

Supplemental Information

Genetic background determines allele-specific *PfKelch13*-mediated artemisinin tolerance and persistence in *Plasmodium falciparum*

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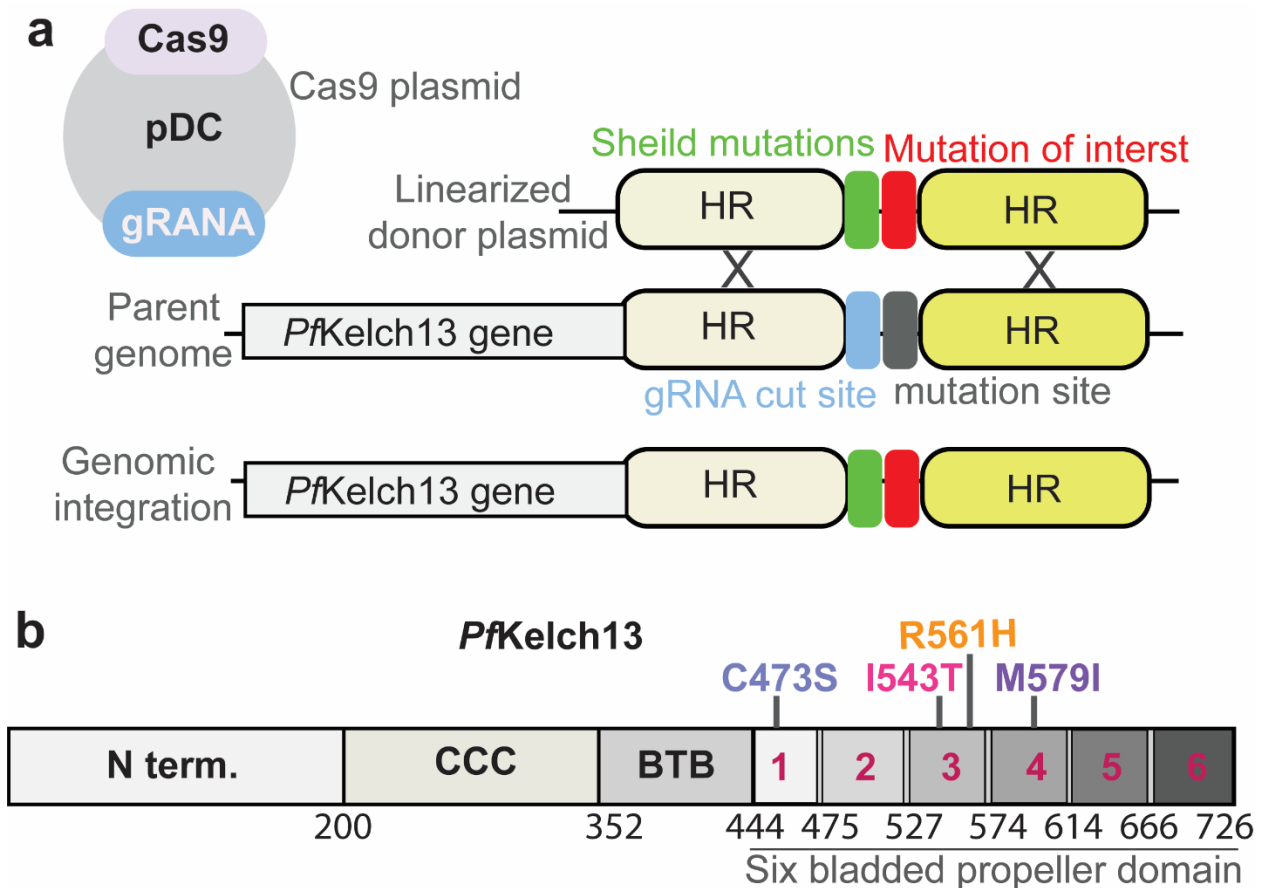
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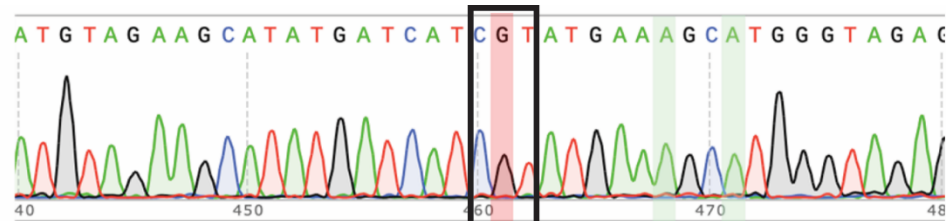


Supplementary Fig. 1: CRISPR–Cas9 editing strategy and domain organization of *Pfkkelch13*.

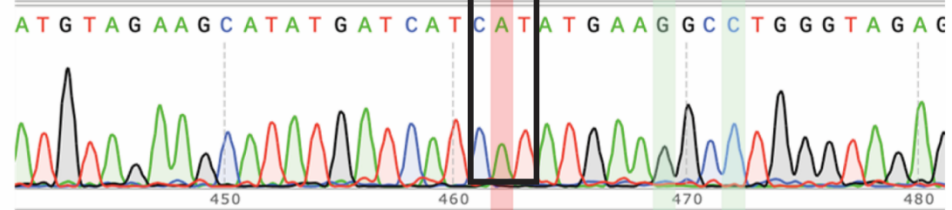
(a) Schematic of the plasmid used to introduce point mutations into the *Plasmodium falciparum* *Pfkkelch13* locus. The Cas9 expression plasmid (Cas9, pDC) and the guide RNA (gRNA) expression cassette target a gRNA cut site within the endogenous *Pfkkelch13* gene in the parent genome. A linearized donor plasmid carries two homology regions (HR) flanking the edited sequence. The donor insert contains (i) the desired mutation of interest (red) and (ii) additional synonymous “shielding” mutations (green) that disrupt the protospacer and/or PAM sequence without altering the encoded amino acids, thereby preventing re-cleavage by Cas9 after repair. Following double-strand break formation at the gRNA cut site and homology-directed repair using the donor plasmid, the edited allele is integrated at the native *Pfkkelch13* locus, yielding a parasite line in which the mutation of interest and its associated shielding mutations are fixed in the genome.

(b) Linear map of the *Pfkkelch13* showing the N-terminal region (N term.), the coiled-coil–containing (CCC) region, and the BTB/POZ domain, followed by the six-bladed β -propeller domain. Amino-acid positions corresponding to the boundaries between regions are indicated below the diagram. The six blades of the propeller domain are numbered (1–6), with the approximate residue ranges shown beneath each blade. The specific residues mutated in this study (C473S, I543T, R561H, and M579I) are indicated above their corresponding locations in the propeller domain.

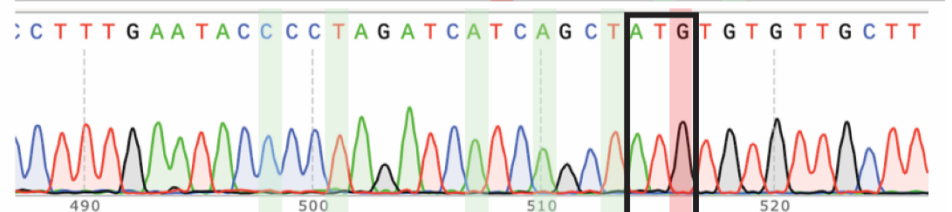
SenTH15.14
Parent



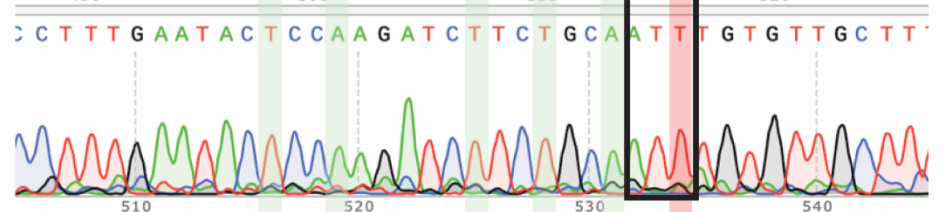
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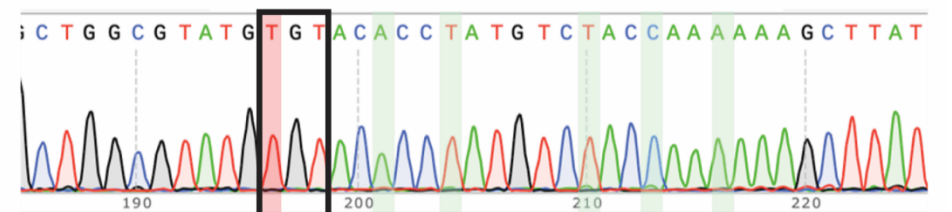
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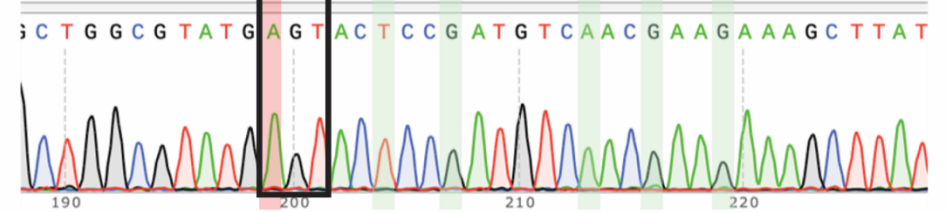
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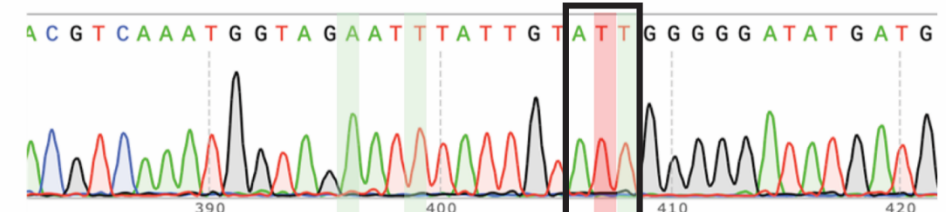
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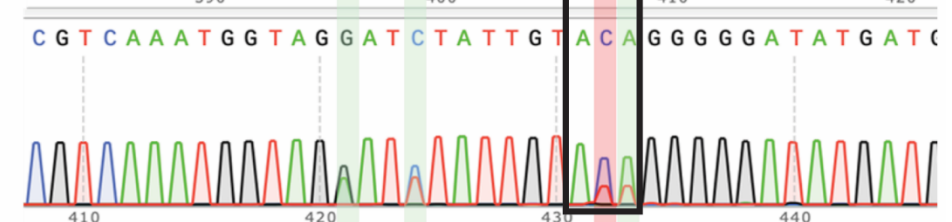
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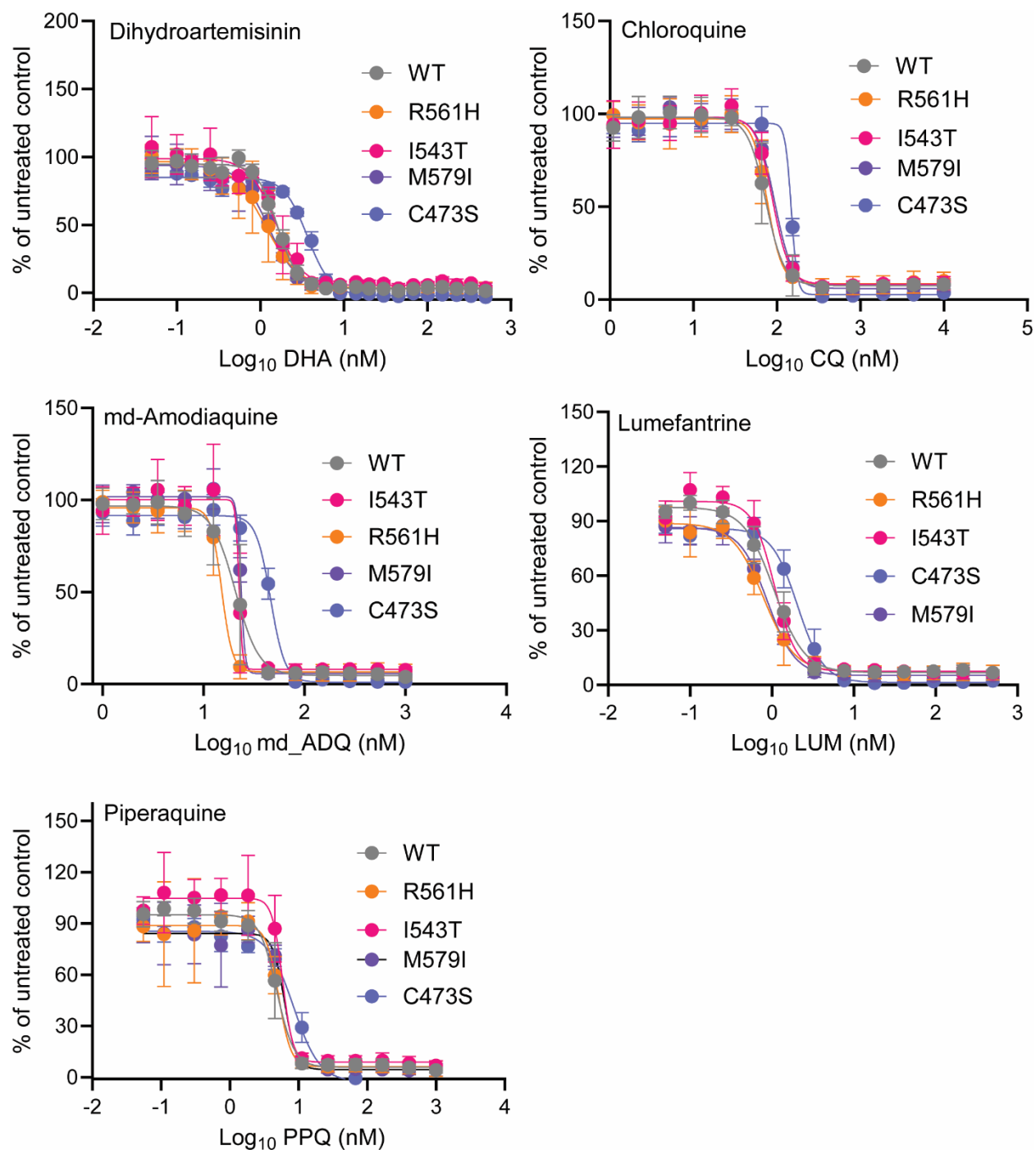
SenTH15.14
Parent



Bulk CRISPR
transfection
I543T

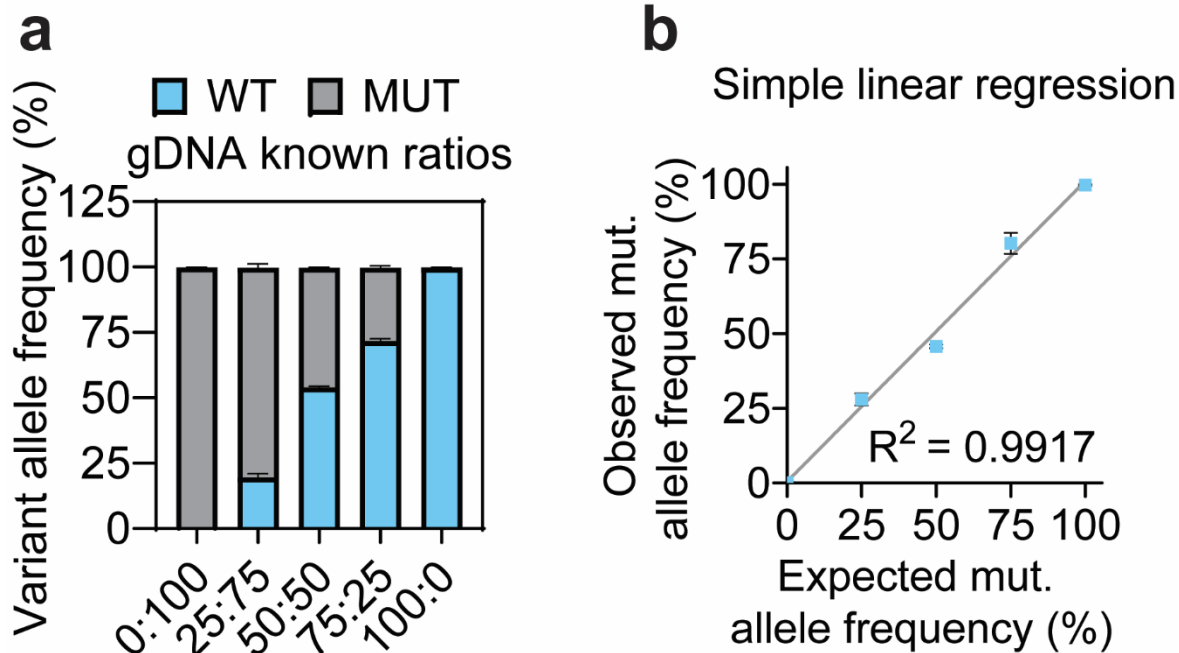


Supplementary Fig. 2. Sequencing confirmation of CRISPR–Cas9 editing at the endogenous *Pfkelch13* locus in the SenTH15.14 background. Representative sequencing traces are shown for the SenTH15.14 WT parent and the corresponding clonal CRISPR-edited lines carrying the *Pfkelch13* mutations R561H, M579I, and C473S. The targeted codons are indicated by red-shaded vertical bands, and linked synonymous shielding substitutions introduced to disrupt the gRNA protospacer and/or PAM without altering the encoded amino acid are indicated by green-shaded bands. The engineered mutant codons are CAT for R561H, ATT for M579I, and AGT for C473S. For the I543T edit, sequence evidence from bulk-edited parasites was obtained by Nanopore amplicon sequencing, as indicated; the engineered mutant codon is ACA. The amino acid substitutions are boxed. Compared with the WT parent, the clonal edited lines show clean base calls at the targeted positions together with the linked shielding substitutions, consistent with precise allelic replacement at the endogenous *Pfkelch13* locus. The bulk I543T sample reflects a heterogeneous population containing both WT and edited alleles.



Supplementary Fig. 3: *In vitro* dose-response (EC_{50}) curves for antimalarial drugs in *SenTH15.14* WT and isogenic *Pfk13*-edited lines. Growth inhibition dose-response curves are shown for dihydroartemisinin (DHA), chloroquine (CQ), monodesethylamodiaquine (mdADQ), lumefantrine (LUM), and piperaquine (PPQ) against the *SenTH15.14* WT parent and CRISPR-engineered *Pfk13* mutant lines (R561H, I543T, M579I, and C473S), all in the same genetic background. Drug concentrations are plotted as log₁₀-transformed nanomolar (nM) values on the x-axis, and responses are normalized to the untreated control (% of untreated control) on the y-axis.

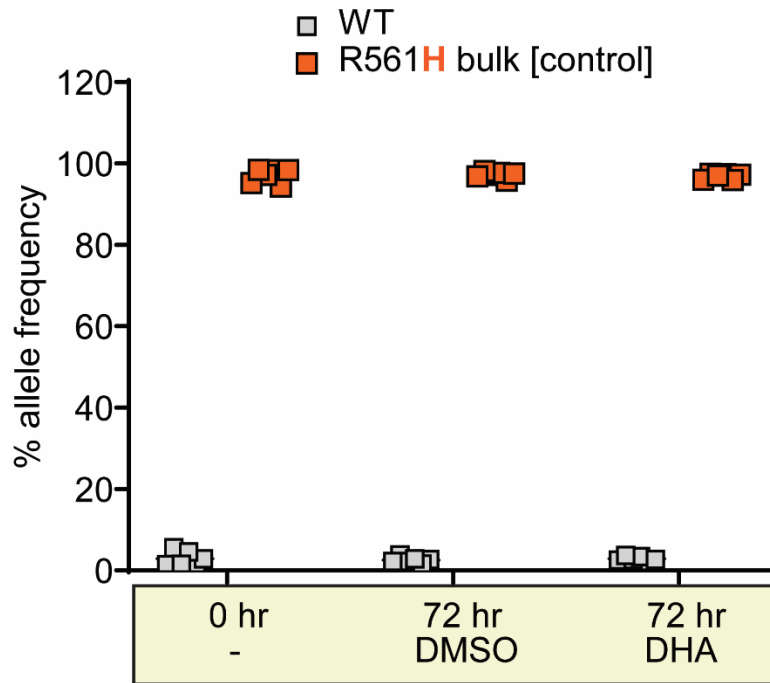
axis. Symbols show the mean response and error bars indicate $\pm$ SD from three independent biological replicates; solid lines represent the best-fit nonlinear regression for each parasite line (fit model as described in Methods). Corresponding EC_{50} values derived from these fits are summarized in Supplementary Table 2. Source data are provided.



Supplementary Fig. 4: Validation of k13 variant allele frequency quantification by nanopore sequencing.

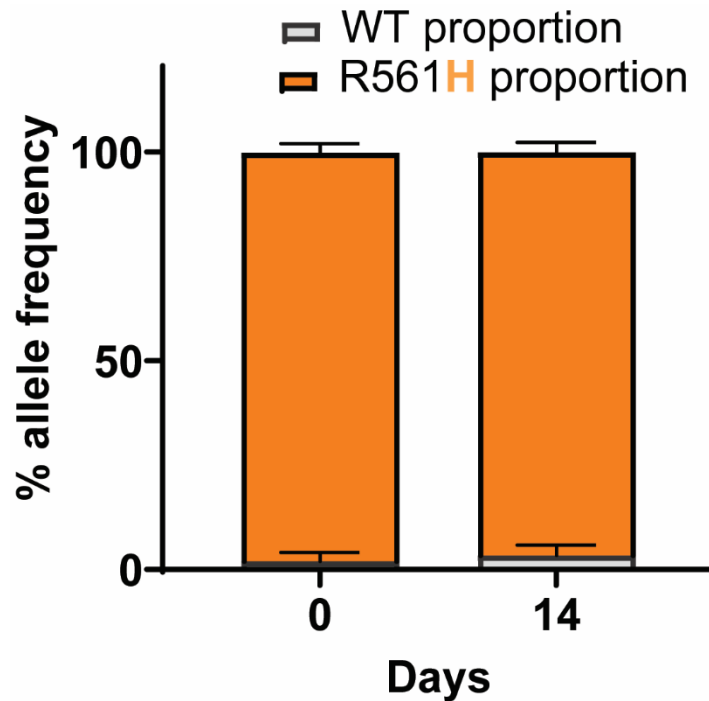
a, Known mixtures of wild-type (WT) and mutant (MUT) genomic DNA were prepared at predefined WT:MUT ratios (0:100, 25:75, 50:50, 75:25, and 100:0) and subjected to the targeted k13 amplicon nanopore sequencing pipeline. Genomic DNA was sourced from the parental SenTh15.14 isolate (WT) and a clonal PfKelch13-edited line (MUT). Stacked bars display the mean observed variant allele frequency (%) of WT (blue) and MUT (grey) sequence reads. Error bars indicate standard deviation (s.d.) across independent mixture preparations ($n = 3$).

b, Simple linear regression comparing the expected mutant allele frequency based on the prepared gDNA ratios against the observed mutant allele frequency measured by the nanopore assay. Points represent the mean $\pm$ s.d. The highly linear correlation ($R^2 = 0.9917$) demonstrates excellent quantitative accuracy and confirms that the amplicon sequencing pipeline does not introduce significant amplification or sequencing bias toward either allele.



Supplementary Fig. 5: Allele frequency of a bulk-edited R561H control population remains stable during the nanopore-assisted RSA protocol.

Variant allele frequency (VAF) of a bulk-edited *PfKelch13* R561H control population was quantified by nanopore sequencing of the *k13* amplicon. Samples were assessed at 0 hr (immediately prior to treatment) and at 72 hr following either a 6 hr 700 nM DHA pulse or a DMSO vehicle control. Grey squares represent the WT allele proportion; orange squares represent the R561H mutant allele proportion. In contrast to the unfit I543T mutation (Fig. 1b), the R561H allele frequency remained highly stable (~97%) throughout the 72-hour assay timeframe. No significant difference was observed between the matched 72 hr DHA and 72 hr DMSO samples ($P = 0.585$, two-tailed paired t-test), confirming that the nanopore-assisted RSA protocol does not introduce artifactual shifts in allele frequency for stable *k13* variants.



Supplementary Fig. 6: Allele frequency of a bulk-edited R561H control population remains completely stable during 14 days of drug-free culture.

Drug-free bulk culture dynamics of a *PfKelch13* R561H control population. Bulk-edited cultures carrying the R561H mutation were maintained under standard *in vitro* conditions without drug selection and sampled at day 0 and day 14. WT (grey) and mutant (R561H, orange) variant allele frequencies (VAFs) were quantified by k13 amplicon nanopore sequencing. Stacked bars show the mean proportion (WT + mutant = 100%) $\pm$ s.d. across biologically independent bulk cultures ($n = 2$). In stark contrast to the severe fitness defect and rapid decline observed in bulk-edited I543T populations over the identical 14-day timeframe (Fig. 1d), the R561H allele frequency remained entirely stable. This confirms that the rapid counter-selection of I543T is a mutation-specific biological fitness penalty in the SenTh15.14 background, rather than a general artifact of maintaining bulk-edited mixed cultures over prolonged periods.

Supplementary Table 1. Primers and gRNA oligonucleotides used for CRISPR-Cas9 editing of *Pfkelch13*. Sequences are shown 5'–3'. Donor templates were generated using approximately 500 bp 5' (upstream) and 3' (downstream) homology arms flanking the target codon; 5' and 3' are defined relative to the *Pfkelch13* 3D7 reference sequence used for donor design. Homology-arm primers include 5' cloning extensions and *Xba*I and *Sal*I restriction sites for insertion into the pUC19 donor backbone. Mutagenesis primers introduce the indicated nonsynonymous SNP (highlighted in red) together with synonymous shield mutations (highlighted in green) to prevent Cas9 re-cutting following HDR-mediated integration. Sequencing-confirmation primers amplify the edited region for Sanger verification. gRNA oligonucleotides are listed as forward/reverse annealing pairs with *Bbs*I-compatible overhangs for directional cloning into the Cas9/gRNA expression plasmid.

Primer ID	Sequence 5'-3'	Use
K13_5HA1_Fwd	gatcTCTAGAAAGAAGAACATAGGAAAC GATTG	C473S donor: 5' homology arm forward primer
K13_3HA1_Rev	atgcGTGACATTATCAATACCTCCAAC AACATATAT	C473S donor: 3' homology arm reverse primer
K13_C473S_3HA_Fwd	AGTACTCCGATGTCAACGAAGAAAGC TTATTTTGGAAAGTGCTGTAT	C473S: edit-site (mutagenesis) forward primer
K13_C473S_5HA_Rev	TCTTCGTTGACATCGGAGTACTCATA GCCAGCATTGTTGACT	C473S: edit-site (mutagenesis) reverse primer
K13_5HA2_Fwd	atgcTCTAGACTTAGATAGGGATAGTGA GTTATT	543/561/579 donors: 5' homology arm forward primer
K13_3HA2_Rev	atgcGTGACTTATATATTTGCTATTAAA ACGGAGTGA	543/561/579 donors: 3' homology arm reverse primer
K13_I543T_3HA_Fwd	AGGATCTATTGTACAGGGGGATATGAT GGCTCTTC	I543T: edit-site (mutagenesis) forward primer
K13_I543T_5HA_Rev	TCCCCCTGTACAATAGATCCTACCATT TGACGTAACACCAC	I543T: edit-site (mutagenesis) reverse primer
K13_R561H_3HA_Fwd	TCATCATATGAAGGCCTGGGTAGAGG TGGCACCTT	R561H: edit-site (mutagenesis) forward primer
K13_R561H_5HA_Rev	CCCAGGCCTTCATATGATGATCATATG CTTCTACATTC	R561H: edit-site (mutagenesis) reverse primer
K13_M579I_3HA_Fwd	TCCAAGATCTTCTGCAATTGTGTTGC TTTTGATAATAAAATTTATGT	M579I: edit-site (mutagenesis) forward primer
K13_M579I_5HA_Rev	ACACAATGCGAGAAGATCTGGAGT ATTCAAAGGTGCCACCTCTA	M579I: edit-site (mutagenesis) reverse primer

K13_5HA3_Fwd	CTTAGATAGGGATAGTGAGTTATTTA G	Sequencing confirmation forward primer
K13_3HA3_Rev	GAACATAACATATTAGATTCCGTTGAA C	Sequencing confirmation reverse primer
Selected guides for gRNA insertion into Cas9 plasmids for <i>Pfkelch13</i> gene editing		
K13_gRNA1_Fwd	TATTGTAAGCTTTTTTGGTAGACAT	C473S gRNA oligo (forward)
K13_gRNA1_Rev	AAACATGTCTACCAAAAAAGCTTAC	C473S gRNA oligo (reverse)
K13_gRNA2_Fwd	TATTGGGTAGAATTTATTGTATTGG	I543T gRNA oligo (forward)
K13_gRNA2_Rev	AAACCCAATACAATAAATTCTACCC	I543T gRNA oligo (reverse)
K13_gRNA3_Fwd	TATTGTGATCATCGTATGAAAGCAT	R561H gRNA oligo (forward)
K13_gRNA3_Rev	AAACATGCTTTCATACGATGATCAC	R561H gRNA oligo (reverse)
K13_gRNA4_Fwd	TATTGACACATAGCTGATGATCTAG	M579I gRNA oligo (forward)
K13_gRNA4_Rev	AAACCTAGATCATCAGCTATGTGTC	M579I gRNA oligo (reverse)

Supplementary Table 2: *In vitro* drug susceptibility of the SenTH15.14 parent line and isogenic *Pfkelch13*-edited derivatives.

Half-maximal effective concentration (EC_{50}) values were determined for dihydroartemisinin (DHA), chloroquine (CQ), monodesethylamodiaquine (mdADQ), lumefantrine (LUM), and piperazine (PPQ) in the SenTH15.14 WT parent line and CRISPR-engineered *Pfkelch13* mutant lines (R561H, I543T, M579I, and C473S) generated in the same genetic background. Values are presented as mean $\pm$ SD from three independent biological replicates. EC_{50} values for the CRISPR-edited lines did not differ significantly from those of the SenTH15.14 WT parent for the tested compounds. Dose–response (EC_{50}) curves for each drug and parasite line are shown in **Supplementary Fig. 3**.

	DHA	CQ	mdADQ	LUM	PPQ
<i>Parent (WT)</i>	1.63 $\pm$ 0.2	76.9 $\pm$ 25.8	18.0 $\pm$ 5.3	1.08 $\pm$ 0.3	4.66 $\pm$ 1.0

<i>R561H</i>	1.01 ± 0.6	82.7 ± 24.5	15.8 ± 2.9	0.80 ± 0.2	5.43 ± 1.1
<i>I543T</i>	1.07 ± 0.6	80.7 ± 17.5	18.3 ± 4.4	1.12 ± 0.1	5.45 ± 0.8
<i>M579I</i>	1.12 ± 0.2	94.5 ± 7.5	23.5 ± 0.2	0.89 ± 0.1	6.26 ± 0.4
<i>C473S</i>	1.06 ± 0.6	91.7 ± 34.4	19.5 ± 6.1	0.74 ± 0.1	4.90 ± 0.7