Video Article

CRISPR/Cas9 Gene Editing to Make Conditional Mutants of Human Malaria Parasite *P. falciparum*

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Abstract

Malaria is a significant cause of morbidity and mortality worldwide. This disease, which primarily affects those living in tropical and subtropical regions, is caused by infection with *Plasmodium* parasites. The development of more effective drugs to combat malaria can be accelerated by improving our understanding of the biology of this complex parasite. Genetic manipulation of these parasites is key to understanding their biology; however, historically the genome of *P. falciparum* has been difficult to manipulate. Recently, CRISPR/Cas9 genome editing has been utilized in malaria parasites, allowing for easier protein tagging, generation of conditional protein knockdowns, and deletion of genes. CRISPR/Cas9 genome editing has proven to be a powerful tool for advancing the field of malaria research. Here, we describe a CRISPR/Cas9 method for generating *glmS*-based conditional knockdown mutants in *P. falciparum*. This method is highly adaptable to other types of genetic manipulations, including protein tagging and gene knockouts.

Video Link

The video component of this article can be found at https://www.jove.com/video/57747/

Introduction

Malaria is a devastating disease caused by protozoan parasites of the genus *Plasmodium*. *P. falciparum*, the most deadly human malaria parasite, causes approximately 445,000 deaths per year, mostly in children under the age of five[1]. *Plasmodium* parasites have an intricate life cycle involving a mosquito vector and a vertebrate host. Humans first become infected when an infected mosquito takes a blood meal. Then, the parasite invades the liver where it grows, develops, and divides for approximately one week.After this process, the parasites are released into the bloodstream, where they undergo asexual replication in red blood cells (RBCs). Growth of the parasites within the RBCs are directly responsible for the clinical symptoms associated with malaria[2].

Until recently, production of transgenic *P. falciparum* was a laborious process, involving several rounds of drug selection that took many months and had a high failure rate. This time-consuming procedure relieson the generation of random DNA breaks in the region of interest and the endogenous ability of the parasite to mend its genome though homologous repair[3,4,5,6]. Recently, Clustered Regularly Interspaced Palindromic Repeat/Cas9 (CRISPR/Cas9) genome editing has been successfully utilized in *P. falciparum*[7,8]. The introduction of this new technology in malaria research has been critical for advancing understanding of the biology of these deadly *Plasmodium* parasites. CRISPR/Cas9 allows for specific targeting of genes through guide RNAs (gRNAs) that are homologous to the gene of interest. The gRNA/Cas9 complex recognizes the gene through the gRNA, and Cas9 then introduces a double-strand break, forcing the initiation of repair mechanisms in the organism[9,10]. Because *P. falciparum* lacks the machinery to repair DNA breaks through non-homologous end joining, it utilizes homologous recombination mechanisms and integrates transfected homologous DNA templates to repair the Cas9/gRNA-induced double-strand break[11,12].

Here, we present a protocol for the generation of conditional knockdown mutants in *P. falciparum* using CRISPR/Cas9 genome editing. The protocol demonstrates usage of the *glmS* ribozyme to conditionally knockdown protein levels of PfHsp70x (PF3D7_0831700), a chaperone exported by *P. falciparum* into host RBCs[13,14]. The *glmS* ribozyme is activated by treatment with glucosamine (which is converted to glucosamine-6-phosphate in cells) to cleave its associated mRNA, leading to a reduction in the protein[14]. This protocol can be easily adapted to utilize other conditional knockdown tools, such as destabilization domains or RNA aptamers[4,5,15]. Our protocol details the generation of a repair plasmid consisting of a hemagglutinin (HA) tag and *glmS* ribozyme coding sequence flanked by sequences that are homologous to the PfHsp70x open reading frame (ORF) and 3'-UTR. We also describe the generation of a second plasmid to drive expression of the gRNA. These two plasmids, along with a third that drives expression of Cas9, are transfected into RBCs and used to modify the genome of *P. falciparum* parasites. Finally, we describe a polymerase chain reaction (PCR)-based technique to verify integration of the tag and *glmS* ribozyme. This protocol is highly adaptable for the modification or complete knockout of any *P. falciparum* genes, enhancing our ability to generate new insights into the biology of the malaria parasite.

Protocol

Continuous culture of *P. falciparum* requires the use of human RBCs, and we utilized commercially purchased units of blood that were stripped of all identifiers and anonymized. The Institutional Review Board and the Office of Biosafety at the University of Georgia reviewed our protocols and approved all protocols used in our lab.

1. Choosing a gRNA Sequence

1. Go to CHOPCHOP (http://chopchop.cbu.uib.no/) and select *'Fasta Target'*. Under *'Target'*, paste the 200 base pairs from the 3' end of the open reading frame (ORF) of a gene and 200 base pairs from the start of the gene's 3'-UTR. Under *'In'*, select *'P. falciparum'* (3D7 v3.0), and select *'CRISPR/Cas9'* under *'Using'*. Next, click *'Find Target Sites'*.
2. Select a gRNA sequence from the options presented, giving preference to the most efficient gRNA that is closest to the site of modification and that has the fewest off-target sites.
   NOTE: Potential gRNA sequences are identified because they are immediately upstream of a Protospacer Adjacent Motif (PAM), which is required for recruitment of Cas9 to DNA. The sequence that is cloned into pMK-U6, the vector that drives gRNA expression, is the 20 bases immediately upstream of the PAM. The PAM specific for *S. pyogenes* Cas9 is the nucleotide sequence NGG and should not be included in the sequence that is cloned into pMK-U6.
   NOTE: CHOPCHOP visually ranks the gRNA sequences, displaying the best options in green, the less ideal options in amber, and the worst options in red. CHOPCHOP gives each gRNA sequence an efficiency score that is calculated using the most up-to-date parameters found in the literature, and they predict off-target sites that could be recognized by the gRNA. Two or three gRNA sequences may need to be attempted to find the gRNA best suited to a particular gene.
3. Purchase the gRNA sequence and its reverse-complement as Polyacrylamide Gel Electrophoresis-purified oligos. The gRNA sequence used to target PfHSP70x can be found in **Figure 1B**.
   NOTE: This oligo should include 15 base pairs homologous to the gRNA-expressing plasmid, which are necessary for sequence and ligation-independent cloning (SLIC) into the pMK-U6 vector[16].

2. Cloning the gRNA Sequence into pMK-U6

1. **Digest the pMK-U6 with BtgZI.**
   1. Digest 10 µg of pMK-U6 with 5 µL of BtgZI enzyme (5,000 units/mL) for 3 h at 60 °C. Follow the enzyme manufacturer's protocol for reaction conditions.
   2. After the 3 h incubation period, add an additional 3 µL of BtgZI to the reaction to ensure complete digestion of the plasmid. Digest for an additional 3 h, still following the manufacturer's instructions for ensuring the correct reaction conditions.
   3. To purify the digested pMK-U6 from the reaction, use a column-based PCR cleanup kit according to the manufacturer's instructions.
   4. Separate the digested DNA using a 0.7% agarose gel and extract the 4,200-base pair band.

2. **Anneal the oligos containing the gRNA sequence.**
   1. Reconstitute the PAGE-purified oligos to a concentration of 100 µM using nuclease-free water.
   2. Combine 10 µL of each oligo with 2.2 µL of 10x buffer 2 (see **Table of Materials**). Ensure that the total reaction volume is 22.2 µL.
   3. Run the gRNA annealing program in a thermocycler: Step 1: 95 °C, 10 min; step 2: 95 °C, 1 s, with a reduction in temperature of 0.6 °C/cycle; step 3: Go to step 2, 16 times; step 4: 85 °C, 1 min; step 5: 85 °C, 1 s, with a reduction in temperature of 0.6°C/cycle; step 6: Go to step 5, 16 times; step 7: 75 °C, 1 min; step 8: 75 °C, 1 s, with a reduction in temperature of 0.6 °C/cycle; step 9: Go to step 8, 16 times. Steps 10 to 21: Repeat the procedure used in Steps 4–9 until the temperature reaches 25 °C; step 22: 25 °C, 1 min.

3. **Insert the annealed gRNA oligos into the BtgZI-digested and gel-purified pMK-U6 plasmid.**
   1. Combine 100 ng of digested pMK-U6 with 1 µL of 10x buffer 2.1 and 3 µL of annealed gRNA oligos. Increase the volume to 9.5 µL with nuclease-free water.
   2. Add 0.5 µL of T4 polymerase and incubate the reaction at room temperature for 2.5 min.
   3. Move the reaction to ice and incubate for 10 min.
   4. Immediately transform 5 µL of the reaction into competent *E. coli* according to the bacteria supplier's instructions. Plate the bacteria on Lysogeny Broth (LB) agar plates containing 100 µg/mL Ampicillin.
   5. Allow the transformed bacteria to grow at 37 °C overnight, then select colonies and extract DNA with a commercially available plasmid miniprep kit.

3. Designing Homology Regions of the Repair Template

1. **Design shield mutations within the homology repair template to prevent re-cutting of the DNA that is integrated into the genome.**
   NOTE: A shield mutation typically consists of introducing a silent mutation to alter the PAM so that Cas9 will not induce a break in the repair template. The PAM required for the Cas9 used in this protocol is the nucleotide sequence "NGG", where "N" is any nucleotide. If possible, change one of the G nucleotides to an A, C, or T.
   1. If the PAM cannot be silently mutated, introduce at least 2 silent mutations into the 6 base pairs directly adjacent to the PAM[7,8].
      NOTE: These mutations will prevent recognition of the repair template by the gRNA and prevent re-cutting of the repaired locus by the Cas9/gRNA complex. The shield mutations can be introduced into the homology region by amplifying the DNA with primers that contain the mutation.

2. **Amplify the ORF homology region for the repair template.**

1. Using PCR, amplify 800 base pairs from the 3' end of the target gene's ORF. Design the primers that will be used to exclude the stop codon from this amplicon.
2. Design the primers for insertion of this amplicon into the pHA-*glmS* that has been digested with SacII and AfeI through either a DNA ligation reaction or SLIC[16].

3. **Amplify the 3'-UTR homology region for the repair template.**
    1. Using PCR, amplify the 800 base pairs immediately following the stop codon of the target gene. Design primers for insertion of this amplicon into the pHA-*glmS* that has been digested with HindIII and NheI through either a DNA ligation reaction or SLIC[16].
    NOTE: The high AT content of the *P. falciparum* genome can make amplification of regions such as UTRs difficult. An alternative approach to using PCR is synthesizing the homology regions.

4. Cloning Homology Regions into the Repair Plasmid

1. **Insert the ORF homology region into the pHA-*glmS*.**
    1. Digest the pHA-*glmS* with SacII and AfeI, according to the enzyme manufacturer's instructions. Insert the ORF homology region PCR product into the digested plasmid using SLIC[16].
    2. Transform into competent *E. coli* as performed in steps 2.3.4 and 2.3.5.

2. **Insert the 3'-UTR homology region into a pHA-glmS plasmid that already contains the ORF homology region (see step 4.1).**
    1. Digest the plasmid with HindIII and NheI according to the enzyme manufacturer's instructions. Insert the 3'-UTR homology region amplicon into the digested plasmid using SLIC[16].
    2. Transform into competent *E. coli* and extract the plasmid DNA (steps 2.3.4 and 2.3.5).

5. Precipitating DNA for Transfection

1. Add 40 µg each of pMK-U6, pUF1-Cas9, and pHA-*glmS* DNA (for a total of 120 µg of DNA) into a sterile 1.5 mL microcentrifuge tube.
2. Add 1/10th the volume of DNA of 3 M sodium acetate in water (pH 5.2) to the tube and mix it well using a vortex (*e.g.,* if the volume in step 5.1 was 100 µL, add 10 µL of sodium acetate).
3. Add 2.5 times the volume of 100% ethanol to the tube and mix it well using a vortex for at least 30 s (*e.g.,* if the volume in 5.1 was 100 µL, add 250 µL of 100% ethanol).
4. Place the tube on ice or at -20 °C for 30 min.
5. Centrifuge the tube at 18,300 x *g* for 30 min at 4 °C.
6. Carefully remove the supernatant from the tube. Do not disturb the pellet.
7. Add 3 times the volume of 70% ethanol to the tube and mix it briefly using a vortex (*e.g.*, if the volume in 5.1 was 100 µL, add 300 µL of 70% ethanol).
8. Centrifuge the tube at 18,300 x *g* for 30 min at 4 °C.
    NOTE: This step should be performed under sterile conditions in a biological safety cabinet.
9. Carefully remove the supernatant from the tube. Do not disturb the pellet. Leave the tube open and allow the pellet to air-dry for 15 min.
10. Store the precipitated DNA at -20 °C until it is needed for transfection.

6. Isolating Human RBCs from Whole Blood in Preparation for Transfection

1. Aliquot fresh blood into sterile 50 mL conical tubes (approximately 25 mL per tube).
2. Centrifuge the tubes at 1,088 x *g* for 12 min, with centrifuge brakes set to 4.
3. Aspirate off the supernatant and buffy coat. Resuspend the RBC pellet with an equal volume of incomplete RPMI.
    Note: Incomplete RPMI is prepared by supplementing RPMI 1640 with 10.32 µM thymidine, 110.2 µM hypoxanthine, 1 mM sodium pyruvate, 30 mM sodium bicarbonate, 5 mM HEPES, 11.1 mM glucose, and 0.02% (v/v) gentamicin.
4. Repeat steps 6.2–6.3 twice. After the last wash, resuspend the RBCs in an equal volume of incomplete RPMI and store the tubes at 4 °C.

7. Transfecting RBCs with the CRISPR/Cas9 Plasmids (To Be Done Aseptically)

NOTE: *P. falciparum* cultures are maintained as described in other reports[17]. Maintain all the cultures at 37 °C under 3% O_2, 3% CO_2, and 94% N_2 unless stated otherwise. Whenever blood is used in this protocol, it is referring to the pure red blood cells prepared in step 6. The blood used should not be older than 6 weeks, as there is typically a decrease in parasite proliferation in older blood. The following steps describe pre-loading RBCs with DNA and adding a parasite culture to the transfected cells. Other established transfection protocols are compatible with transfecting these constructs[18,19].

1. Prepare a 1x cytomix buffer in water (120 mM KCl, 0.15 mM $CaCl_2$, 2 mM EGTA, 5 mM $MgCl_2$, 10 mM K_2HPO_4, 25 mM HEPES, pH 7.6). Filter-sterilize the buffer using a 0.22 µm filter.
2. Add 380 µL of the 1x cytomix to the DNA precipitated in step 5, and vortex to dissolve. Allow the DNA to dissolve in the 1x cytomix for 10 minutes, vortexing every 3 min for 10 s.
3. In a sterile 15 mL conical tube, combine 300 µL of RBCs (50% hematocrit, from step 6) in the incomplete RPMI with 4 mL of 1x cytomix.
4. Centrifuge the RBCs from step 7.3 at 870 x *g* for 3 min, and then remove the supernatant from the RBC pellet.
5. Resuspend the RBC pellet with the DNA/cytomix mixture from step 6.2 and transfer to a 0.2 cm electroporation cuvette.
6. Electroporate the RBCs using the following conditions: 0.32 kV, 925 µF, capacitance set to "High Cap", and resistance set to "Infinite".

7. Following electroporation, transfer the contents from the cuvette to a 15 mL conical containing 5 mL of complete RPMI (cRPMI). Centrifuge the tube at 870 x g for 3 min at 20 °C, and then decant the supernatant.
    NOTE: cRPMI is prepared through the same method as incomplete RPMI with the addition of 0.25% (w/v) lipid-rich bovine serum albumin.
8. Resuspend the pellet in 4 mL of cRPMI and transfer to one well in a 6-well tissue culture plate. Add 400 μL of a high-schizont culture (7–10% schizont parasitemia is ideal) to the transfected RBCs.
    NOTE: Parasitemia is defined as the percentage of parasite-infected RBCs.
9. The next day, wash the culture with 4 mL of cRPMI. Centrifuge the culture at 870 x g for 3 min and aspirate the supernatant. Resuspend the culture in 4 mL of cRPMI.
10. 48 h after completing step 7.6, wash the culture with 4 mL of cRPMI. Then resuspend the culture in cRPMI containing 1 μM DSM1 to select for the Cas9 plasmid.
11. **Continue washing the cultures each day with cRPMI until parasites are no longer visible by blood smear. After this point, replace the culture medium with fresh cRPMI plus 1 μM DSM1 every 48 h.**
    1. To make a blood smear, pipette 150 μL of culture into a 0.6 mL centrifuge tube. Pellet the cells by centrifugation at 1,700 x g for 30 s.
    2. Aspirate off the supernatant. Use a pipette to transfer the pelleted cells to a glass slide. Using a second glass slide held at a 45° angle to the first slide, smear the blood droplet. Stain the slide using a commercially available staining kit according to the manufacturer's protocol.
    3. View the parasites using a 100X oil immersion objective.

12. Beginning 5 days post-transfection (step 7.6), remove 2 mL of the culture with RBCs resuspended in the culture medium. Add back 2 mL of fresh medium (cRPMI plus 1 μM DSM1) and blood at 2% hematocrit. Add fresh blood in this manner once a week until parasites reappear, as determined by thin blood smear (step 7.11).
    NOTE: If integration is successful, parasites generally reappear in the culture by one-month post-transfection.
13. Once parasites reemerge, remove DSM1 drug pressure. Alternatively, remove drug pressure after parasites have been cloned out.

8. Checking Parasites for Integration of the Repair Template

1. When parasites are visible again by thin blood smear, isolate DNA from the culture using an appropriate kit.
2. **Use PCR to amplify the modified region of the genome to determine if the targeted locus has been successfully altered and if the unmodified wild-type locus (indicative of wild-type parasites) is detectable.**
    1. To detect parasites that have integrated the repair template, use a forward primer that sits at the beginning of the ORF, outside of the cloned homology region. Use a reverse primer that sits in the 3'-UTR.
        NOTE: As this amplification includes the sequences of the HA tags and *glmS* ribozyme, amplicons from integrated parasites will be longer than the same region amplified in wild-type parasites.

9. Cloning Parasites by Limiting Dilution

1. **Perform serial dilutions of the parasite culture from step 7.13 to achieve a final concentration of 0.5 parasites/200 μL. Add 200 μL of the diluted culture to the wells of a 96-well tissue culture plate.**
    NOTE: Because parasitemia is defined as the percentage of infected RBCs and the hematocrit is also a defined number (2%), the number of parasites per unit volume is easily inferred.
    1. Prepare 1 mL of culture in cRPMI at 5% parasitemia and 2% hematocrit (at these parasitemia and hematocrit levels, the culture contains 1×10^7 parasites/mL).
    2. Dilute this culture 1:100 with cRPMI. Dilute again 1:100 with cRPMI.
    3. Dilute 1:400. Perform this dilution by adding 62.5 μL of culture to 25 mL of cRPMI and 1 mL of blood. This dilution results in the desired concentration of 0.5 parasites/200 μL.

2. **Maintain the cloning plate until parasites are detectable in the wells.**
    1. Every 48 h, replace the medium in the 96-well plate with fresh medium.
    2. Once a week, starting 5 days after beginning the cloning plate (step 9.1), remove 100 μL from each well and add back 100 μL of fresh medium + blood (2% hematocrit).

3. **Identify any wells containing parasites.**
    1. Place the 96-well plate at a 45° angle for approximately 20 min, allowing the blood to settle at an angle within the plate.
    2. Place the 96-well plate on a light box. Notice that the wells containing parasites contain media that is yellow in color, compared to the pink media of parasite-free wells, due to acidification of the medium by the parasites.
    3. Using a serological pipette, move the contents of the parasite-containing wells to a 24-well tissue culture plate to allow expansion of the parasitemia.
    4. Using PCR analysis as described in step 8, check these clonal parasite lines for correct integration.

10. Knockdown of the Protein by Treating Parasites with Glucosamine and Confirmation via Western Blot Analysis

1. Prepare a 0.5 M GlcN (glucosamine) stock solution, which can be stored at -20° C.
2. Add GlcN to the *glmS* parasite cultures and allow them to grow in the presence of GlcN.

NOTE: The final concentration and timing of GlcN treatment depends on the experiment and parasite line. GlcN can impact parasite growth, so the parental parasite strain should be exposed to a range of GlcN concentrations to determine its sensitivity to the compound. Often, a concentration of 2.0-7.5 mM GlcN is used[13,14,20].

3. Isolate protein samples from the GlcN-treated parasites[13].
4. **Use protein samples for western blot analysis to detect reductions in the protein[13].**
    1. Use an anti-HA antibody according to the manufacturer's instructions to detect the HA-glmS-tagged protein. Compare the HA band to a loading control, such as PfEF1α.

Representative Results

A schematic of the plasmids used in this method as well as an example of a shield mutation are shown in **Figure 1**. As an example of how to identify mutant parasites after transfection, results from PCRs for checking integration of the HA-*glmS* construct are shown in **Figure 2**. A representative image of a cloning plate is shown in **Figure 3** to demonstrate the color change of the medium in the presence of parasites. Results from an immunofluorescence assay and western blotting experiments are shown in **Figure 4** to demonstrate the functionality of the HA tag and *glmS*-based reductions of proteins in the parasites. **Figure 5** demonstrates the inability of short homology arms on PCR products to modify the parasite genomes and obtain viable mutants.
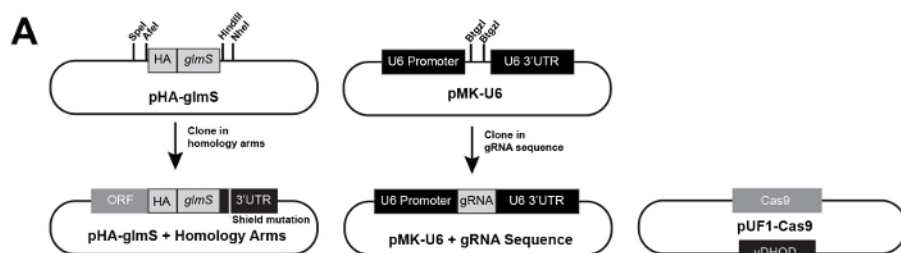

Figure 1: Summary of our three-plasmid approach to CRISPR/Cas9 and examples of a gRNA oligo and shield mutation. (A) Schematics of empty pHA-glmS and pMK-U6 are shown with the restriction enzyme sites used for cloning. Also shown are pHA-glmS and pMK-U6 after the homology arms and gRNA sequences have been cloned into them, respectively. Finally, pUF1-Cas9 is shown. yDHOD = yeast dihydrofolate reductase, the resistance marker to DSM1. **(B)** The forward oligo used for cloning the PfHsp70x gRNA sequence into pMK-U6 is shown, with the gRNA sequence in capital letters and the pMK-U6 homology arms necessary for cloning in lowercase letters (top). The genomic target of the PfHsp70x gRNA is shown as the downstream PAM, in red (middle). The shield mutation in the PfHsp70x gRNA PAM is shown in red (bottom). Please click here to view a larger version of this figure.
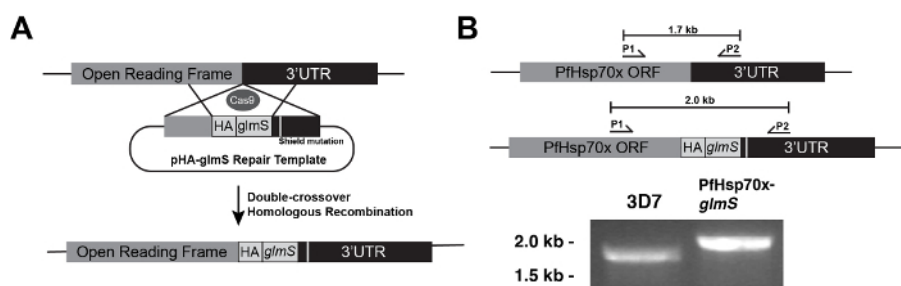
Figure 2: Schematic of CRISPR/Cas9 genome modification using pHA-glmS and a strategy for confirming integration. (A) Cas9, guided to a genomic locus by a gRNA, induces a double strand break in the DNA. The parasite repairs the damage through double crossover homologous repair, using the pHA-glmS plasmid as a template and introducing the HA-glmS sequence into the genome. **(B)** A PCR test to identify correct integration of the HA-glmS sequence. Using primers P1 and P2, the 3' ORF of wild-type PfHsp70x and PfHsp70x-*glmS* mutants are amplified[13]. The amplicon from PfHsp70x-glmS is longer than wild-type due to insertion of the HA-glmS sequence. Please click here to view a larger version of this figure.
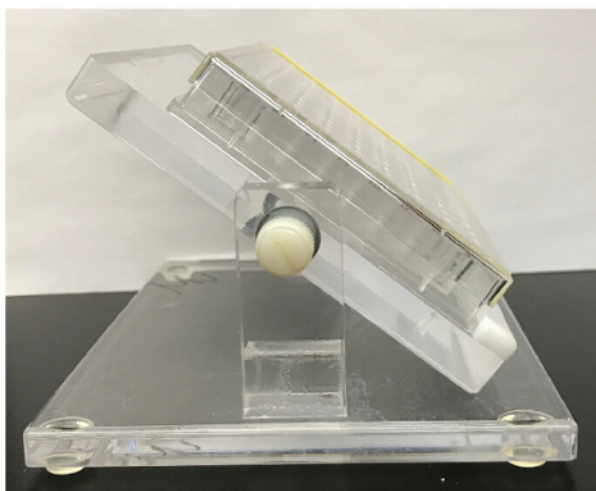
**Figure 3: Identification of wells containing parasites in a 96-well cloning plate.** (**A**) The 96-well plate is set at a 45° angle for approximately 20 min to allow the blood to settle at an angle in the plate. (**B**) The well on the left contains a parasite culture, indicated by the yellow color of the medium in comparison to the pink medium of the parasite-free well on the right. Please click here to view a larger version of this figure.
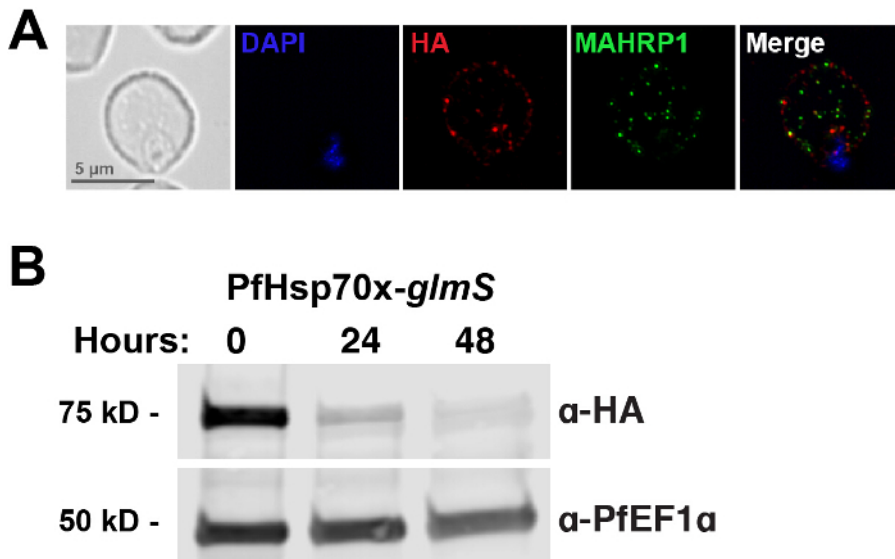
Figure 4: An immunofluorescence assay shows the correct HA-tagging of PfHsp70x and Western blotting shows reduction of PfHsp70x protein levels during treatment with glucosamine. (**A**) PfHsp70x-*glmS* parasites were fixed and stained with DAPI (nucleus marker) and antibodies to HA and MAHRP1 (Membrane Associated Histidine Rich Protein 1, a marker of protein export to the host RBC)[13]. (**B**) PfHsp70x-*glmS* parasites were treated with 7.5 mM glucosamine, and whole-parasite lysates were used for Western blotting analysis[13]. The membrane was probed with antibodies for HA and PfEF1α as a loading control[13]. As expected, glucosamine treatment resulted in a reduction of the protein. Please click here to view a larger version of this figure.
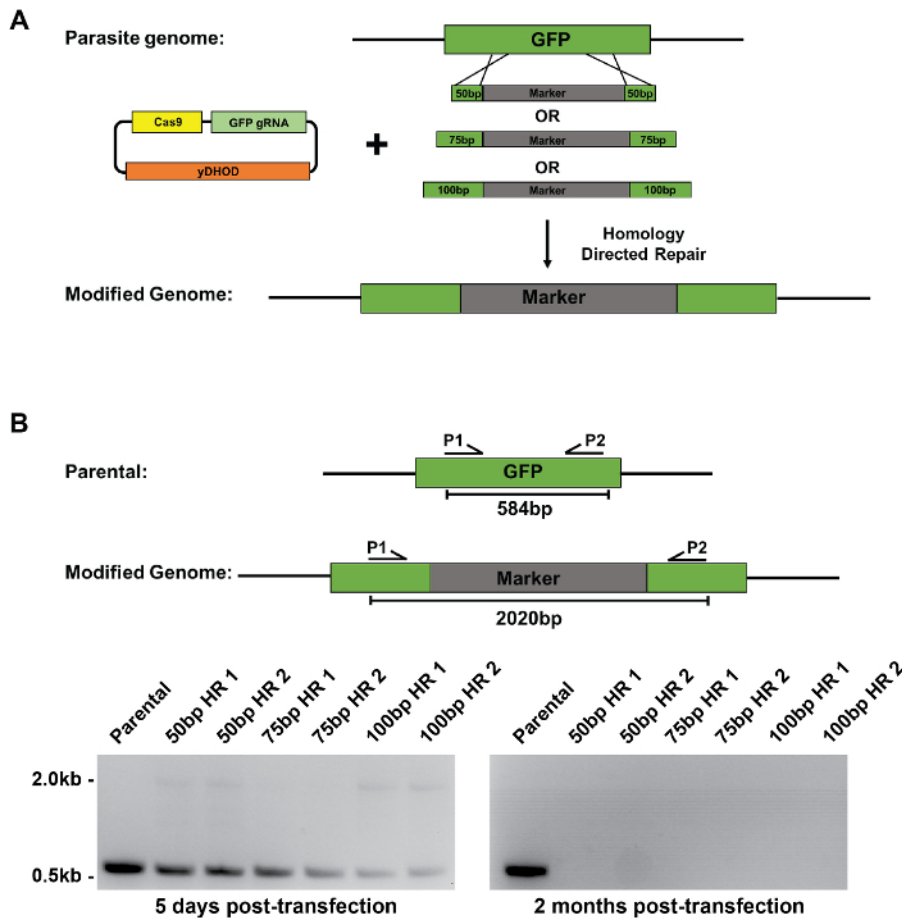
Figure 5: Using short homology sequences for repair. (**A**) Schematic representation showing knockout of GFP in B7 parasites[21]. B7 parasites are a derivative of 3D7 in which Plasmepsin II has been tagged with GFP. PCR products containing 50, 75, or 100 base pairs of GFP homology regions flanking a blasticidin S resistance cassette (labeled "marker"), along with pUF1-Cas9-eGFP-gRNA, a plasmid expressing Cas9 and a GFP gRNA, were transfected into B7 parasites. Each transfection was carried out twice. DSM1 drug pressure was applied 2 days post-transfection. (**B**) Shown here are PCR tests on DNA isolated from transfected parasites 5 days post-transfection and 2 months post-transfection. Primers used to test integration of the BSD resistance cassette will yield a 584-base pair product for B7 parental parasites and a 2020-base pair product for parasites that have integrated the marker. Please click here to view a larger version of this figure.

Discussion

The implementation of CRISPR/Cas9 in *P. falciparum* has both increased the efficiency of and decreased the amount of time needed for modifying the parasite's genome, compared to previous methods of genetic manipulation. This comprehensive protocol outlines the steps necessary for generating conditional mutants using CRISPR/Cas9 in *P. falciparum*. While the method here is geared specifically for the generation of HA-*glmS* mutants, this strategy can be adapted for a variety of needs, including the tagging of genes, gene knockouts, and the introduction of point mutations.

A critical early step in this protocol is the selection of a gRNA sequence. When selecting a gRNA, there are several points to consider such as where the gRNA sits, how efficient it is, and whether or not it has the potential for off-target effects. The gRNA sequence should be as close as possible to the site of modification, ideally within 200 base pairs. This decreases the likelihood of the parasites using the repair template to fix their genome without integrating the tag. The tool used here to locate the gRNA was a free, online service called CHOPCHOP[22]. Another online tool, Eukaryotic Pathogen CRISPR guide RNA/DNA Design Tool (EuPaGDT; http://grna.ctegd.uga.edu/), can also be used[23]. EuPaGDT provides additional characterization of gRNA sequences, including prediction of off-target hits and potential issues that may prevent transcription of the gRNA. EuPaGDT also has tools for batch processing of gRNAs to target multiple genes or whole genomes. The selected gRNA should be one that sits closest to the site of modification with the highest efficiency and minimal off-target hits. An important limitation of CRISPR/Cas9 gene editing that may arise is the inability to design a suitable gRNA to target the gene of interest. In such cases, a trial-and-error approach may be needed, using multiple sub-optimal gRNA sequences until the best option is found, and successful gene editing has occurred.

Another important factor to consider when generating *P. falciparum* mutants using CRISPR/Cas9 is the length of the homology regions used in the repair template. This protocol recommends that the homology regions should be approximately 800 base pairs each, but we have also been successful in using smaller regions numbering 500 base pairs[3]. Successful genome modification using CRISPR/Cas9 and short homology arms on PCR products have also been used in other protozoan parasites such as *Toxoplasma gondii* and *Trichomonas vaginalis*[24,25]. We tested the feasibility of using smaller homology arms on PCR products (50, 75, or 100 base pairs) by attempting to knockout GFP in B7 parasites using a blasticidin resistance cassette[21]. We saw some integration of the blasticidin resistance cassette at 5 days post transfection; however, these

parasites never recovered from transfection. For these transfections, we selected for the Cas9-expressing plasmid using DSM1. A different selection method, such as treating transfected cultures with blasticidin S alone or in combination with DSM1, may improve the chances of parasites reappearing when using shorter homology regions for repairing the Cas9/gRNA-induced breaks. In this case, we did not select with blasticidin S since we wanted to test if short homology arms could be used in instances where a drug resistance cassette is not being integrated into the genome, such as when a protein is being tagged.

The core components of CRISPR/Cas9 gene editing discussed are the Cas9 endonuclease, the gRNA, and the repair template. We describe a three-plasmid approach to introduce these components into the parasites, where Cas9, the gRNA, and the repair template are found in separate plasmids. In addition to this approach, our lab has been successful in using a two-plasmid approach in which Cas9 and gRNA expression are driven by a single plasmid and the repair template is found in a second plasmid[3]. Similar two-plasmid approaches have also been successfully employed by other labs to generate mutants[7,8,26,27,28,29]. Furthermore, several labs are using a strain of *Plasmodium* (NF54[attB]) which constitutively expresses Cas9 and a T7 RNA polymerase to drive expression of gRNAs[30]. In this case, a single plasmid containing the repair template and the gRNA are transfected into NF54[attB] parasites[31,32]. Finally, a plasmid-free approach utilizing a purified Cas9-gRNA ribonucleoprotein complex has been used to insert mutations into the genome, as well[33]. The success of these different approaches demonstrates flexibility of the methods in which researchers can introduce Cas9/gRNA components into the parasite.

Finally, the choice of drug pressure to apply to transfected parasites can be altered, depending on constructs used. Here, we show successful generation of mutants by transiently selecting for the Cas9-expressing plasmid using DSM1 until parasites reappear. To generate PfHsp70x knockout parasites, *pfhsp70x* was replaced with the human dihydrofolate reductase gene, and parasites were then selected using WR99210[13]. The recently described TetR-PfDOZI knockdown system relies on integration of a plasmid containing a blasticidin S resistance gene, allowing for selection of parasites using blasticidin S[15,31].

Overall, CRISPR/Cas9 gene editing of *P. falciparum* has proven to be a powerful tool in malaria research, and the protocol here details the methods for generating conditional knockdown mutants[3,7,8,13,20,28]. This protocol is highly adaptable to individual research interests.

Disclosures

The authors have nothing to disclose.

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