SUPPLEMENTAL MATERIAL

Pfs25 library preparation and sequencing. Barcoded Pfs25 polymerase chain reaction (PCR) products from individual pools and a 3D7 control were purified using PureLink PCR Purification and quantified using Qubit fluorometer (Invitrogen, Carlsbad, CA). PCR products were pooled equimolarly into a single aliquot for Ion Xpress Plus Fragment Library preparation (Invitrogen), and the library was prepared as per protocol. Library quantity and quality was assessed on the Tapestation 2200 (Agilent, Santa Clara, CA). The prepared library was further pooled with Pfs48/45 libraries (described below), and the final pool was sequenced on an Ion Torrent Personal Genome Machine using 400-bp chemistry and a 314 chip.

Pfs25 analysis. Because of the differing lengths of the Pfs25 and Pfs48/45 under consideration, we adopted different approaches—both previously published—to analyze these sequence data.[5,6] In the case of Pfs25, we used SeekDeep software (http://baileylab.umassmed.edu/SeekDeep/) to cluster full-length Pfs25 reads. This iterative clustering method identifies and corrects errors arising from PCR and sequencing by comparing clustering results across PCR replicates and allowing for the base insertion errors that are common in Ion Torrent sequencing data. Because of our moderate sequencing coverage (average depth: 4,168 [range: 430–,835]), we set a 2.00% detection limit, allowing us to detect any allele that exists at > 2.00% in a population but eliminating potential noise that occurs at lower frequencies.

Pfs48/45 library preparation and sequencing. Because of their extended length, Pfs48/45 PCR products were amplified with non-barcoded primers. Products were sheared to an average length of 300 base pairs (bp) using the Covaris S2 Acoustic Ultrasonicator (Covaris, Woburn, MA). Sheared PCR fragments from each pool, as well as from a 3D7 control and from a sequencing control, were prepared individually for Ion Torrent sequencing as described above. Individual prepared libraries were pooled with the Pfs25 library, and the pooled libraries were run on an Ion Torrent Personal Genome Machine using 400-bp chemistry and a 314 chip.

Pfs48/45 analysis. Similar to our previous work, we enforced a suite of nonsequential quality controls on the reads and their constituent bases to overcome PCR and sequencing error.[6] Reads were initially mapped to the reference sequence using *bwa-mem* with default unpaired read settings. Custom python scripts were then used to filter each base for inclusion and to calculate final frequency for minor allele detection.[6] Bases were censored if 1) they had poor mapping ($q < 10$), 2) reads were less than 200 bp, 3) bases were within 10 bp of the end of the read, 4) the read had poor alignment score (≤ 80), 5) minor alleles did not occur on both strands, or 6) the base had a Phred score quality of < 34 (based on our previous work).

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SUPPLEMENTAL TABLE 1
Samples used for study

Country	Number	Year collected	Patient population	Reference
India	24	2000	Isolates collected from patients presenting with uncomplicated malaria at the clinic of S.C.B. Medical College	Unpublished
Cambodia	81	2009–2011	Isolates collected from adults presenting with uncomplicated malaria in several sites in Cambodia	1
Malawi	60	2010	Isolates collected from children and adults presenting with uncomplicated malaria in Lilongwe	2
Tanzania	43	2005	Isolates collected from children presenting with uncomplicated malaria in Fukayosi, Bagamoyo District	3
DRC	100	2013	Isolates collected from asymptomatic children from multiple sites in the DRC in the Demographic Health Survey	Unpublished
Madagascar	21	2004	Isolates collected from adults presenting with uncomplicated malaria in Sainte Marie	4

DRC = Democratic Republic of Congo.