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An improved method for introducing site-directed point mutation into the *Toxoplasma gondii* genome using CRISPR/Cas9

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Abstract

Toxoplasma gondii is an obligate intracellular parasite in the phylum Apicomplexa. Due to the ease of genetic manipulations in *T. gondii* it serves as a model organism for intracellular parasites. We utilized CRISPR/Cas9, which can be designed to target a specific genomic locus, to introduce site-directed point mutations directly into the *T. gondii* genomes. This paper contains step-by-step protocols for: (1) designing the guide RNA sequence; (2) constructing the CRISPR/Cas9 construct for a target gene and preparing a donor sequence; and (3) transfection of the CRISPR/Cas9 modules into the parasite and selecting the parasite with the desired point mutation. In brief: *T. gondii* strains PRU *ku80 hvgprt* or RH *ku80 hvgprt* were nucleofected with pDHFRSAG1::Cas9-U6::sgGeneA and a mutation donor sequence at a molar ratio of ~1:3. After 10 days of 1 μ M pyrimethamine selection the parasite population was enriched for parasites with the desired point mutation. This technique was also applied to evaluate the importance of genes of interest by introducing either knock out or silence mutations at the same time and then tracking the population kinetics of the resultant *T. gondii* strains. In addition to previously established high efficient knock out and knock in strategies in *Toxoplasma*, the site directed point mutation technique presented in this manuscript provides another powerful tool set for *T. gondii* research.

Keywords

CRISPR/Cas9; Genetic manipulation; Site-directed mutation; *Toxoplasma gondii*

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Background

Toxoplasma gondii is an obligate intracellular protozoan pathogen in phylum Apicomplexa that causes disease in humans and other animals. Conventional forward genetic approaches employing sexual crosses of parental lines in the cat have been used to elucidate critical virulence factors in this pathogen [1,2]. In addition, forward genetic approaches introducing random mutations throughout the whole genome followed by analysis of responsible SNPs by next generation sequence have been used for the identification of genes responsible for drug resistance [3,4] and essential genes for the secretion of parasite proteins [5]. Following identification of the genes of interest by forward genetic approaches or other biochemical approaches, reverse genetic approaches are usually applied to investigate the functions of these genes. In *T. gondii* conventional gene knock out using ~1 kbp homologous region flanking non-essential genes has become a well established genetic analysis technique [6]; however, the efficiency of making transgenic parasites is decreased due to non-homologous integration of exogenous DNA fragments into the parasite genome in a sequence independent fashion [7]. To minimize this problem strains that have a knockout of the responsible gene, TgKU80, for random insertion of the exogenous DNA into the parasite genome have been developed and these KU80 strains display highly efficient homologous recombination [8,9]. The currently available reverse genetic techniques for *T. gondii* utilize the insertion of a selectable marker at the target locus to enrich transgenic parasites. A limitation of these techniques has been the difficulty in introduction of a SNP into the target locus without also introducing a selectable marker at the target locus. A recent breakthrough in the manipulation of *T. gondii* is the use of CRISPR/Cas9 techniques for gene manipulation in this parasite [10,11]. The CRISPR/Cas9 system utilizes Cas9 nuclease guided by an RNA sequence which recognizes a complement sequence [12]. In this paper, we report improved step-by-step protocols that utilize the CRISPR/Cas9 system to introduce site-directed point mutations combined with drug selection to enrich the mutated parasite population without inserting a selectable marker into the target locus.

Objective

The objective of the protocol herein is to allow the creation of a site-directed point mutation in the *Toxoplasma gondii* genome (Figure 1), as an example we show the results of the site-directed point mutation of 'Target Gene A'.

A second objective is to utilize this point mutation technique to evaluate the importance of a target gene by tracking the population dynamics of the parasite after introducing several point mutations into the target gene at the same time. For example, studies of the population dynamics that demonstrated a decrease of a mutant allele for which a premature stop codon was introduced compared to a silent mutation allele provide evidence that the knock out of the target gene affects the growth of the parasite.

Methods

Materials

Parasites and DNA plasmids—RH *ku80 hxgprt* (ATCC: PRA-319) [8] or PRU *ku80 hxgprt* [9] were used in this protocol. Detailed protocols for maintaining parasite cultures with host cells have been previously published [13]. The parental plasmid pSAG1::Cas9-U6::sgUPRT [10] can be obtained through Addgene (Addgene #54467). We created the plasmid pDHFRSAG1::Cas9-U6::sgUPRT (Figure 2A) which has a pyrimethamine resistant type *Toxoplasma gondii* DHFR-TS (M2M3) marker [14] in the parental plasmid for these studies. To introduce the DHFR selectable marker into pSAG1::Cas9-U6::sgUPRT, the parental plasmid was linearized with primers 5'-ATCGATAAGCTTATTTAAATTCCTGCAAGTGCATAGAAG-3' and 5'-ATTACAGCCTGGCGTTTACATCCGTTGCCTTTTC-3', and the selectable DHFR marker was amplified with primers 5'-ATTTAAATAAGCTTATCGATACCGT-3' and 5'-CGCCAGGCTGTAAATCCCGT-3' from the pLIC-3HA-DHFR (Generous gift from Dr. Michael White) and ligated to create the new plasmid using Gibson Assembly® Master Mix (E2611S) (New England BioLabs, Ipswich, MA, USA) [15]. Briefly, PCR amplified fragments were purified with NucleoSpin® Gel and PCR Clean-up kit (Clontech, Mountain View, CA, USA), around 10 ng per 1 kbp DNA of plasmid and equal molar insert (90 ng for the 9 kbp parental plasmid and 40 ng 4 kbp DHFR insert) were mixed in 1 µl DNA elution buffer (5mM Tris-HCl, pH 8.5), followed by addition of 1 µl 2 × Gibson Assembly Master Mix and by incubation at 50 °C for 60 min. Assembled DNA was used for transforming *Escherichia coli* DH5alpha with standard techniques.

Procedures

Step 1. Designing the guide RNA (gRNA) sequence for a target gene—The gRNA designing tool for eukaryotic pathogens [16] <http://grna.ctegd.uga.edu/> was used to find a high efficiency gRNA target sequence followed by the Protospacer Adjacent Motif (PAM) nucleotide sequence of NGG. For “Target Gene A” at least 100 bp around the targeted Ser 25 site was searched for gRNA candidates using the following parameters; (1) PAM sequence allows NGG only; and (2) background genome uses *Toxoplasma gondii* genome (genotype I GT1 strain for searching gRNA for RH strain, or genotype II ME49 strain for Pru strain). For “Target Gene A” the gRNA sequence of GCTTGGAAGATGACGCCTTT adjacent to the target Ser 25 site was chosen.

Step 2. Constructing a Cas9 and single guide RNA (sgRNA) expressing plasmid and donor sequence plasmid—To make a plasmid with the appropriate gRNA sequence for the target sequence, primers was designed as described in Figure 2B. Briefly, a primer of 20 bp of target sequence followed by 20 bp designed to anneal to the sgRNA scaffold region of the parental template plasmid (red sequence in Figure 2B) was used as a forward primer. The reverse primer was designed to anneal to 20 bp before the gRNA targeting site (shown in blue sequence in Figure 2B). As a plasmid template, plasmids with any target gRNA sequence can be used for construction as the target gRNA of parental plasmid will be replaced with the gRNA sequence of interest in this step. Replacing the gRNA sequence was performed with the designed primers and the Q5® Site-Directed

Mutagenesis Kit (New England BioLabs, E0552S). Briefly, 1.25 µl 2 × KLD Reaction Buffer, 0.25 µl PCR reaction, 0.75 µl distilled water and 0.25 µl KLD Enzyme Mix were mixed and incubated at room temperature for 5 min and used for the transformation of *E. coli* DH5alpha with standard techniques. The resultant plasmid was designated pDHFR-SAG1::Cas9-U6::sgGeneA. From this plasmid, cas9 nuclease and sgRNA were expressed in the transfected parasite to form the RNA guided nuclease complex at the target genome locus to make a double strand DNA break.

To introduce the specified mutation at the target site, we prepared the donor sequences with the desired mutations. Briefly, 850 bp region of gene A around the target site was amplified by PCR with Donor_F and Donor_R from the gDNA of the parental strain (Figure 2C). For the cloning of the amplified region, we used the CloneJet PCR cloning system (#K1231) (Thermo Fisher Scientific, MA, USA) according to the manufacture's instructions. After cloning of the wild type sequence, the mutation was introduced to replace the PAM sequence without replacing the coding amino acid (as depicted in Figure. 2C the CGG motif in the antisense sequence was mutated to CGT and the mutation site in Donor_mut_Reverse is shown in red text). Another mutation was introduced to replace the target amino acid coding codon using the primers (as depicted in Figure 2C the mutation sites are shown in red text in Donor_mut_S25Ser_F and Donor_mut_S25Stop_F) using the Q5 site-directed mutation kit to produce pDonor-GeneA-mut-[S25Ser or S25Stop]. These mutations in the guide RNA target sequence prevent the cas9 nuclease from breaking the already mutated sequence, extensively.

Step 3. Transfection and selection of the mutated parasites—The overall transfection steps are shown in the Figure 3. For transfection, the donor plasmid pDonor-GeneA-mut-[S25Ser or S25Stop] was amplified with PCR primers Donor_F and Donor_R. Following this PCR, 10 µg of circular pDHFR-SAG1::Cas9-U6::sgGeneA plasmid and the linearized donor DNA 1 µg each (total 2µg) was used for the transfection. The molar ratio of the pDHFR-SAG1::Cas9-U6::sgGeneA (13,234 bp) and donor DNA (850 bp) was 1:3. Mixed DNA was ethanol precipitated and washed once with 70 % ethanol and the precipitated DNA was then resuspended in 5 µl TE buffer. Parasites were nucleofected using the Basic Parasite Nucleofector® Kit 1 (Lonza, Basel, Switzerland) according to manufacture's instructions. Briefly, parasites (Pru *ku80 hxgprt* or RH *ku80 hxgprt*) and infected host cells were harvested from a 50% lysed out culture and passaged through a 27G needle three times to make sure that all host cells were lysed. For transfection from 10⁶ to 10⁷ parasites can be used and host cell debris does not need to be removed. Harvested parasites were pelleted by centrifugation at 2,000 × g for 5 min at room temperature, the supernatant was aspirated completely, and then the parasites were re-suspended in 100 µl of nucleofection buffer (82 µl Basic Parasite Nucleofector® solution 1 supplemented with 18 µl Supplement P1). A 100 µl of the parasite suspension was mixed with DNA solution and transferred to a cuvette (provided as a component of the kit). Parasites were nucleofected using program U-033 and parasites were immediately inoculated into confluent HFF cells grown in a T25 flask containing selection medium (1 µM pyrimethamine in the 10% FBS DMEM) at 37°C. Parasites were then incubated at 37°C for several days until parasite growth could be seen.

Step 4. Checking the mutation—After pyrimethamine resistant parasites visibly start growing (around 4 days with RH *ku80 hxgprt* and 7 days with Pru *ku80 hxgprt*) drug selection was removed. The parasite population was then examined for the presence of the mutation. Genomic DNA was purified from parasite infected host cells using conventional methods at day 0 and day 10 and day 25 after nucleofection (Figure. 3) using Wizard® Genomic DNA Purification Kit (A1120) (Promega, Madison, WI, USA). Briefly, infected host cells were scraped from T25 and centrifuged to get the cell pellet in a microcentrifuge tube. The cell pellet was vortexed to break the packed pellet, followed by the addition of 100 µl Nuclei Lysis Solution and then vortexed for 30 sec to cause cell lysis. Following cell lysis, 33 µl Protein Precipitation Solution was added, the sample was vortexed for 30 sec, then incubated on ice for 5 min and the solution was clarified by centrifugation at $15,000 \times g$ for 5 min at 4 °C. DNA was then precipitated from the cleared lysates by adding 100 µl isopropanol to the supernatant, followed by centrifugation at $15,000 \times g$ for 15 min at room temperature, and then the resultant DNA pellet was washed with 70 % ethanol once before dissolved in 25 µl TE buffer. PCUR was performed on the isolated DNA using primers Donor_F and Outside_R which amplify 1 kbp around the target locus (Figure. 2C) and the amplified DNA was purified and used for direct sequencing.

Step 5. Tracking the mutation allele population in the competitive assay—The results in Figure 4 demonstrate the chromatogram of DNA sequences of Pru *ku80 hxgprt* nucleofected with pDHFR-SAG1::Cas9-U6::sgGeneA and two types of donor sequence (one carrying the mutation which introduce a premature stop codon TAA and one carrying a silent mutation TCC for the wild type Ser 25 TCA). As shown in Figure 4 in the blue box, by day 10 after nucleofection the target locus had been replaced with mutated donor sequence as indicated in the replaced base in the PAM region from CCG (Day 0) to ACG (Day 10), (PAM sequence was located on anti-sense strand, thus the PAM sequence of CGG had been replaced with CGT). Thus, 10 days was enough to enrichment for the mutants. The day 10 chromatogram demonstrates that almost half of the population is replaced with the S25Ser donor and half is replaced with the S25stop donor as indicated in the mixed base of C (from the Ser 25 codon donor) and A (from the stop codon donor) at the second codon base of Ser 25 (Figure. 4. red box). This is consistent with the equal amount of each plasmid used in this transfection, i.e. a 1:1 ratio of these two mutations was used. However, by day 25, one can see a decrease in the amount of parasites with an “A” in the second base in the Ser25 codon (Figure 4 bottom panel), suggesting that the introducing a stop codon TAA in Gene A has affected the ability of this parasite to grow in standard culture conditions and that the TCC (neutral mutation in the same base) or TCA (wild type codon) are becoming the dominant parasites in culture (i.e. a time dependent demonstration of a lack of fitness of the stop codon mutant).

Special Remarks/Comments

When several mutation type donors are transfected at the same time followed by tracking which type of mutation(s) is enriched over time, one can use this technique to evaluate the fitness of different mutant alleles, even if the function of the gene is quite important (i.e. ‘essential’). In conventional genetic manipulation an inability to obtain a gene knock out

was considered an indication of the essentiality of the gene; however, in this system the importance of the gene can be directly demonstrated by following the kinetics of the mutation as a disappearing population of the knock out parasite (or mutated parasite), providing clear evidence of gene fitness that is not just inferred from a lack of obtaining a transformant parasite line (which could be due to other issues besides a gene being essential).

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Highlights

- CRISPR/Cas9 makes it possible to introduce point mutation(s) into the *Toxoplasma gondii* genome
- A point mutation can be introduced without inserting the selectable marker into the gene locus
- Drug selection with a marker on the Cas9 construct enriched transgenic parasites
- A competitive assay herein provides a quick evaluation of the effects of a target gene mutation on population dynamics.

Figure 1. A scheme for introducing site-directed point mutations into the *Toxoplasma gondii* genome

A brief scheme of the protocol is shown. A guide RNA (gRNA) target sequence is selected (Step 1) and introduced into the pDHFR-SAG1::Cas9-U6::sgGOI (Step 2A) plasmid. Donor sequences carrying the mutation (Red or Green) at the target site (Step 2B) are used for co-transfection into parental parasite (step 3). To enrich the parasite population that contains the desired DNA by transfection, pyrimethamine selection is used. Following this selection, analysis of the mutated site is performed using PCR and sequencing (4A and 4B).

Figure 2. Preparing Cas9 and sgRNA expressing plasmid and constructing the mutation donor plasmids

(A) A schematic diagram of the pDHFR-SAG1::Cas9-U6::sgGOI is shown. DHFR drug selectable marker is inserted head to head to the GFP fused Cas9 protein expression cassette flanked by the gene expression regulatory sequence of SAG1. The sgRNA expression cassette is located after the Cas9 protein expression cassette.

(B) The scheme of designing the primers to introduce the 20 bp target gRNA sequence for geneA in the parental plasmid pDHFR-SAG1::Cas9-U6::sgGOI is shown. 20 bp gRNA sequence region is flanked by U6 promoter (shown in blue text) and sgRNA scaffold (shown in red text). The gRNA target region is underlined and is located in the anti-sense strand of the Gene A coding sequence. The PAM sequence is shown in green text.

(C) A schematic diagram of the mutation donor sequence is shown. Primers which are used to amplify the donor sequence region (Donor_F and Donor_R) and the primers which are used to amplify the mutated genome locus (Donor_F and Outside_R) are shown above the genomic sequence of gene A. Mutation sites are shown in red text. Target amino acid site Ser 25 is shown in *italicized and underlined* text with translation.

Figure 3. Co-transfection and selection of the mutant parasites

A scheme of co-transfection and drug selection is shown. PCR was used to amplify two mutation donor sequences that were then transfected with the pDHFR-SAG1::Cas9-U6::sgGeneA plasmid. Parasite samples before incubation and those after incubation for 10 days and 25 days were collected to purify gDNA to evaluate the dynamics of the mutation frequency at the target locus.

Figure 4. Sequence chromatogram of the mutated locus

DNA fragment amplified from gDNA samples at various time points after transfection with primers Donor_F and Outside_R (Figure2C) were sequenced. A representative sequence chromatogram around the target site is shown. The gRNA target sequence in the anti-sense strand is shown with green arrow. The PAM sequence in the anti-sense strand is shown in a blue box. Codon for the target amino acid site Ser25 is shown in a red box. Each base A, T, G and C is shown respectively as a Green, Red, Blue or Black peaks.