a low frequency of homologous recombination relative to the very high frequency of nonhomologous recombination in wild-type strains of *T. gondii* even when extensive DNA homology is provided in DNA target molecules used in genetic studies².

We recently genetically blocked the major pathway of nonhomologous recombination in type I and type II strains of *Toxoplasma gondii* by deleting the gene encoding the KU80 protein^{1,2}. The resulting type I and type II $\Delta ku80$ strains exhibit normal growth rates, size and behavior both *in vitro* and *in vivo* during tachyzoite and bradyzoite stages *in vitro* and *in vivo*, however, these strains exhibit essentially a 100% frequency of homologous recombination and this phenotype increases the likelihood of rapidly isolating desired progeny of targeted genetic manipulations by several hundred to several thousand-fold^{1,2,4}. Recent community-wide use of type I and type II $\Delta ku80$ strains has significantly ramped up the pace, the variety, as well as the success rate of targeted genetic approaches in *T. gondii*^{1-4,8-13}. Here we describe a comprehensive protocol for targeted gene deletion, gene replacement, and gene tagging in $\Delta ku80$ strains of *T. gondii*. We demonstrate how to design and reliably target genetic manipulations to specific genes or genetic loci in $\Delta ku80$ strains of *Toxoplasma gondii*.

Protocol

1. Gene Targeting to Delete a Gene of Interest

This protocol is designed for the efficient generation of a mutant strain that has a targeted gene deletion at a defined genetic locus. The previously described *T. gondii* hypoxanthine-xanthine-guanine phosphoribosyltransferase (*HXGPRT*) selectable marker is used in this protocol for safe and reliable marker insertion following mycophenolic acid and xanthine (MPA + X) selection¹⁴⁻¹⁶. This protocol uses a validated and cost-effective method for constructing DNA targeting molecules based on yeast recombinational cloning^{17,18}. Several alternative protocols are commercially available for constructing recombinant targeting molecules by using bacterial recombinational cloning methods, *in vitro* recombinational cloning methods, or fusion PCR. The protocol described below provides the highest overall efficiency and reliability of recovering cloned parasites possessing a targeted gene deletion in $\Delta ku80$ strains of *T. gondii*.

1.1 Preparation of targeting DNA

1. Access ToxoDB⁶ (<http://www.toxodb.org>) to obtain genomic sequences for the gene of interest (GOI).

Note: Genomic sequences should be obtained from the Type I, II or III genome sequence in the ToxoDB database that is closest to the strain being manipulated. For example: GT1 for RH and ME49 for Pru.

2. Design specific overlap primers that amplify the 5' and 3' genomic targeting flanks of the gene of interest (GOI) incorporating a 33 bp overlap with the yeast shuttle vector pRS416 (or alternatively pRS426) and incorporate a 33 bp overlap with the selectable marker (*HXGPRT*) (**Figures 1A-B**). Add a unique restriction enzyme site in each primer at both ends of the 5' and 3' genomic targeting flanks, placed between the 33 bp overlap and the *T. gondii*-specific primer sequence(s) (**Figures 1A-B**).

Note: A greater than 30 bp overlap is required for efficient yeast recombination cloning. The 5' and 3' genomic targeting flanks are designed to amplify specific DNA fragments between 800 and 1,400 bp that define a targeted deletion. Be aware that shorter flanks will significantly reduce targeting efficiency in the $\Delta ku80\Delta hxgpRT$ background².

3. PCR amplify the 5' target flank using primers F1 and R1, and PCR amplify the 3' target flank using primer F2 and R2 (**Figures 1A-C**) from genomic DNA³. Separately, PCR amplify the ~2 Kbp *HXPGR*T gene including the associated 5' and 3' *dhfr* flanking regions of the *HXGPRT* cDNA pminiHXGPRT cassette¹⁴ using primers HXF and HXR (**Figures 1B-C**).

Note: Amplify target flanks using genomic DNA from the parental strain to be transfected.

Note: Place the *HXGPRT* marker in the forward orientation relative to the 5' and 3' DNA targeting flanks to facilitate subsequent efficient genetic manipulations, such as the targeted removal of *HXGPRT* from the disrupted locus.

4. Verify correct PCR product sizes by agarose gel electrophoresis and estimate the DNA fragment concentration using agarose gel standards or another method for determining DNA concentration.
5. Generate competent yeast aliquots as described in Gietz and Schiestly¹⁷.
6. Combine 50 ng of 5' genomic targeting flank, 50 ng of 3' genomic targeting flank, 100 ng of *HXGPRT* selection marker, and 50 ng of linearized shuttle vector with sterile H₂O to achieve a final volume of 10 - 20 μ l. Transform competent yeast with the three PCR products and yeast-*E. coli* shuttle vector for recombinational cloning using the protocol described by Gietz and Schiestly¹⁷. Plate transformed yeast on uracil-minus minimal medium agar plates and incubate at 30 °C for 2 - 3 days.

Note: The ordered assembly of the plasmid, the 5' target flank, the *HXGPRT* marker, and the 3' target flank is facilitated by the engineered 33 bp overlap between the DNA fragments (**Figures 1A-B**) and is mediated by homologous recombination in yeast.

7. Harvest yeast by adding 2 ml of 2x YPAD to the uracil-minus plates and gently scraping to dislodge colonies without disrupting the agar. Centrifuge the yeast solution for 5 min at 5,000 x g and discard the supernatant.
8. Resuspend the yeast pellet in 250 μ l of a resuspension buffer containing RNaseA and add 50 - 100 μ l of acid-washed glass beads to the solution. Vortex for 5 min at the highest speed to smash the yeast cells.
9. Allow the glass beads to settle to the bottom of the tube and transfer the supernatant to a 2 ml Eppendorf tube.
10. Isolate yeast DNA using a miniprep DNA isolation kit, resuspending in a final volume of ~100 μ l.
11. Mix 2 μ l yeast DNA (~50 ng) with 40 μ l of DH10B electroporation competent *E. coli* kept on ice.
12. Transfer the solution to a chilled 2 mm gap electroporation cuvette and electroporate the bacterial cells at 2.4 kV and 129 Ω .
13. Rescue cells by adding 0.8 ml SOC broth to each cuvette and transfer solution to a 14 ml snap-cap Falcon tube. Incubate cells at 37 °C on a roller drum for 40 - 60 min.
14. Plate *E. coli* on 2XYT + ampicillin (AMP) plates and incubate the plates at 37 °C overnight.
15. Select ~6 - 8 single colonies and grow in 3 ml 2XYT + AMP for ~16 hr at 37 °C.
16. Prepare a 30% glycerol freezer stock of each *E. coli* clone.
17. Isolate plasmid p Δ GOI from *E. coli* clones using a miniprep kit, resuspending in a final volume of ~100 μ l.
18. Validate the p Δ GOI using restriction enzyme digests to measure the DNA plasmid size and verify the expected p Δ GOI DNA banding patterns.
19. Finalize validation of p Δ GOI by DNA sequencing of the 5' and 3' genomic targeting flanks to verify 100% DNA sequence based on genome sequence data in <http://www.ToxoDB.org>.

Note: Near perfect sequence homology is essential for maximizing gene targeting efficiency³ since each base pair difference constitutes a corresponding cut in the length of perfect homology.

Note: The genome sequence of the type II *Δku80* strain based on the Pru parental strain is not available at this time. This work uses the type II ME49 genome sequence as the surrogate genome for the type II *Δku80* strain. Based on sequence data at a number of genetic loci, it is estimated that the ME49 genome and the Pru genome exhibit single nucleotide polymorphisms at a frequency of ~1 per 10,000 nucleotides, or less.

20. Generate >200 μg of pΔGOI stock by inoculating a ~250 ml 2XYT + AMP overnight culture with glycerol stocks from a validated *E. coli* miniprep clone.
21. Isolate pΔGOI plasmid DNA from the large culture using a maxiprep DNA isolation kit, resuspending in a final volume of ~1,000 μl.
22. Repeat restriction enzyme digests, or DNA sequencing of the pΔGOI maxiprep DNA to verify the correct targeting DNA molecule prior to transfection.
23. Linearize ~15 μg pΔGOI at the 5' end using the unique restriction enzyme X (RE.X) digest site built into the 5' target flank (**Figures 1B**).

Note: Gene targeting in *T. gondii* requires a minimum of ~10 μg of targeting DNA to obtain an efficient frequency of targeting events at the gene locus of interest².

24. Validate linearization of pΔGOI and determine the DNA concentration by agarose gel electrophoresis or an alternative method.

Note: If restriction enzyme digestion at the 5' flank does not yield complete linearization, the designed 3' unique RE.Y digest site can be used as an alternative site for linearization of pΔGOI.

Note: A completely linearized targeting DNA molecule is essential for successful gene targeting by homologous recombination in the *Δku80* strains.

25. Neutralize the restriction enzyme by incubating at 68 °C for 15 - 20 min.
26. Prepare at least 15 μg of linearized plasmid in 100 μl of sterile H₂O. Store linearized pΔGOI at -20 °C until the time of transfection.

1.2 Preparation of *T. gondii* parasites, transfection, selection, subcloning, validation, archival and maintenance of genetically manipulated strains

General methods for the culture and manipulation of *T. gondii* in human foreskin fibroblast (HFF) cells are described¹⁹⁻²¹. The *Δku80Δhxgprt* strains replicate normally in parasite infection medium (Eagles Minimal Essential Medium (EMEM) growth medium supplemented with 1% fetal bovine serum (FBS) and 1% Antimycotic-Antibiotic diluted from a 100x stock)^{1,2}. All work with *Toxoplasma gondii* must be performed using biosafety level 2 procedures. The *T. gondii* RH*Δku80*² parental strain was generated from the RH*Δhxgprt* strain from the Roos Lab¹⁴. The Pru*Δku80*¹ parental strain was generated from Pru*Δhxgprt* (Prugniaud strain BSG-4²²) that contains a stably integrated CAT selectable marker and a green fluorescent protein (GFP) reporter under the control of the bradyzoite stage specific promoter LDH2.

1. Inoculate a 25 cm² flask containing confluent HFF cells with 1 x 10⁶ viable type I RH *Δku80Δhxgprt* parasites, or inoculate two 25 cm² flasks with 2 x 10⁶ viable type II Prugniaud (Pru) *Δku80Δhxgprt* parasites.

Note: Viable parasites are defined as extracellular parasites that have freshly lysed out.

Note: This scale provides a sufficient number of viable parasites for three transfection experiments.

2. Inspect the *Δku80Δhxgprt* infected cultures ~68 - 72 hr post-infection. Verify the presence of viable parasites and ~90 - 95% lysis of HFF cells to plan the precise timing of transfection.

Note: The isolation of freshly egressed parasites is essential for the success of this protocol because low parasite viability will abolish gene-targeting efficiency.

3. Agitate viable parasites into solution by closing the plug-seal lid on the 25 cm² flask and vigorously shake the flask back and forth to agitate the parasites off of the growth surface without splashing liquid onto the inside of the lid or the neck of the flask.

Note: Parasite harvest does not require a syringe-needle release protocol for the type I or type II *Δku80* strains.

4. Transfer the parasite solution to a 10 ml syringe attached to a filter holder containing a 3 μm membrane and place the filter unit on top of an open 15 ml screw-cap tube. Syringe filter the parasites into the 15 ml screw-cap tube.

Note: Filtering the parasites through the membrane removes infected cells and cellular debris.

5. Determine the parasite concentration (tachyzoite forms per ml) using a hemocytometer.

Note: Optional: Using the parasite solution, set up a plaque forming unit (PFU) assay²¹ that will be read 7 - 8 days later to determine adequate viability of the transfected parasites (PFU to tachyzoite ratio of ≥0.2).

6. Pellet parasites for 7 min at 1400 x g and aspirate supernatant to ~0.4 ml without disturbing the parasite pellet. Spin for 2 min at 1,400 x g and aspirate remaining supernatant to ~0.01 ml without disturbing the parasite pellet.
7. Resuspend the parasite pellet by flicking the bottom of the tube and immediately add cytomix²³ buffer (1.33x concentration) to the parasite pellet to obtain a parasite concentration of 4 - 5 x 10⁷ parasites/ml cytomix.

Note: Omit the addition of ATP and glutathione to cytomix since these additions do not improve the efficiency of gene targeting.

8. Thaw the 100 μl aliquot of the linearized pΔGOI from protocol 1.1 (step 26).

9. Transfer 0.3 ml of the parasite cytomix solution ($\sim 1.3 - 1.6 \times 10^7$ parasites) to the 100 μ l of linearized p Δ GOI from protocol 1.1 (step 26), and immediately transfer the entire 0.4 ml parasite + p Δ GOI mix to a chilled 2 mm gap electroporation cuvette.
10. Electroporate the parasites at 1.4 kV and 24 Ω .
11. Rest the transfection cuvette at room temperature for ~ 5 min then transfer the entire contents of the transfection cuvette into a 150 cm² flask containing confluent HFF cells with 30 ml infection medium and incubate the infected culture overnight.
12. Begin selection in mycophenolic acid + xanthine (MPA + X) ~ 20 hr post-transfection (**Figure 2A**) by replacing the infection medium with MPA + X selection medium (MPA (25 μ g/ml) and xanthine (50 μ g/ml) in infection medium).

Note: Do not disturb the incubating MPA + X selection flask.

13. Estimate the total number of PFUs in the MPA + X selection flask ~ 8 days post-transfection by visually inspecting the monolayer. Verify the presence of developing PFUs and healthy zones of infection by light microscopy.

Note: If plaques are not visible by day 8, maintain the selection and monitor for signs of infection since mutant strains may have a reduced replication rate.

Note: The $\Delta ku80\Delta hxgprt$ parasite strains have an extremely low background of undesired nontargeted events, which allows for the successful isolation of mutant strains with quite severe, but not lethal growth defects. If a gene is essential and it cannot be deleted, the primary transfection, or subsequently passed parasite population, may reveal a phenotype where the parasites cease to replicate during the selection as the population resolves to targeted knockouts.

14. Shake the flask as in step 3 to dislodge extracellular parasites from the monolayer [after visible plaques have developed] to generate a parasite solution. Transfer ~ 0.5 ml of the parasite solution from the MPA + X selection flask to a 25 cm² MPA + X selection flask containing confluent HFF cells (designate this culture pass 1A).
15. Dislodge parasites in the original 150 cm² MPA + X selection flask ~ 3 days later and transfer ~ 0.5 ml parasite solution to a second 25 cm² MPA + X selection flask containing confluent HFF cells (designate this culture pass 1B).
16. Allow zones of infection to develop in pass 1A and/or pass 1B flasks for ~ 5 days. Visually verify by light microscopy that pass 1A and 1B flasks contain a minimum of 25 viable PFUs with healthy zones of infection.
17. Choose either the pass 1A or pass 1B flask for continued passage in MPA + X selection medium in 25 cm² flasks. Maintain passage under MPA + X selection.

Note: Parasites must be cultured for a sufficient number of generations to delete nonintegrated persisting episomes from the population prior to subcloning. Do not perform subcloning prior to 20 days post-transfection for type I RH $\Delta ku80\Delta hxgprt$, or prior to 25 days post-transfection for type II Pru $\Delta ku80\Delta hxgprt$ parasites to allow sufficient time to dilute nonintegrated episomes^{1,2}.

18. Subclone parasites in a 96-well tray containing confluent HFF cells with MPA + X selection medium. Prepare one tray at a concentration of 1 parasite/well and a second tray at a concentration of 2 parasites/well.

Note: Subclone parasites that have recently lysed ($\sim 90 - 95\%$ of the HFF cells in the 25 cm² flask) to ensure that the parasites have high viability.

Note: The optimal time frame post-transfection for subcloning was established by measuring how rapidly successfully targeted parasites arise at a high frequency in the selected population¹.

19. Continue to passage the primary population of transfected parasites in MPA + X selection medium using a weekly passage schedule of infecting a 25 cm² HFF flask with 10 μ l of the parasite solution.

Note: If clones carrying the targeted gene deletion (knockouts) are not obtained from the first subcloning in step 18, resubclone the parasites from the continuously maintained population $\sim 10 - 12$ weeks post-transfection.

Note: One of the reasons a gene deletion is not obtained arises from the episomal persistence of some p Δ GOI plasmids. Episomal persistence is determined by the sequences contained in the 5' and the 3' genomic targeting flanks and does not appear to arise from the *HXGPRT* genetic element. Approximately 5 - 10% of targeting plasmids exhibit significant episomal persistence that necessitates a later time of subcloning to allow the parasite population a sufficient number of generation times to dilute the persisting episomes and resolve the population to primarily stable integrants.

20. Score the 96-well tray 6 - 7 days post-subcloning (type I parasites) or 7 - 8 days post-subcloning (type II parasites) for wells that contain a single PFU, identified as a single zone of infection in light microscopy at either 40X or 60X power. Mark a small dot on the 96-well tray lid to designate the location of the PFU in the well.
21. Mix the contents of each well containing a single PFU using a pipette set at 50 μ l (200 μ l tip) by directing fluid flow over the PFU to disperse parasites in the well.

Note: Mixing the well accelerates parasite lysis of the HFF monolayer. Type I RH strains will lyse the well ~ 4 days after mixing, type II Pru parasites will lyse the well ~ 5 days after mixing.

22. Select a dozen lysed wells (clones) and scratch across the bottom of each of these lysed wells using a 0.5 - 10 μ l pipette tip while simultaneously drawing-up 6 μ l of parasite solution. Transfer the 6 μ l of parasite solution to a well in a 24-well tray containing confluent HFF cells in 1 ml MPA + X selection medium.

Note: It is essential to scratch the bottom of the well to keep the opening bore of the pipette tip in constant contact with parasites residing at the bottom of the well.

23. Monitor the 24-well tray for lysis of the HFF cells.

Note: Type I RH parasites will typically lyse the well in the 24-well format in ~4 days. Type II Pru parasites will typically lyse the well in ~5 days.

24. Transfer 2 μ l of viable parasite solution scratched from the bottom of each well in the 24-well format to the corresponding well of a new 24-well tray containing confluent HFF cells and 1 ml MPA+X selection medium per well.

Note: All parasites clones and lines can be continuously maintained by passing 2 μ l of viable parasite solution every 7 days in this 24-well tray format.

25. Verify that parasites were successfully transferred to a new 24-well tray by visually inspecting with light microscopy ~18 hr post-passage.

26. Harvest parasites from the lysed wells of the 24-well tray from step 23 - 24 using either a 1 ml or 10 ml pipette and transfer the parasite solution to an Eppendorf tube.

27. Pellet the parasites at 1,400 \times g for 7 min, rinse once in 1 ml phosphate buffered saline (PBS), and pellet again at 1,400 \times g for 3 min.

28. Aspirate PBS from the pellet, then add 200 μ l of PBS to the pellet to resuspend the parasites. Freeze the parasite solution at -80 $^{\circ}$ C until DNA isolation.

29. Isolate parasite DNA from each clone using a tissue DNA isolation minikit.

30. Validate the targeted gene deletion (knockout) by PCR using validation primers (**Figures 2A-C**). Test for the absence of the functional coding region of the gene of interest and using the upstream CxF and downstream CxR genomic DNA primers (**Figures 2A-B**) test for the presence of PCR products that span the genomic targeting flanks and *HXGPRT* to demonstrate correct 5' and 3' targeted integration of the *HXGPRT* gene into the deleted gene locus.

Note: Primers for the validation must be unique to the gene of interest or a false positive result can be obtained. Primer design can be validated on <http://www.toxodb.org> by blasting primer sequences to the genome(s) to verify primers are unique.

31. Passage parasite clones on a weekly schedule as described in step 24. Once the targeted deletion has been validated, continuing selection in MPA+X is optional.

32. Archive parasite clones by preparing freezer stocks. Pellet extracellular parasites from lysed HFF cells in a 25 cm^2 culture flask, remove media and gently resuspend parasite pellet in cell culture freezing medium at a parasite concentration $>4 \times 10^7$ parasites per ml. Transfer aliquots to cryovials and store parasites indefinitely in liquid nitrogen, or at -80 $^{\circ}$ C.

2. Deletion of *HXGPRT*

This protocol is designed for removing the *HXGPRT* marker from its integration site in the genome of a genetically manipulated $\Delta ku80$ strain. Removal of *HXGPRT* allows for marker recovery and the generation of strains with multiple genetic manipulations using only selections based on *HXGPRT*¹⁻³. While the protocol detailed below describes the method to re-target a locus to delete *HXGPRT*, it should be noted that removal of *HXGPRT* at the gene locus of interest also permits simultaneous re-integration of the wild-type gene (complementation), re-integration of a mutant gene, as well as re-integration of a tagged gene (N- or C-terminal GFP, HA tag, etc.), allowing for a variety of genetic manipulations. The mechanisms of action²⁴ and targeting protocols using 6-thioxanthine selections have been previously described^{1-3,15}.

2.1 Delete *HXGPRT*

1. Excise the *HXGPRT* selectable marker from p Δ GOI by digesting with RE.Z to cut at the unique restriction enzyme sites flanking the *HXGPRT* (**Figure 1B**).

2. Verify complete digestion of DNA by agarose gel electrophoresis. Isolate the larger of the two bands from the agarose gel.

Note: The larger of the two bands contains the plasmid along with the 5' and 3' DNA target flanks, but not the *HXGPRT* gene.

3. Place the agarose section containing the larger DNA band in a spin column laying the gel flat on the membrane. Spin the column at 13,000 \times g for 4 min at RT. Add sterile H₂O to the flow-through to bring the volume to 100 μ l.

Note: Other commercial methods are available to isolate DNA from agarose.

4. Concentrate DNA by EtOH precipitation, resuspending the DNA in a final volume of 18 μ l sterile H₂O.

5. Mix 1 μ l of concentrated DNA with 7 ml H₂O, 1 μ l 10 \times ligation buffer and 1 μ l T4 DNA ligase (5 units) and place the reaction at 4 $^{\circ}$ C overnight to generate p Δ GOIc (p Δ GOIclean) (**Figure 3A**).

Note: DNA fragments with RE.Z containing DNA ends can be designed and included at this step to create plasmids suitable for targeted re-integration of the wild-type gene (complementation), targeted re-integration of a mutant gene as well as targeted re-integration of a tagged gene (N- or C-terminal GFP, HA tag, etc.).

6. Dilute the reaction 2 \times with sterile H₂O prior to electroporation.

7. Transform p Δ GOIc into *E. coli* as in protocol 1.1 to subclone, isolate, and validate the targeting plasmid. Then linearize p Δ GOIc in preparation for transfection (see steps 1.1.11 to 1.1.26).

8. Repeat the parasite transfection protocol as outlined in protocol 1.2 (steps 1 to 11).

Note: Prior to conducting steps 1 to 8, verify that targeted removal of *HXGPRT* is feasible in the mutant strain. Infect a 150 cm^2 flask of confluent HFF cells with 1×10^6 tachyzoites of the mutant strain using 6-thioxanthine (6TX) selection medium (200 μ g/ml 6TX in infection medium) and place the flask in a PFU assay. Inspect the flask 8 days later to verify that no (or very few; <10) PFU are visible, which is essential to establish that the potential gene targeting efficiency will exceed any potential spontaneous reversion of the mutant strain to a phenotype with reduced *HXGPRT* expression (a 6TX resistance phenotype).

Note: Approximately 5 - 10 % of *HXGPRT* integration sites exhibit a significant and spontaneous frequency of 6TX resistance even though the *HXGPRT* marker is still integrated at the targeted site (see Representative Results section for explanation).

9. Begin 6TX selection ~20 hr post-transfection by changing the medium in the 150 cm² flask to 6TX selection medium.

Note: Do not disturb the flask for ~10 days post-transfection.

10. Inspect the flask for PFU formation at day 10 - 12 post-transfection (type I parasites) or day 10 - 16 post-transfection (type II parasites).

Note: Parasites being targeted for the deletion of *HXGPRT* will not begin to grow under 6TX selection until the *HXGPRT* gene and mRNA is deleted, and the residual *HXGPRT* protein is inactivated.

11. Shake the selection flask to create a parasite solution, and transfer 0.5 - 1.0 ml of the parasite solution to a new 25 cm² flask containing confluent HFF cells and 5 ml 6TX selection medium. Repeat the transfer from the primary 150 cm² to new 25 cm² flasks once a day for two more days.

Note: Multiple sampling increases the chances of capturing viable targeted parasites that have lost the *HXGPRT* gene.

12. Inspect the 25 cm² flasks ~5 days after infection to verify the presence of developing PFUs with zones of healthy replicating parasites. Pick one or two flasks containing zones of infection.

Note: Parasites deleted of the *HXGPRT* gene replicate at a normal growth rate in 6TX selection.

13. Continue to passage the parasites in 6TX selection medium.

14. Subclone the parasite population after 25 - 30 days of selection. Set up one 96-well tray with ~ 1 parasite/well and another with ~ 2 parasites/well.

15. Prepare parasite DNA from isolated clones and maintain clones in a 24-well format culture according to protocol 1.2 (steps 19 to 29).

16. Validate deletion of *HXGPRT* by PCR using the strategy outlined in **Figures 3A-B**.

3. C-terminal Tagging of Proteins

This protocol is designed for C-terminal tagging of proteins by targeting the tag for integration via double cross over homologous recombination at the genomic locus of the gene using MPA + X selection^{2,4}. This protocol works efficiently because the 5' *dhfr* sequence of the *HXGPRT* marker is a fully validated functional 3' untranslated region for other genes^{2,4}.

3.1 Direct C-terminal tagging of proteins at endogenous genetic loci

1. Create targeting pΔGOI tag plasmid construct using yeast recombinational cloning (**Figure 4**) and methods described in protocol 1.1. The 5' genomic targeting flank contains the last 800 to 1,200 bp of coding region (or genomic DNA) of the GOI, except the termination codon is moved to a position 3' to the tag of choice (HA tag, Myc tag, His tag, etc.)
2. Create the targeted insertion of the C-terminal tag at the endogenous locus of the protein-coding gene by following the steps in protocol 1.1 and 1.2 using the strategy outlined (**Figure 4**).
3. Verify the insertion of the C-terminal tag at the endogenous gene locus using a PCR strategy.
4. Retarget the gene locus using methods described in protocol 1 and protocol 2 to delete the *HXGPRT* selectable marker to create a precisely regulated endogenous gene locus that expresses a tagged protein. This protocol also recovers the *HXGPRT* selectable marker that can be used again to target another locus in the tagged strain (see protocol 1).

Representative Results

A detailed template is provided for constructing a targeting plasmid to delete a gene, including the placement of restriction enzyme sites and generation of the primers that facilitate genetic targeting and validation of gene targeting, as well as plasmid construction for the subsequent deletion of *HXGPRT* in a single-step process (**Figures 1A-C, Figure 2A, Figure 3A**). A general schematic is presented for making a targeted gene deletion (**Figure 2A**), the primer pairs used to validate the deletion of a knockout, for example, type I *rop18* (**Figure 2B**), and representative results of the PCR validation are shown (**Figure 2C**). This representative result is shown to illustrate the range of results that can be obtained at genetic loci that are relatively difficult to target. A successfully targeted gene deletion will result in the absence of the gene of interest PCR product (PCR 1), the presence of the 3' genomic targeting flank (PCR2), and the presence of the *HXGPRT* selectable marker properly integrated between the 5' and 3' genomic targeting flanks that define the deletion (PCR3 and PCR4). Clones 3, 4, 5, 6, 8, 9, and 11 are validated targeted gene deletions (knockouts) where *HXGPRT* has replaced the GOI (**Figure 2C**).

A clone that does not contain a gene deletion can be represented by a variety of banding patterns. Typically, a clone without a gene deletion will mirror the parental strain (parental pattern) with bands observed for PCR1 and PCR2, but not for PCR3 or PCR4 as seen for clones 1 and 2 (**Figure 2C**). This "parental pattern" arises from a few potential mechanisms. Occasionally, MPA resistant selected parasites carry nonintegrated persisting episomes of the pΔGOI targeting plasmid, and we note that continued selection often forces these episomes to integrate. We also observe some rare background in the type I Δ*ku80* strain due to unintended integration of the pΔGOI targeting plasmid, which contains a *HXGPRT* cDNA expressed via *DHFR* 5' and 3' promoter elements, into either the *DHFR* locus or into the partially deleted *HXGPRT* locus¹⁴. While this rare event is a targeted integration, it was not the intended integration and serves as a key point to remember in designing any gene replacement strategy. DNA homology greater than 120 bp carried on your pΔGOI targeting plasmid may provide an alternative site for recombination in Δ*ku80* strains² that could give back an undesired background. In the type II Pru Δ*ku80* strain this background is reduced or nonexistent compared to type I because the *HXGPRT* selectable marker is based on type I sequences that have nucleotide polymorphisms when compared with type II DNA which greatly reduces this rare background in experiments using the type II Pru Δ*ku80* strain¹. If a gene locus

is essential (cannot be deleted), or has an extremely low gene targeting frequency at the locus, or if persisting [nonintegrated] episomes are not easily eliminated via growth and selection, this parental pattern will dominate the pattern observed in MPA resistant clones. Alternatively, the targeting DNA molecule is sometimes observed to integrate at only the 5' or the 3' genomic targeting flank and a band is observed for either PCR3 or PCR4 (but not both) along with PCR1 and PCR2 as seen for clone 10 (5' integration) and clone 7 (3' integration) (Figure 2C). These patterns suggest the infrequent occurrence of a single cross over integration of the targeting plasmid at the locus that integrates *HXGPRT* but this targeted integration does not delete the gene of interest. This pattern (lanes 7 & 10 in Figure 2C) emphasizes the need to report PCR data to verify that targeted integration occurred instead of a single homologous cross over and a second nonhomologous integration of the targeting molecule. Rarely, we observe a genetic mixture represented by clone 12 (Figure 2C) that represents both a parental pattern and a targeted deletion pattern. This pattern most likely arises on occasion from two parasite genotypes that are present at the same location in a cloning well.

A general schematic for the removal of *HXGPRT* from $\Delta ku80\Delta goi::HXGPRT$ to delete *HXGPRT* from the strain is shown (Figure 3A). The primer pair used to validate the removal of *HXGPRT* from $\Delta ku80\Delta gra2::HXGPRT$ (Figure 3B) amplifies a unique ~1.2 Kbp band in PCR5 as seen in clones 4, 7, 9, 11 and 12 (Figure 3C). If *HXGPRT* is not removed from the locus, a ~3.4 Kbp band is observed (clones 1, 2, 3, 6, 8, 10, and the parental control). Several mechanisms act to make the removal of *HXGPRT* (6TX selection) more challenging and less efficient than the integration of *HXGPRT* (MPA + X selection). MPA selection is simply more efficient than 6TX selection^{14,16}. In addition, higher levels of *HXGPRT* expression are needed for 6TX selection than for MPA selections^{16,24}. Consequently, mutations that may reduce *HXGPRT* enzyme activity or mutations or epigenetic changes that reduce the expression level of *HXGPRT* at a gene's locus can potentially abolish the effectiveness of 6TX selection. Even with these challenges of 6TX selection, the success rate of 6TX selection in $\Delta ku80$ strains is greater than 90% on the first attempt¹⁻³.

A general scheme for direct C-terminal tagging of protein-coding genes is shown (Figure 4). This scheme uses direct integration via double cross over homologous recombination of *HXGPRT* 3' of the tagged gene and also employs validation strategies similar to those described above. When a gene is suspected to be an essential gene this can be verified using a second transfection with an independently isolated targeting DNA plasmid. Alternative methods, such as schemes for regulated gene expression, are available for further verifying if a gene is essential²⁵.

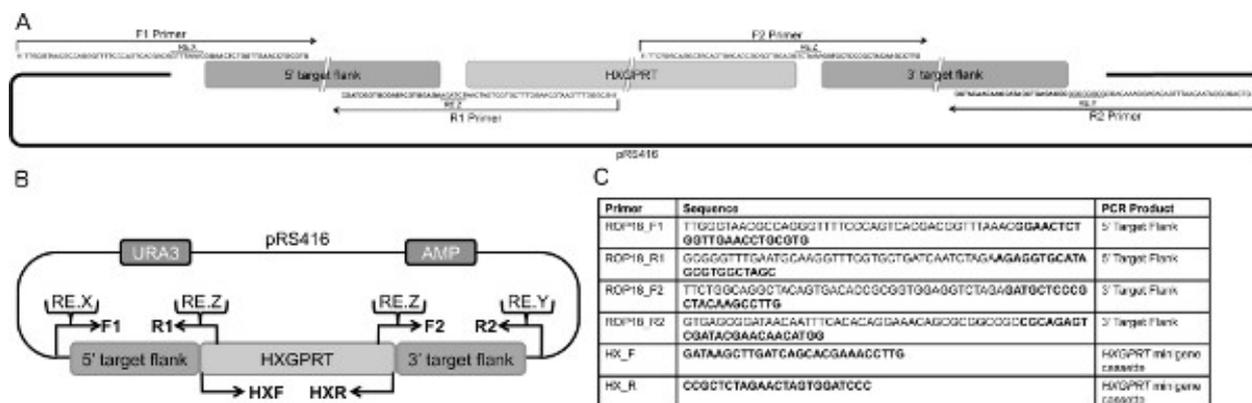


Figure 1. Overview of the design of a targeting DNA plasmid. **A.** Schematic for designing overlap primers used to PCR amplify the 5' and 3' target flanks with 33 bp overlaps for the pRS416 shuttle vector and *HXGPRT* minigene cassette. Primers depicted in the schematic were used to generate the 5' and 3' target flanks of p Δ ROP18¹⁰. **B.** Overall strategy for designing a targeting DNA molecule. The backbone of p Δ GOI is the pRS416 shuttle vector containing uracil (URA) and ampicillin (AMP) selectable markers. Inserted into pRS416 is a ~1 Kbp 5' DNA target flank amplified from genomic DNA with overlaps for the *HXGPRT* minigene cassette and pRS416 using the F1 and R1 primers, a ~1 Kbp 3' DNA target flank is amplified from genomic DNA with overlaps for the *HXGPRT* minigene cassette and pRS416 using the F2 and R2 primers and the *HXGPRT* minigene cassette. The following unique restriction enzyme digest sites are added to the primers: RE.X (restriction enzyme X cut site) in the F1 primer to cut the plasmid at the 5' end of the 5' DNA target flank, RE.Y in the R2 primer to cut the plasmid at the 3' end of the 3' DNA target flank and RE.Z in the R1 and F2 primers to excise the *HXGPRT* minigene cassette. **C.** Primer sequences used to generate the targeting construct for the deletion of *rop18*. The **bold** regions correspond to *T. gondii* genomic sequence, and the nonbold regions correspond to the restriction enzyme sites and sequences that overlap with pRS416 and the *HXGPRT* minigene cassette. The HX_F and HX_R primer pair amplifies the *HXGPRT* minigene cassette¹⁴. Primers read from 5' to 3'. Table adapted from Fentress *et al*¹⁰. [Click here to view larger figure.](#)

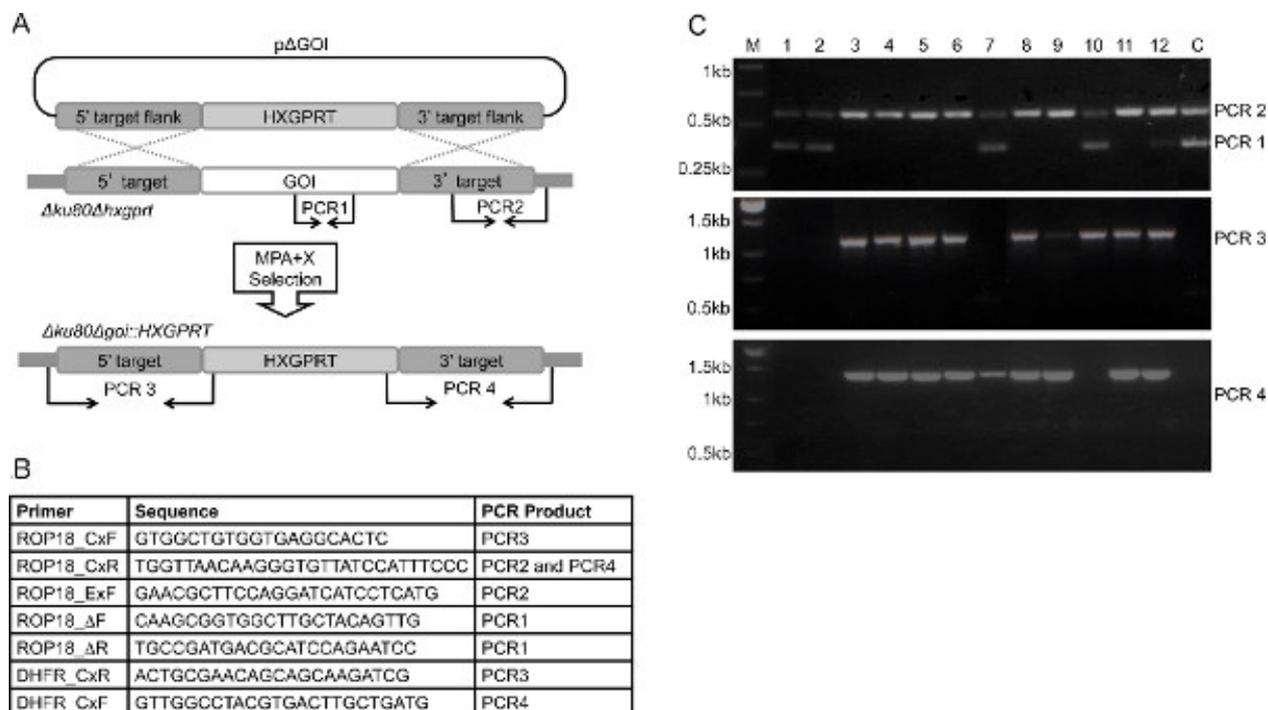


Figure 2. Overview of the protocol for the deletion of a gene using HXGPRT. **A.** Disruption of a GOI in the $\Delta ku80\Delta hxgprt$ strain by a double crossover homologous recombination event using a ~1 Kbp 5' DNA target flank and a ~1 Kbp 3' DNA target flank on plasmid pΔGOI. A PCR strategy using PCR1, PCR2, PCR3, and PCR4 (not to scale) is shown that enables genotype verification to validate clones with targeted integration of HXGPRT and deletion of the GOI locus. **B.** Representative primer pairs designed to validate the deletion of *rop18*. ROP18_DF and ROP18_DR amplify PCR1, ROP18_ExF and ROP18_CxR amplify PCR2, ROP18_CxF and DHFR_CxR amplify PCR3 and DHFR_CxF and ROP18_CxR amplify PCR4 (see **Figure 2A**). Primers read from 5' to 3'. Table adapted from Fentress *et al*¹⁰. **C.** Representative results of validation of a targeted gene deletion (*rop18*) using HXGPRT. Following transfection of plasmid pΔROP18 into $\Delta ku80\Delta hxgprt$ and selection in MPA+X, MPA+X resistant clones were assayed for the deletion of *rop18*. The parental strain control is positive for PCR 1 (~400 bp) and PCR 2 (~650 bp) product and negative for PCR 3 (~1,200 bp) and PCR 4 (~1,300 bp) product. A targeted GOI knockout is positive for the PCR2, PCR3 and PCR4 products and is negative for the PCR 1 product (see **Figure 2A**). Shown are representative panels of the results of PCR1 and PCR2 (*top panel*), PCR3 (*middle panel*), and PCR4 (*bottom panel*). Clones 3, 4, 5, 6, 8, 9 and 11 exhibit the correct banding pattern of PCR 1, PCR2, PCR3 and PCR4 that defines a targeted gene deletion event in the clone. Clones 1, 2, 7, 10 and 12 are not gene deletions, and correspond to other potential representative results. (C = parental control strain $\Delta ku80\Delta hxgprt$, M = size markers). [Click here to view larger figure.](#)

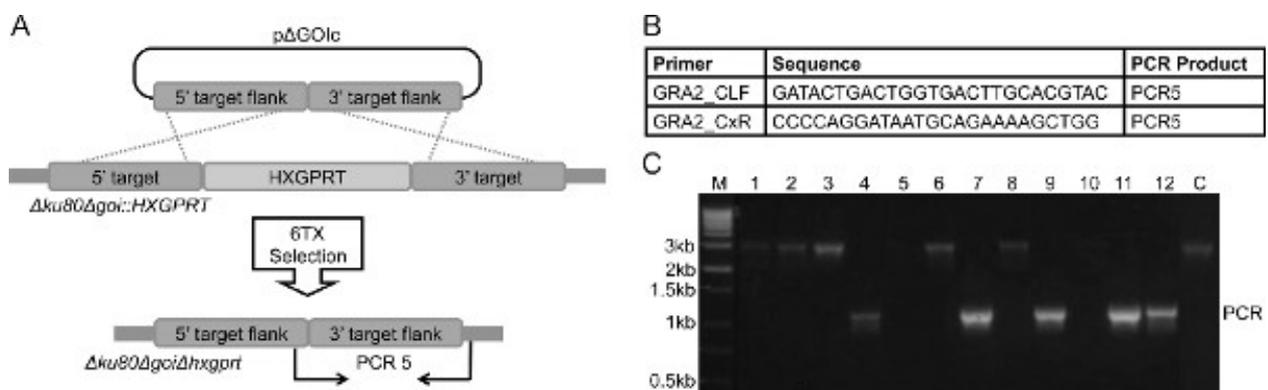


Figure 3. Overview of the protocol for re-targeting the gene of interest to delete HXGPRT. **A.** Strategy for removal of the HXGPRT selectable marker by a double crossover homologous recombination event in the strain $\Delta ku80\Delta goi::HXGPRT$ using a ~1 Kbp 5' DNA target flank and a ~1 Kbp 3' DNA target flank on plasmid pΔGOIc. The PCR strategy for genotype verification is depicted using a primer pair to assay for a PCR product (PCR5) that spans the deletion (not to scale). **B.** A representative primer pair designed to validate the removal of the HXGPRT selectable marker from the *gra2* locus in strain $\Delta ku80\Delta gra2::HXGPRT$. Primers GRA2_CLF and GRA2_CxR amplify PCR5. Primers read from 5' to 3'. **C.** Representative panel of 6TX-resistant clones that may be obtained following transfection of plasmid pΔGOIc into $\Delta ku80\Delta goi::HXGPRT$ and selection in 6TX. Clones 4, 7, 9, 11 and 12 exhibit the correct banding pattern of PCR5 that corresponds to targeted deletion of the HXGPRT marker (~1.2 Kbp product that spans the 3' flank with slight overlap with the 5' flank and outside the 3' flank). Clones 1, 2, 3, 6, 8, and 10 (this band is light) represent the expected banding pattern of around ~3.4 Kbp that corresponds to the parental clone genotype, even though these clones exhibited a degree of resistance to 6TX that permitted their selection (this phenotype can occasionally arise spontaneously by shut-down of HXGPRT expression (see note in protocol 2.1 (step 8) and Representative Results section). (C = parental control strain $\Delta ku80\Delta goi::HXGPRT$, M = size markers). [Click here to view larger figure.](#)

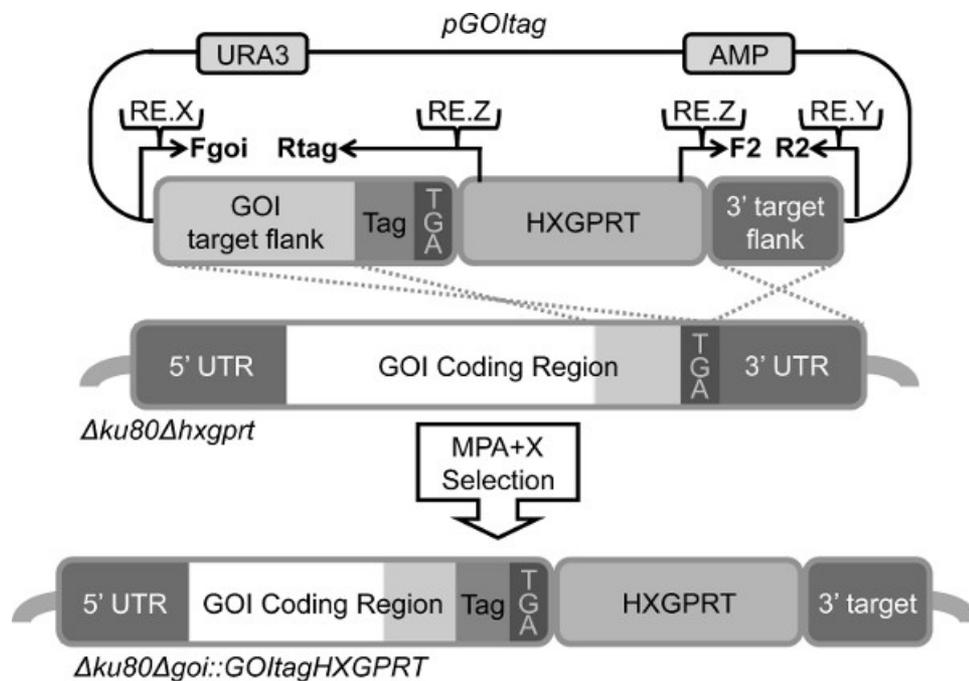


Figure 4. Design of a targeting DNA molecule to tag the C-terminal end of a protein. The strategy is based on the ability of the *HXGPRT* marker to also function as a 3' downstream regulatory region. The backbone of pΔGOLtag is the pRS416 shuttle vector containing uracil (URA) and ampicillin (AMP) selectable markers. Inserted into pRS416 is a ~1 Kbp 5' DNA target flank amplified from genomic DNA with overlaps for the *HXGPRT* minigene cassette and pRS416 using the Fgoi and Rtag primers, a ~1 Kbp 3' DNA target flank is amplified from genomic DNA with overlaps for the *HXGPRT* minigene cassette and pRS416 using the F2 and R2 primers and the *HXGPRT* minigene cassette. The 5' DNA target flank replaces the termination codon with the tag (HA, Myc, His, etc.) followed by a replacement termination codon. MPA + X selection integrates the C-terminal tag and a downstream *HXGPRT*, and moves the 3' UTR to a position following the *HXGPRT* marker. The following unique restriction enzyme digest sites are added to the primers: RE.X (restriction enzyme X cut site) in the Fgoi primer to cut the plasmid at the 5' end of the 5' DNA target flank, and RE.Y in the R2 primer to cut the plasmid at the 3' end of the 3' DNA target flank, and RE.Z in the Rtag and F2 primers to excise the *HXGPRT* minigene cassette for use for plasmid construction to re-target the tagged gene locus to delete the *HXGPRT* marker which will restore the placement of the 3' UTR.

Discussion

Here we provide a protocol for efficient gene targeting in *Δku80* parasite strains to enable the efficient recovery of genetically manipulated progeny that possess targeted gene deletions, gene replacements, and/or tagged genes. The sequential execution of these methods provides a reliable approach for the isolation of parasite mutants that contain single or multiple targeted genetic manipulations¹⁻³. While the generation of a targeted deletion depends on multiple factors, the use of the *Δku80* strain with the presented strategy and protocol greatly increases the efficiency and ease of making precisely defined mutant strains of *T. gondii* for functional genomic studies.

The genome of *T. gondii* during asexual stages is haploid. Thus a consideration to make when planning to delete a gene is that the gene must not be essential *in vitro*. Nonetheless, the efficiency of targeting gene knockouts in *Δku80* strains is extremely high, and this enables the isolation of mutant strains with significantly impaired replication rates. If a targeted gene is essential, parasites often cease to replicate during selection. Another critical factor in targeted genetic manipulation is that perfect homology with at least 620 bp of the genomic targeting flank is required to obtain detectable targeted integrations². Longer regions of homology produce higher efficiencies of targeting and using targeting DNA flanks of approximately 1,000 bp is a reliable and efficient approach¹⁻⁴. For this reason it is important that genomic targeting flanks are generated from the same strain of *T. gondii* (RH, Pru) as the strain that is being genetically manipulated. Lack of sufficient homology can frequently result in the targeted integration of one genomic targeting flank, but not the other. Incomplete integration or failure to obtain a knockout may also be due to episomal persistence. Immediately after transfection, all of the targeting molecules are nonintegrated episomes, and sometimes targeting molecules can persist as episomes for many generations. Using target DNA flanks of ~1 Kbp and continuing to pass parasites under selection prior to re-subcloning frequently resolves problems associated with episomal persistence.

Once a knockout is validated and the *HXGPRT* marker is removed, the gene targeting strategy can be repeated on a second GOI to generate multiple gene deletions in a single parasite strain¹⁻³. The generation of deletions, replacements, or tagged genes can also be accomplished by using different selectable markers (*bleomycin*, *CAT*, pyrimethamine resistant *DHFR*, etc.). Although the *CAT* selectable marker currently is present in the type II *Δku80* Pru genome¹, this marker can be removed using the *HXGPRT* selection protocol described here. In our hands, *HXGPRT* selection is the safest and the most efficient marker with the highest targeting efficiency where a single-copy targeted insertion is sufficient for robust selection in MPA + X medium. *HXGPRT* also provides the only currently useful approach for targeted deletion of a selectable marker (Figure 3) using 6-thioxanthine (6TX) selection^{2,3}. Thus this protocol provides the only current approach for developing defined mutant strains with several targeted genetic manipulations.

This protocol provides a valuable and efficient method for targeted genetic manipulation in *Δku80* strains of *Toxoplasma gondii* and is applicable to analysis of a single gene, a family of genes, or genome-wide functional genomic studies. Prior to the availability of *Δku80* strains^{1,2}, these

approaches were not widely feasible due to the inefficiency of these methods. Consequently, this protocol is widely applicable for targeted genetic manipulation of *Toxoplasma gondii* and is accessible to all investigators interested in addressing a range of questions on parasite biology, host response, and functional genomics.

Disclosures

The authors declare that they have no competing financial interests.

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