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TITLE:

CRISPR/Cas9 gene editing to make conditional mutants of the human malaria parasite *Plasmodium falciparum*

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CRISPR, *Plasmodium*, *glmS,* knockdown, malaria, genetics

SHORT ABSTRACT:
We describe here a method for generating *glmS*-based conditional knockdown mutants in *P. falciparum* using CRISPR/Cas9 genome editing.

LONG 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 better 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, but 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*. The method is highly adaptable to other types of genetic manipulations, including protein tagging and gene knockouts.

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45 **INTRODUCTION:**
46 Malaria is a devastating disease caused by protozoan parasites of the genus *Plasmodium*. *P.*
47 *falciparum*, the most deadly human malaria parasite, causes approximately 445,000 deaths per
48 year, mostly in children under the age of five[1]. *Plasmodium* has an intricate life cycle involving a
49 mosquito vector and a vertebrate host. Humans become infected when an infected mosquito
50 takes a blood meal. The parasite first invades the liver where they grow, develop, and divide for
51 approximately one week. After this time, the parasites are released in the bloodstream where
52 they undergo asexual replication in red blood cells (RBC). Growth of the parasites within the red
53 blood cells are directly responsible for all of the clinical symptoms associated with malaria[2].
54
55 Until recently, production of transgenic *P. falciparum* was a laborious process, involving several
56 rounds of drug selection that took many months and had a high rate of failure. This time-
57 consuming procedure relies on the generation of random DNA breaks in the region of interest
58 and the endogenous ability of the parasite to mend its genome though homologous repair[3-6].
59 Recently, Clustered Regularly Interspaced Palindromic Repeat/Cas9 (CRISPR/Cas9) genome
60 editing has been successfully utilized in *P. falciparum*[7,8]. The introduction of this new
61 technology in malaria research has been critical for advancing understanding of the biology of
62 these deadly *Plasmodium* parasites. CRISPR/Cas9 allows for specific targeting of genes through
63 the use of guide RNAs (gRNAs) that are homologous to the gene of interest. The gRNA/Cas9
64 complex recognizes the gene through the gRNA and Cas9 introduces a double-strand break,
65 forcing the organism to initiate repair mechanisms[9,10]. Because *P. falciparum* lacks the
66 machinery to repair DNA breaks via non-homologous end joining, it utilizes homologous
67 recombination mechanisms and integrates transfected homologous DNA templates to repair
68 the Cas9/gRNA induced double-strand break[11,12].
69
70 Here we present a protocol for the generation of conditional knockdown mutants in *P.*
71 *falciparum* using CRISPR/Cas9 genome editing. The protocol demonstrates usage of the *glmS*
72 ribozyme to conditionally knockdown protein level of PfHsp70x (PF3D7_0831700), a chaperone
73 exported by *P. falciparum* into the host red blood cell (RBC)[13,14]. The *glmS* ribozyme is activated
74 by treatment with glucosamine (which is converted to glucosamine-6-phosphate within cells) to
75 cleave its associated mRNA, leading to reduction in protein[14]. This protocol is easily adapted to
76 utilize other conditional knockdown tools such as destabilization domains or RNA aptamers[4,5,15].
77 Our protocol details the generation of a repair plasmid consisting of a hemagglutinin (HA) tag
78 and *glmS* ribozyme coding sequence flanked by sequences homologous to the PfHsp70x open
79 reading frame (ORF) and 3'UTR. We also describe the generation of a second plasmid to drive
80 expression of the gRNA. These two plasmids, along with a third plasmid that drives expression
81 of Cas9, are transfected into RBCs and used to modify the genome of *P. falciparum* parasites.
82 Finally, we describe a polymerase chain reaction (PCR)-based technique to verify integration of
83 the tag and *glmS* ribozyme. This protocol is highly adaptable for the modification or complete
84 knockout of any *P. falciparum* genes, enhancing our ability to generate new insights into the
85 biology of the malaria parasite.
86
87 **PROTOCOL:**

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88 **Ethics Statement:** Continuous culture of *P. falciparum* requires the use of human RBCs and we
89 utilize commercially purchased units of blood that are stripped of all identifiers and
90 anonymized. The Institutional Review Board and the Office of Biosafety at the University of
91 Georgia have reviewed our protocols and approved all protocols used in our lab.
92
93 **1. Choose gRNA sequence**
94
95 1.1. Go to CHOP CHOP (http://chopchop.cbu.uib.no/) and select FASTA target**.** Under "Target",
96 paste the 200 base pairs from the 3' end of the open reading frame (ORF) of a gene and 200
97 base pairs from the start of the gene's 3'UTR. Under "In", select the species to be *P. falciparum*
98 (3D7 v3.0) and select CRISPR/Cas9 under "Using". Next, click "Find Target Sites".
99
100 1.2. Select a gRNA sequence from the options presented, giving preference to the most efficient
101 gRNA that is closest to your site of modification and that has the fewest off-target sites.
102
103 Note: Potential gRNA sequences are identified because they are immediately upstream of a
104 Protospacer Adjacent Motif (PAM), which is required for recruitment of Cas9 to DNA. The
105 sequence that is cloned into pMK-U6, the vector that drives gRNA expression, is the 20 bases
106 immediately upstream of the PAM. The PAM specific for *S. pyogenes* Cas9 is the nucleotide
107 sequence NGG and should not be included in the sequence that is cloned into pMK-U6.
108
109 CHOP CHOP visually ranks the gRNA sequences, displaying the best options in green, the less
110 ideal options in amber, and the worst options in red. CHOP CHOP gives each gRNA sequence an
111 efficiency score that is calculated using the most up-to-date parameters found in the literature,
112 and they predict off-target sites that could be recognized by the gRNA. Two or three gRNA
113 sequences may need to be tried to find the gRNA best suited to a particular gene.
114
115 1.3. Purchase the gRNA sequence and its reverse-complement as Polyacrylamide Gel
116 Electrophoresis-purified oligos; the gRNA sequence used to target PfHSP70x can be found in
117 **Figure 1B**.
118
119 Note: This oligo should include 15 base pairs homologous to the gRNA-expressing plasmid,
120 which are necessary for sequence and ligation-independent cloning (SLIC) into the pMK-U6
121 vector[16].
122
123 **2. Clone gRNA sequence into pMK-U6**
124
125 2.1. Digest pMK-U6 with BtgZI.
126
127 2.1.1. Digest 10 µg of pMK-U6 with 5 µL of BtgZI enzyme (5000 units/mL) for 3 h at 60 °C.
128 Follow the enzyme manufacturer's protocol for reaction conditions.
129

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130 2.1.2. After the 3 h incubation, add an additional 3 µL BtgZI to the reaction to ensure complete
131 digestion of the plasmid. Digest for an additional 3 h, again following manufacturer's
132 instructions for ensuring the correct reaction condition.
133
134 2.1.3. To purify the digested pMK-U6 from the reaction, use a column-based PCR cleanup kit
135 according to manufacturer's instructions.
136
137 2.1.4. Separate the digested DNA using a 0.7% agarose gel and extract the 4,200 base pair band.
138
139 2.2. Anneal the oligos containing the gRNA sequence.
140
141 2.2.1. Reconstitute the PAGE-purified oligos to a concentration of 100 µM using nuclease free
142 water.
143
144 2.2.2. Combine 10 µL of each oligo with 2.2 µL 10x buffer 2 (see the Table of Materials); the
145 total reaction volume will be 22.2 µL.
146
147 2.2.3. Run the gRNA annealing program in a thermocycler: Step 1- 95 °C, 10 min; step 2- 95 °C, 1
148 s, with a reduction in temperature of 0.6 °C/cycle; step 3- Go to step 2, 16 times; step 4- 85 °C,
149 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
150 times; step 7- 75 °C, 1 min; step 8- 75 °C, 1 s, with a reduction in temperature of 0.6 °C/cycle;
151 step 9- Go to step 8, 16 times. Steps 10-21- Repeat the procedure used in Steps 4-9 until the
152 temperature reaches 25 °C; step 22- 25 °C, 1 min.
153
154 2.3. Insert the annealed gRNA oligos into the BtgZI-digested and gel-purified pMK-U6 plasmid.
155
156 2.3.1. Combine 100 ng digested pMK-U6 with 1 µL 10x buffer 2.1 and 3 µL annealed gRNA
157 oligos. Bring the volume up to 9.5 µL with nuclease-free water.
158
159 2.3.2. Add 0.5 µL T4 polymerase and incubate reaction at room temperature for 2 min 30 s.
160
161 2.3.3. Move the reaction to ice and incubate for 10 min.
162
163 2.3.4. Immediately transform 5 µL of the reaction into competent *E. coli* according to
164 manufacturer's instructions and plate bacteria on Lysogeny Broth (LB) agar plates containing
165 100 µg/mL Ampicillin.
166
167 2.3.5. Allow transformed bacteria to grow at 37 °C overnight, then select colonies for DNA
168 extraction with a commercially available plasmid miniprep kit.
169
170 **3. Design homology regions of the repair template**
171
172 3.1. Design shield mutations within the homology repair template to prevent re-cutting of DNA
173 that is integrated into the genome.

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174
175 Note: The shield mutation typically consists of introducing a silent mutation to alter the
176 protospacer adjacent motif (PAM) so that Cas9 will not induce a break in the repair template.
177 The PAM required for the Cas9 used here is the nucleotide sequence NGG, where N is any
178 nucleotide. If possible, change one of the G nucleotides to an A, C, or T.
179
180 3.1.1. If the PAM cannot be silently mutated, introduce at least two silent mutations into the six
181 base pairs directly adjacent to the PAM[7,8].
182
183 Note: These mutations will prevent recognition of the repair template by the gRNA and prevent
184 re-cutting of the repaired locus by the Cas9/gRNA complex. The shield mutations can be
185 introduced into the homology region by amplifying the DNA with primers that contain the
186 mutation.
187
188 3.2. Amplify the ORF homology region for the repair template.
189
190 3.2.1. Using PCR, amplify 800 base pairs from the 3' end of the target gene's ORF. Design the
191 primers to be used to exclude the stop codon from this amplicon.
192
193 3.2.2. Additionally, design the primers to insert this amplicon into pHA-*glmS* that has been
194 digested with SacII and AfeI, either through a DNA ligation reaction or SLIC[16]
195
196 3.3. Amplify the 3'UTR homology region for the repair template.
197
198 3.3.1. Using PCR, amplify the 800 base pairs immediately following the stop codon of the target
199 gene. The primers used should be designed to insert this amplicon into pHA-*glmS* that has been
200 digested with HindIII and NheI, either through a DNA ligation reaction or SLIC[16].
201
202 Note: The high AT content of the *P. falciparum* genome often makes amplification of regions
203 such as UTRs difficult. An alternative approach to using PCR is to synthesize the homology
204 regions.
205
206 **4. Clone homology regions into the repair plasmid**
207
208 4.1. Insert ORF homology region into pHA-*glmS*.
209
210 4.1.1. Digest pHA-*glmS* with SacII and AfeI, according to enzyme manufacturer's instructions,
211 and insert the ORF homology region PCR product into the digested plasmid using SLIC [16].
212
213 4.1.2. Transform into competent *E. coli*.
214
215 4.2. Insert 3'UTR homology region into pHA-glmS plasmid that already contains the ORF
216 homology region (see 4.1).
217

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218 4.2.1. Digest the plasmid with HindIII and NheI according to enzyme manufacturer's
219 instructions, and then insert the 3'UTR homology region amplicon into the digested plasmid
220 using SLIC[16].
221
222 4.2.2. Transform into competent *E. coli*. See steps 2.3.4 and 2.3.5 above.
223
224 **5. Precipitate DNA for transfection**
225
226 5.1. Add 40 µg each of pMK-U6, pUF1-Cas9, and pHA-*glmS* DNA (for a total of 120 µg of DNA)
227 into a sterile 1.5 mL microcentrifuge tube.
228
229 5.2. Add 1/10th the volume of DNA of 3M sodium acetate in water (pH 5.2) to the tube and mix
230 well using a vortex. For example, if the volume in step 5.1 was 100 µL, add 10 µL sodium
231 acetate.
232
233 5.3. Add 2.5 volumes of 100% ethanol to the tube and mix well using a vortex for at least 30 s.
234 For example, if the volume in 5.1 was 100 µL, add 250 µL 100% ethanol.
235
236 5.4. Place the tube on ice or at -20 °C for 30 min.
237
238 5.5. Centrifuge the tube at 18,300g for 30 min at 4 °C.
239
240 5.6. Carefully remove the supernatant from the tube. Do not disturb the pellet.
241
242 5.7. Add 3 volumes of 70% ethanol to the tube and mix briefly using a vortex. For example, if
243 the volume in 5.1 was 100 µL, add 300 µL 70% ethanol.
244
245 5.8. Centrifuge the tube at 18,300g for 30 min at 4 °C.
246
247 Note: This step should be performed under sterile conditions in a biological safety cabinet.
248
249 5.9. Carefully remove the supernatant from the tube. Do not disturb the pellet. Leave the tube
250 open and allow the pellet to air dry for 15 min.
251
252 5.10. Store the precipitated DNA at -20 °C until it is needed for transfection.
253
254 **6. Isolate human RBCs from whole blood in preparation for transfection.**
255
256 6.1. Aliquot fresh blood into sterile 50 mL conical tubes (approximately 25 mL per tube).
257
258 6.2. Centrifuge tubes at 1088g for 12 min, with centrifuge brakes set to 4.
259
260 6.3. Aspirate off supernatant and buffy coat.
261

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262 6.4. Resuspend RBC pellet with equal volume incomplete RPMI.
263
264 Note: Incomplete RPMI is prepared by supplementing RPMI 1640 with 10.32 µM thymidine,
265 110.2 µM hypoxanthine, 1 mM sodium pyruvate, 30 mM sodium bicarbonate, 5 mM HEPES,
266 11.1 mM glucose, and 0.02% (v/v) gentamicin.
267
268
269 6.5. Repeat steps 6.2-6.4 twice.
270
271 6.6. After the last wash, resuspend RBCs in equal volume incomplete RPMI and store at 4 °C.
272
273 **7. Transfect RBCs with the CRISPR/Cas9 plasmids (to be done aseptically)**
274
275 Note: Plasmodium falciparum cultures are maintained as described[17]. Whenever blood is used
276 in the protocol, it is referring to pure red blood cells prepared in Step 6. Blood used should not
277 be older than 6 weeks, as we see a decrease in parasite proliferation in older blood. We
278 describe here a protocol for pre-loading RBCs with DNA and then adding parasite culture to the
279 transfected cells. Other established transfection protocols would be compatible with
280 transfecting these constructs[18,19].
281
282 7.1. Prepare 1x cytomix buffer in water (120 mM KCl, 0.15 mM $CaCl_2$, 2mM EGTA, 5mM $MgCl_2$,
283 10mM K_2HPO_4, 25mM HEPES, pH 7.6). Filter-sterilize the buffer using a 0.22 µM filter.
284
285 7.2. Add 380 µL of 1x cytomix to the DNA precipitated in step 5 and vortex to dissolve. Allow
286 the DNA to dissolve in 1x cytomix for 10 minutes, vortexing every 3 minutes for 10 seconds.
287
288 7.3. In a sterile 15 mL conical tube, combine 300 µL of red blood cells (RBCs, 50% hematocrit,
289 from Step 6) in incomplete RPMI with 4 mL of 1x cytomix.
290
291 7.4. Centrifuge the RBCs from 7.3 at 870g for 3 min, then remove the supernatant from the RBC
292 pellet.
293
294 7.5. Resuspend the RBC pellet with DNA/cytomix mixture from step 6.2 and transfer to a 0.2 cm
295 electroporation cuvette.
296
297 7.6. Electroporate the RBCs using the following conditions: 0.32kV, 925 µF, capacitance set to
298 "High Cap", and resistance set to "infinite".
299
300 7.7. Following electroporation, transfer the contents from the cuvette to a 15 mL conical
301 containing 5 mL of complete RPMI (cRPMI). Centrifuge the tube at 870g for 3 min at 20 °C, then
302 decant the supernatant.
303
304 Note: cRPMI is prepared as described for incomplete RPMI in 7.4 with the addition of 0.25%
305 (w/v) lipid-rich bovine serum albumin.

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7.8. Resuspend the pellet in 4 mL cRPMI and transfer to a well of 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.

7.9. The next day, wash the culture with 4 mL of cRPMI.

7.9.1. Centrifuge the culture at 870g for 3 min and aspirate the supernatant. Resuspend the culture in 4 mL cRPMI.

7.10. 48 h after 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.

7.11. Continue washing the cultures each day with cRPMI until parasites are no longer visible by blood smear. After this point, the culture medium should be replaced with fresh cRPMI + 1 µM DSM1 every 48 h.

7.11.1. To make a blood smear, pipette 150 µL of culture into an 0.6 mL eppendorf tube. Pellet the cells by centrifugation at 1700g for 30 s.

7.11.2. 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.

7.11.3 View parasites using a 100x oil immersion objective.

7.12. Beginning 5 days post-transfection (Step 7.6), remove 2 mL of the culture, with RBCs resuspened in the culture medium, and add back 2 mL fresh medium (cRPMI + 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.

Note: If integration is successful, parasites generally reappear in culture by one-month post-transfection.

7.13. Once parasites reemerge, remove drug pressure. Alternatively, remove drug pressure after parasites have been cloned out.

8. Check parasites for integration of the repair template

8.1. When parasites are visible again by thin blood smear, isolate DNA from the culture.

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349 8.2. Use PCR to amplify the modified region of the genome to determine whether the targeted
350 locus has been successfully altered and whether the unmodified *wild-type* locus (indicative of
351 *wild-type* parasites) is detectable.
352
353 8.2.1. To detect parasites that have integrated the repair template, use a forward primer that
354 sits at the beginning of the ORF, outside of the cloned homology region. Use a reverse primer
355 that sits in the 3'UTR.
356
357 Note: As this amplification includes the sequences of the HA tags and *glmS* ribozyme, amplicons
358 from integrated parasites will be longer than the same region amplified in *wild-type* parasites.
359
360 **9. Clone parasites by limiting dilution**
361
362 9.1 Perform serial dilutions of the parasite culture from step 7.13 to achieve a final
363 concentration of 0.5 parasites/200 µL.
364
365 9.1.1. Prepare 1 mL culture in cRPMI at 5% parasitemia and 2% hematocrit; at this parasitemia
366 and hematocrit, the culture contains 1×10^7 parasites/mL.
367
368 9.1.2. Dilute this culture 1:100 with cRPMI. Dilute again 1:100 with cRPMI.
369
370 9.1.3. Dilute 1:400. Perform this dilution by adding 62.5 µL culture to 25 mL cRPMI and 1 mL
371 blood. This dilution results in the desired concentration of 0.5 parasites/200 µL.
372
373 9.1.5. Add 200 µL of the diluted culture to the wells of a 96-well tissue culture plate.
374
375 9.2. Maintain the cloning plate until parasites are detectable in the wells.
376
377 9.2.1. Every 48 h, replace the medium in the 96-well plate with fresh medium.
378
379 9.2.2. Once a week, starting 5 days after beginning the cloning plate (Step 8.1.5), remove 100 µL
380 from each well and add back 100 µL of fresh medium + blood (2% hematocrit).
381
382 9.3. Identify wells containing parasites.
383
384 9.3.1. Place the 96-well plate at a 45° angle for approximately 20 min, allowing the blood to
385 settle at an angle within the plate.
386
387 9.3.2. Place the 96-well plate on a light box. Observe that the wells containing parasites will
388 contain medium that is yellow in color, compared to the pink medium of parasite-free wells,
389 due to acidification of the medium by the parasites.
390
391 9.3.3. Using a serological pipette, move the contents of the parasite-containing wells to a 24-
392 well tissue culture plate to allow expansion of parasitemia.

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393
394 9.3.4. Using PCR analysis as described in Step 8, check these clonal parasite lines for correct
395 integration.
396
397 **10. Knockdown protein by treating parasites with GlcN and confirm knockdown by Western**
398 **blot analysis.**
399
400 10.1. Prepare 0.5 M GlcN stock solution; the stock can be stored at -20° C.
401
402 10.2. Add GlcN to *glmS* parasite cultures and allow to grow in the presence of GlcN.
403
404 Note: The final concentration and timing of GlcN treatment to be used is dependent upon the
405 experiment and parasite line. GlcN can impact parasite growth, so the parental parasite strain
406 should be exposed to a range of GlcN concentrations to determine sensitivity to the compound.
407 Often, a concentration of 2.0-7.5 mM GlcN is used[13,14,20].
408
409 10.3. Isolate protein samples from GlcN-treated parasites[13].
410
411 10.4. Use protein samples for Western blot analysis to detect reduction in protein[13].
412
413 10.4.1. Use an anti-HA antibody according to manufacturer's instructions to detect the HA-
414 glmS-tagged protein, and compare the HA band to a loading control, such as PfEF1α.
415
416 **REPRESENTATIVE RESULTS:**
417 A schematic of the plasmids used in this method as well as an example of a shield mutation are
418 shown in **Figure 1**. As an example of how to identify mutant parasites after transfection, results
419 from PCRs to check integration of the HA-*glmS* construct is shown in **Figure 2**. A representative
420 image of a cloning plate is shown in **Figure 3** to demonstrate the color change of the medium in
421 the presence of parasites. Results from an immunofluorescence assay and Western blotting
422 experiments are shown in **Figure 4** to demonstrate the functionality of the HA tag and *glmS*-
423 based reduction of protein in the parasites. **Figure 5** demonstrates the inability of short
424 homology arms on PCR products to modify the parasites genome and obtain viable mutants.
425
426 **FIGURE LEGENDS:**
427
428 **Figure 1: Summary of our three-plasmid approach to CRISPR/Cas9 and examples of a gRNA**
429 **oligo and shield mutation.** A) Schematics of empty pHA-glmS and pMK-U6 are shown with the
430 restriction enzyme sites used for cloning. Also shown are pHA-glmS and pMK-U6 after the
431 homology arms and gRNA sequences have been cloned into them, respectively. Finally, pUF1-
432 Cas9 is shown. yDHOD: yeast dihydrofolate reductase, the resistance marker to DSM1. B) The
433 forward oligo used for cloning the PfHsp70x gRNA sequence into pMK-U6 is shown, with the
434 gRNA sequence in capital letters and the pMK-U6 homology arms necessary for cloning shown
435 in lower case (Top). The genomic target of the PfHsp70x gRNA is shown as well as the

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436 downstream PAM, in red (Middle). The shield mutation in the PfHsp70x gRNA PAM is shown in
437 red (Bottom).
438
439 **Figure 2: Schematic of CRISPR/Cas9 genome modification using pHA-glmS and strategy for**
440 **confirming integration.** A) Cas9, guided to a genomic locus by a gRNA, induces a double strand
441 break in the DNA. The parasite repairs the damage through double crossover homologous
442 repair, using the pHA-glmS plasmid as a template and introducing the HA-glmS sequence into
443 the genome. B) A PCR test to identify correct integration of the HA-glmS sequence. Using
444 primers P1 and P2, the 3' ORF of *wild-type* PfHsp70x and PfHsp70x-*glmS* mutants are
445 amplified[13]. The amplicon from PfHsp70x-glmS is longer than *wild-type* due to insertion of the
446 HA-glmS sequence.
447
448 **Figure 3: Identification of wells containing parasites in a 96-well cloning plate.** A) The 96-well
449 plate is set at a 45° angle for approximately 20 minutes to allow the blood to settle at an angle
450 in the plate. B) The well on the left contains a parasite culture, indicated by the yellow color of
451 the medium in comparison to the pink medium of the parasite-free well on the right.
452
453 **Figure 4: An immunofluorescence assay shows the correct HA-tagging of PfHsp70x and**
454 **Western blotting shows reduction of PfHsp70x protein levels during treatment with**
455 **glucosamine.** A) PfHsp70x-*glmS* parasites were fixed and stained with DAPI (nucleus marker)
456 and antibodies to HA and MAHRP1 (Membrane Associated Histidine Rich Protein 1, a marker of
457 protein export to the host RBC)[13].B) PfHsp70x-*glmS* parasites were treated with 7.5 mM
458 glucosamine and whole-parasite lysates were used for Western blotting analysis[13]. The
459 membrane was probed with antibodies for HA and PfEF1α as a loading control[13]. As expected,
460 glucosamine treatment results in a reduction of the protein.
461
462 **Figure 5: Using short homology sequences for repair.** A) Schematic representation showing
463 knockout of GFP in B7 parasites[21]. B7 parasites are a derivative of 3D7 where Plasmepsin II has
464 been tagged with GFP. PCR products containing 50, 75, or 100 base pairs of GFP homology
465 regions flanking a blasticidin S resistance cassette (labeled "marker"), along with pUF1-Cas9-
466 eGFP-gRNA, a plasmid expressing Cas9 and a GFP gRNA, were transfected into B7 parasites.
467 Each transfection was carried out twice. Drug pressure (DSM1) was applied 2-day post
468 transfection. B) PCR test on DNA isolated from transfected parasites five days post-transfection
469 and two months post-transfection. Primers used to test integration of the BSD resistance
470 cassette will yield a 584 base pair product for B7 parental parasites and a 2020 base pair
471 product for parasites that have integrated the marker.
472
473 **DISCUSSION:**
474 The implementation of CRISPR/Cas9 in *P. falciparum* has both increased the efficiency of and
475 decreased the amount of time needed for modifying the parasite's genome, in comparison to
476 previous methods of genetic manipulation. The comprehensive protocol described in this
477 manuscript outlines the steps taken to generate conditional mutants using CRISPR/Cas9 in
478 *Plasmodium falciparum*. While the method here is written specifically for the generation of HA-

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479 *glmS* mutants, this strategy can be adapted for a variety of purposes, including the tagging of
480 genes, gene knockouts, and introduction of point mutations.
481
482 A critical early step in this protocol is the selection of a gRNA sequence. When selecting a gRNA,
483 there are several considerations to keep in mind in regards to where the gRNA sits, how
484 efficient it is, and whether it has the potential for off-target effects. Typically, the gRNA
485 sequence should be as close as possible to the site of modification, ideally within 200 bp. This
486 will decrease the likelihood of the parasites using the repair template to fix their genome
487 without integrating the tag. The tool used here to locate a gRNA was a free online service called
488 CHOP CHOP[22]. Another online tool, Eukaryotic Pathogen CRISPR guide RNA/DNA Design Tool
489 (EuPaGDT, http://grna.ctegd.uga.edu/), can also be used[23]. EuPaGDT provides additional
490 characterization of gRNA sequences, including prediction of off-target hits and potential issues
491 that may prevent transcription of the gRNA. EuPaGDT also has tools for batch processing of
492 gRNAs to target multiple genes or whole genomes. The gRNA chosen should be one that sits
493 closest to the site of modification with the highest efficiency and minimal off-target hits. An
494 important limitation to CRISPR/Cas9 gene editing that may arise is the inability to design a
495 suitable gRNA to target the gene of interest. In such cases, a trial-and-error approach may be
496 needed, using multiple sub-optimal gRNA sequences until the best one is found and successful
497 gene editing has occurred.
498
499 Another important consideration for generating *P. falciparum* mutants using CRISPR/Cas9 is the
500 length of the homology regions used in the repair template. The protocol here states that the
501 homology regions should be approximately 800 base pairs each, but we have also been
502 successful in using smaller regions (500 base pairs)[3]. Successful genome modification using
503 CRISPR/Cas9 and short homology arms on PCR products have been used in other protozoan
504 parasites such as *Toxoplasma gondii* and *Trichomonas vaginalis*[24,25]. We tested the feasibility of
505 using smaller homology arms on PCR products (50, 75, or 100 base pairs) by attempting to
506 knockout GFP in B7 parasites using a blasticidin resistance cassette[21]. We saw some integration
507 of the blasticidin resistance cassette at five days post transfection; however, these parasites
508 never recovered from transfection. For these transfections, we selected for the Cas9-expressing
509 plasmid using DSM1. A different selection method, such as treating transfected cultures with
510 blasticidin S alone or in combination with DSM1, may improve the chances of parasites
511 reappearing when using shorter homology regions for repairing the Cas9/gRNA induced break.
512 We did not select with blasticidin S in this case because we wanted to test whether short
513 homology arms could be used in instances where a drug resistance cassette is not being
514 integrated into the genome, such as when a protein is being tagged.
515
516 The core components of CRISPR/Cas9 gene editing discussed here are the Cas9 endonuclease,
517 the gRNA, and the repair template. We describe a three-plasmid approach to introduce these
518 components into the parasites, where Cas9, the gRNA, and the repair template are found in
519 separate plasmids. In addition to this approach, our lab has been successful in using a two-
520 plasmid approach where Cas9 and gRNA expression are driven by a single plasmid and the
521 repair template is found in a second plasmid[3]. Similar two-plasmid approaches have been
522 successfully employed by other labs to generate mutants[7,8,26-29]. Furthermore, a few labs are

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523 using a strain of *Plasmodium (*NF54^{attB}*)* which constitutively expresses Cas9 and a T7 RNA
524 polymerase to drive expression of gRNA's[30]. In this case, a single plasmid containing the repair
525 template and the gRNA are transfected into NF54^{attB} parasites[31,32]. A plasmid-free approach,
526 utilizing a purified Cas9-gRNA ribonucleoprotein complex, has been used to insert mutations
527 into the genome as well[33]. The success of these different approaches demonstrates flexibility in
528 how researchers can introduce the Cas9/gRNA components into the parasite.
529
530 Finally, the choice of drug pressure to apply to transfected parasites can be altered depending
531 on constructs used. Here, we show successful generation of mutants by transiently selecting for
532 the Cas9 expressing plasmid using DSM1 until parasites reappear. To generate PfHsp70x
533 knockout parasites, *pfhsp70x* was replaced with the human dihydrofolate reductase gene, and
534 parasites were selected using WR99210[13].The recently described TetR-PfDOZI knockdown
535 system relies on integration of a plasmid containing a blasticidin S resistance gene, allowing for
536 selection of parasites using blasticidin S[15,31].
537
538 CRISPR/Cas9 gene editing of *P. falciparum* has proven to be a powerful tool in malaria research,
539 and we have detailed here a method for generating conditional knockdown mutants[3,7,8,13,20,28].
540 The protocol is highly adaptable to individual research interests.
541
542
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550
551 **DISCLOSURES:**
552 The authors have nothing to disclose
553
554 **REFERENCES**
555

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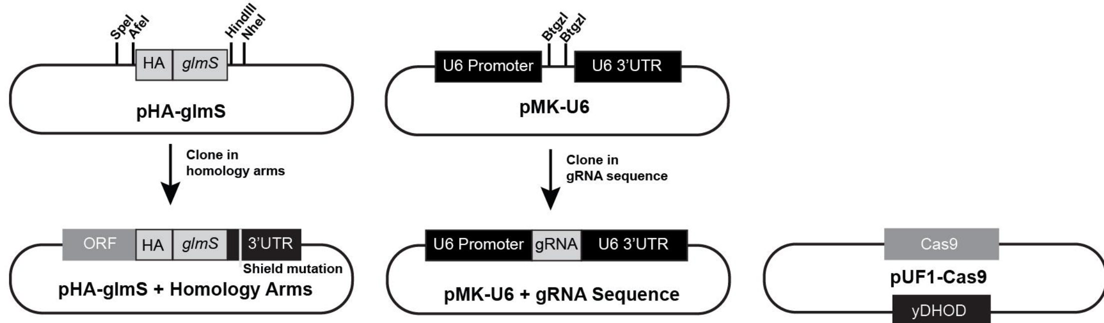


B **PfHsp70x gRNA oligo cloned into pMK-U6:**

5'-taagtatataatattTGCATTATTGTTGTATATTTgttttagagctagaa-3'

Genomic target of PfHsp70x gRNA + the PAM:

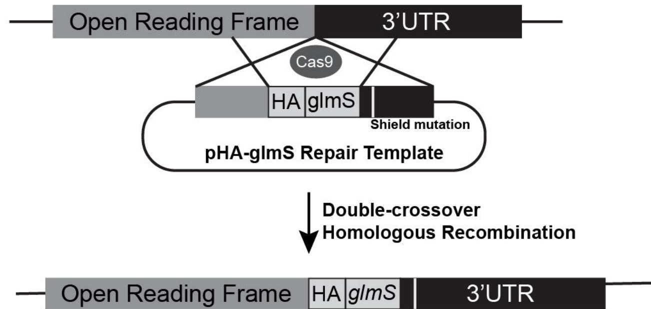
5'-TGCATTATTGTTGTATATTTTGG-3'

PAM

Genomic target of PfHsp70x gRNA + Shield Mutation in the PAM

5'-TGCATTATTGTTGTATATTTTCG-3'

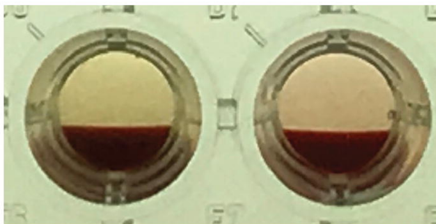
Shield Mutation


A
Open Reading Frame
3'UTR
Cas9
HA
glmS
Shield mutation
pHA-glmS Repair Template
Double-crossover
Homologous Recombination
Open Reading Frame
HA
glmS
3'UTR

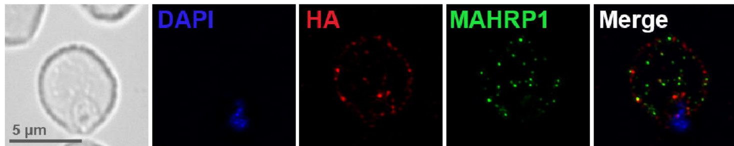
B
1.7 kb
P1
P2
PfHsp70x ORF
3'UTR
2.0 kb
P1
P2
PfHsp70x ORF
HA
glmS
3'UTR
3D7
PfHsp70x-
glmS
2.0 kb -
1.5 kb -

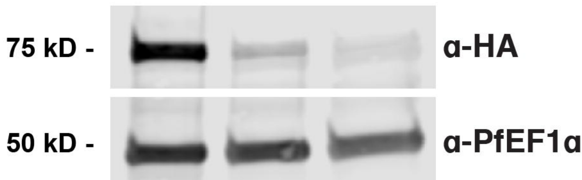
A

B



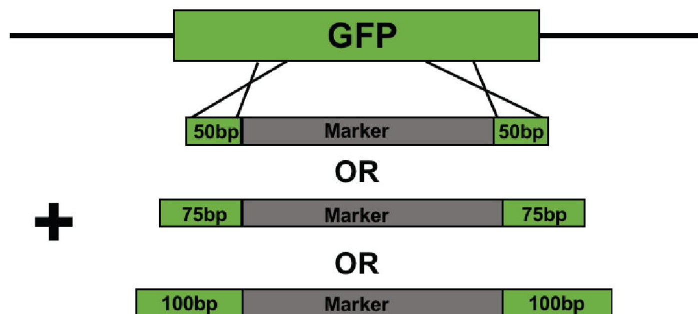
+Parasites -Parasites

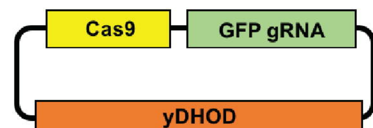

A
DAPI
HA
MAHRP1
Merge
5 µm


B
PfHsp70x-glmS
Hours: 0 24 48
75 kD -
α-HA
50 kD -
α-PfEF1α

A

Parasite genome:





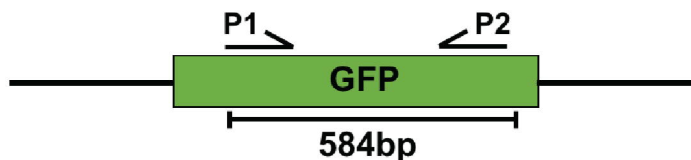
+

Homology Directed Repair

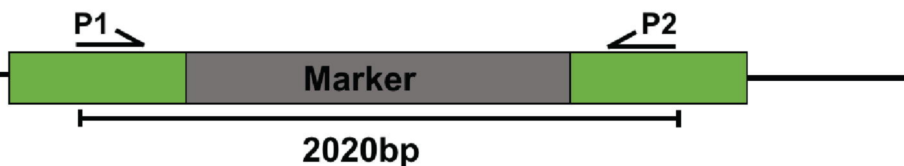
Modified Genome:

B

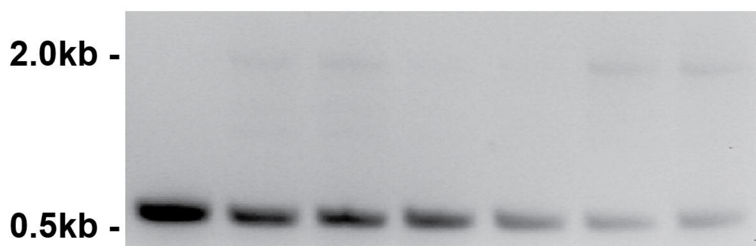
Parental:



Modified Genome:

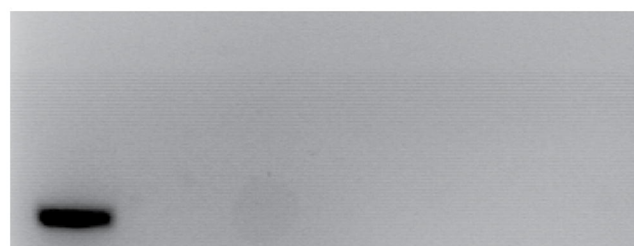


Parental | 50bp HR 1 | 50bp HR 2 | 75bp HR 1 | 75bp HR 2 | 100bp HR 1 | 100bp HR 2



5 days post-transfection

Parental | 50bp HR 1 | 50bp HR 2 | 75bp HR 1 | 75bp HR 2 | 100bp HR 1 | 100bp HR 2



2 months post-transfection