

Supplementary information

***Plasmodium falciparum* is evolving to
escape malaria rapid diagnostic tests in
Ethiopia**

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SUPPLEMENTARY INFORMATION

***Plasmodium falciparum* is evolving to escape malaria rapid diagnostic tests in Ethiopia**

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SUPPLEMENTARY NOTE

Samples subjected to molecular analysis

820 samples with complete demographic and clinical data underwent molecular analysis, including a randomly selected 248/361 (68.7%) of those with the discordant RDT profile of interest (HRP2-, Pf-LDH+), as well as 465/2115 (22.0%) randomly selected *P. falciparum* RDT HRP2 positives (HRP2+, Pf-LDH+) as controls. The remaining 107 samples included 90 with inconclusive HRP2 results (HRP2+ by only 1 RDT, of which 67 were Pf-LDH- and 23 were Pf-LDH+) and 17 negative controls (HRP2-, Pf-LDH-). Quantitative real-time PCR (qPCR) targeting the *P. falciparum* lactate dehydrogenase (*pfl dh*) gene confirmed parasitemia in 731/820 (89%) samples, with a geometric mean (GM) of 1,390.7 parasites/ μ L (geometric standard deviation [geoSD]: 9.8).

*Comparison of *pfhrp2/3* PCR, RDT, and HRP2 bead-based assays*

Concordance between *pfhrp2*-negative PCR results and the discordant RDT profile was good (Cohen's kappa 0.66). Overall, among samples with the discordant RDT profile, 64.8% (95% CI 57-72) were *pfhrp2*-/*3*- and 8.0% (95% CI 5-13) *pfhrp2*-/*3*+, with an additional 15.9% (95% CI 11-22) *pfhrp2*+/*3*- (**Extended Data Figure 2**). Interestingly, of samples HRP2+ by both RDTs, *pfhrp3* could not be amplified in 42.6% (95% CI 38-48).

We observed expected agreement between the results of *pfhrp2/3* PCR assays, RDTs, and a bead-based immunoassay applied to a subset of 456 samples. 93% (95% CI 86-96) of samples *pfhrp2*+/*3*+ by PCR tested positive for HRP2 antigen (GM 40,284 pg/mL HRP2, geoSD 7.5). In comparison, 19% (12-29) of *pfhrp2*-/*3*- samples were HRP2+, with a GM of 2,089 pg/mL HRP2 (geoSD 5.5).

HRP2+ but *pfhrp2*- PCR results are expected in a subset of subjects because HRP2 antigenemia can persist for weeks after clearance of parasitemia¹. 92% (95% CI 88-95) of samples HRP2+, Pf-LDH+ by RDT were HRP2+ by the antigen assay (GM 34,536 pg/mL, geoSD 6.5), compared to 24% (95% CI 18-31) of those with the discordant HRP2-, Pf-LDH+ RDT profile of interest (GM 2,455 pg/mL, geoSD: 7.2) (**Table 2**).

Pf-LDH RDT performance

Despite the prevalence of *pfhrp2*-deleted parasites in this study, use of the SD Bioline's Pf-LDH RDT band rather than its HRP2 band did not improve RDT performance. Among 820 samples with RDT results and *pfdh* real-time PCR results available, overall agreement with PCR was slight regardless of test band used (Cohen's kappa 0.04 for the Pf-LDH band and 0.15 for the HRP2 band). Using *pfdh* PCR as the gold standard, the sensitivity and specificity of the Pf-LDH RDT band were 69% and 38%, respectively. The sensitivity and specificity of the HRP2 RDT band were 62% and 72%, respectively. The poor performance of the Pf-LDH RDT band was unexpected, as the RDTs used in this study passed WHO lot testing prior to shipment. Comparison of RDT results within the larger cohort (**Supplementary Table 11**) suggests several explanations, including decreased performance due to inappropriate storage conditions, operational or administrative user error (Pf-LDH test bands are often fainter than HRP2, especially at lower parasite densities), cross-reactivity with other species (though only in a minority of cases as outlined below, co-infections, and/or immunological factors).

For overall detection of *P. falciparum* parasitemia using any falciparum-specific antigen, the SD and CareStart products had similar levels of agreement with

pfl dh PCR. Compared to PCR, the sensitivity and specificity of the SD RDT (HRP2+ and/or Pf-LDH+) were 93% and 20% (Cohen's kappa 0.14), respectively; the sensitivity and specificity of the CareStart RDT (HRP2+) were 69% and 60% (Cohen's kappa 0.15), respectively. However, because this study purposefully oversampled subjects at high risk for infection by *pfhrp2/3*-deleted parasites for molecular testing, these estimates should not be generalized to routine care in these regions.

Plasmodium vivax co-infection and *pfhrp2/3* deletion

Though analysis of *P. vivax* co-infection was not an objective of this study, analysis of available RDT data did not reveal evidence of selection for *pfhrp2* deletion in the presence of *P. vivax* co-infection. First, the frequency of *pfhrp2* deletion was similar among subjects with versus without *P. vivax* co-infection. Among subjects who underwent *pfhrp2/3* PCR genotyping, *P. vivax* co-infection was identified by the CareStart RDT (Pv-LDH+) in 56 subjects. Of these, 41 (73.2%) were *pfhrp2+* and 15 (26.8%) were *pfhrp2-*. Among all 608 samples with CareStart RDT results who were subjected to *pfhrp2/3* PCR genotyping, 445 (73.2%) were *pfhrp2+* and 163 (26.8%) were *pfhrp2-*. Second, we did not observe an association between *P. vivax* co-infection and subtelomeric deletion structural profile. While there was only a single *pfhrp2* deletion profile observed in this study, three distinct *pfhrp3* deletion profiles were observed. The frequency of *P. vivax* co-infection was similar in the two dominant *pfhrp3* deletion subtelomeric structural profiles. 14 of 134 (10.4%) subjects with the *P. falciparum* chr13-P4 (*pfhrp3* deletion) profile and 6 of 106 (5.7%) with the chr13-P3 (*pfhrp3* deletion) profile had co-infection with *P. vivax*.

Subtelomeric profiling and variant calling using MIPs

The MIP panel was designed using a tiled strategy that involved multiple, overlapping probes spanning each gene target. We detected 244 of 273 targets with sufficient mapping quality and depth across multiple segments of both *pfhrp2* and *pfhrp3* and their flanking regions, spanning positions 1,344,451 to 1,397,773 and 2,780,863 to 2,853,533 of chromosomes 8 and 13, respectively. Fourteen total probes were used to target different segments of *pfhrp2* (n = 8 probes) and *pfhrp3* (n = 6). 241 MIPs mapped to 273 targeted loci on the reference genome, including 32 extra loci accounting for the multicopy genes on chromosome 11.

In total, 43,541,045 reads were devoted to this sample set, or roughly half of a single NextSeq 550 mid-output flow cell. Probes failing to amplify in >90% of the samples were removed from the analysis, leaving 244 loci. 841 of 1014 samples and controls had sequence data after read mapping. 20 of 841 belonged to control strains (positive controls). None of the 20 negative controls had any sequence mapping to the reference genome. Deletion calls were only made in samples with sufficient depth of UMI coverage (see Methods), leaving 375 high-coverage samples from the study cohort and 6 controls in the final call set.

The median parasite density for samples successfully called using MIP sequencing was 5,077 p/μL (SD: 1.6×10^4), compared to 264 p/μL (SD: 5.9×10^3) for samples with failed MIP calls. Among 367 (97.9%) MIP-called samples with matching PCR data, 85 (23.2%) were *pfhrp2*-/*3*- by PCR. Receiver-operator curve analysis indicated the optimal parasite density threshold above which samples had sufficient coverage for MIP calling was approximately 925 p/μL, although this threshold is project-specific and expected to improve with additional sequencing effort (**Extended Data Figure 4**).

Comparison of pfhrp2/3 MIP sequencing to PCR, HRP2 immunoassay, and whole-genome sequencing results

Among 367 (97.9%) MIP-called samples with matching PCR data, 85 (23.2%) were *pfhrp2-/3-* by PCR. MIP sequencing results indicated that 126/367 (34.3%; 95% CI 30-39) were *pfhrp2-*, 264/367 (71.9%; 95% CI 67-76) *pfhrp3-*, and 116/367 (31.6%; 95% CI 27-37) *pfhrp2-/3-* by MIP sequencing. Of samples called *pfhrp2-* by MIP sequencing, 82.0% were *pfhrp2-* by PCR and 73.3% had the discordant RDT profile. Similarly, of samples called *pfhrp3-* by MIP sequencing, 76.7% were *pfhrp3-* by PCR. Differences between genotyping results were apparent and expected due to differences in targets and methodologies (PCR is better suited for samples with low parasite density than MIP sequencing). Comparison of results from MIP and PCR *pfhrp2/3* deletion genotyping revealed excellent agreement between the two approaches for *pfhrp2* (Cohen's kappa 0.82) and good agreement for *pfhrp3* (Cohen's kappa 0.63).

Comparison to the bead-based HRP2 immunoassay results provided additional confidence in the validity of our *pfhrp2/3* deletion calls using MIP sequencing. 175 MIP-called samples also had bead-based antigen detection results available. Despite fundamental differences in the targets of these two approaches (*pfhrp2* gene versus HRP2 antigen, which can linger after clearance of infection),¹ observed agreement between the two methods was consistent with expectation. Of those samples *pfhrp2+* by MIP sequencing, 94.1% were HRP2+ by bead-based antigen immunoassay, whereas 79.5% of those *pfhrp2-* by MIPs were also HRP2- by the immunoassay.

We used whole-genome sequencing (WGS) to evaluate *pfhrp2/3* MIP sequencing results and breakpoint regions in a small validation subset. Among 14

samples subjected to WGS and with MIP calls, median WGS depth of coverage was 20 reads/locus (range 4-38). While the distribution of aligned reads was uneven in the regions flanking *pfhrp2* and *pfhrp3*, visual inspection of WGS coverage supported 13 (93%) *pfhrp2* and 14 (100%) *pfhrp3* deletion calls made using MIP sequencing data (**Figure 4**). For the single discordant *pfhrp2* deletion call (lab ID: 1314), *pfhrp2* PCR results were consistent with the MIP sequencing result. Precise mapping of breakpoint regions using WGS was not possible due to regions of very high coverage (“jackpotting”) resulting from selective whole-genome amplification and low coverage due to ambiguous read mapping to repetitive and paralogous loci. However, breakpoint regions identified using MIPs were consistent with WGS coverage centromeric to *pfhrp2* and *pfhrp3* on chromosomes 8 and 13, respectively, with the exception of calls in chromosome 13’s multi-copy 28S rRNA gene. Discordance in these calls was expected due to ambiguous mapping of short-read sequences to a multi-copy gene. MIP results from well-characterized lab strains 3D7 (*pfhrp2*+/*3*+), DD2 (*pfhrp2*-/*3*+), and HB3 (*pfhrp2*+/*3*-) were consistent with whole-genome alignments of published short-read data. Telomeric deletion breakpoint assessment was limited by a small number of successful MIP targets telomeric to both genes. However, the concordance in *pfhrp2/3* deletion calls and centromeric deletion breakpoint regions by MIP and WGS techniques confirmed the utility of MIPs for identifying *pfhrp2/3* deletions and determining their extent and breakpoint regions.

Association between malaria symptoms, geographical location, and subtelomeric structural variants

We did not observe an association between subtelomeric deletion profile and

the number of symptoms experienced by subjects (**Extended Data Figure 5**). Because the majority of subjects (96.5%, 2620/2714) who tested positive for *P. falciparum* by RDT were febrile, fever alone was not sufficient to evaluate disease severity. Therefore, as a crude metric of disease severity, we calculated the total number of symptoms (six total were assessed: fever, headache, joint pain, feeling cold, nausea, and lack of appetite). No significant differences in the total number of symptoms by deletion profile were revealed for chromosome 8 ($F(3, 334) = 0.29$, $p = 0.83$) or chromosome 13 ($F(2, 335) = 0.33$, $p = 0.72$) by one-way ANOVA. No obvious spatial patterns in subtelomeric deletion profiles were apparent at the regional level (**Supplementary Table 2**).

Extended haplotype homozygosity analysis

91 and 17 biallelic SNPs within the 28kb and 27kb regions centromeric to *pfhrp2* and *pfhrp3* deletions, respectively, were used to calculate EHH statistics. 327 samples with *pfhrp2* deletion calls using MIP sequencing and sufficient variant data were included in the EHH analysis, including 212 *pfhrp2*-intact and 115 *pfhrp2*-deleted haplotypes. 162 samples with *pfhrp3* deletion calls using MIP sequencing and sufficient variant data were included in the EHH analysis, including 37 *pfhrp3*-intact and 125 *pfhrp3*-deleted haplotypes with three distinct subtelomeric structural profiles. No variant calls were made in the 15.5 kb region immediately centromeric to *pfhrp3* to avoid ambiguity in read mapping to the duplicated DNA segment containing multicopy genes including 5.8S, 28S rRNA.

REFERENCES

- 1 Markwalter CF, Gibson LE, Mudenda L, Kimmel DW, Mbambara S, Thuma PE *et al.* Characterization of Plasmodium Lactate Dehydrogenase and Histidine-Rich Protein 2 Clearance Patterns via Rapid On-Bead Detection from a Single Dried Blood Spot. *Am J Trop Med Hyg* 2018; **98**: 1389–1396.

Supplementary Table 1. PCR results by age, sex, and parasite density. P values were calculated using chi-square (gender) and the non-parametric Kruskal-Wallis test (age, parasitemia).

	PCR-based deletion calls				p value*	test statistic
	<i>pfhrp2</i> +/ <i>3</i> +	<i>pfhrp2</i> -/ <i>3</i> -	<i>pfhrp2</i> -/ <i>3</i> +	<i>pfhrp2</i> +/ <i>3</i> -		
N	253	136	27	192		
Sex = Female, N (%)	72 (29%)	48 (35%)	7 (26%)	59 (31%)	0.53	$X^2(3, N=610) = 2.24$
Age, Median (IQR)	19 (13)	18 (14)	16 (14)	19 (14)	0.76	$X^2(3, N=608) = 1.16$
Parasitemia (p/ μ L), Median (IQR)	2824 (7712)	3877 (8191)	2112 (6828)	2946 (8340)	0.14	$X^2(3, N=608) = 5.54$

Supplementary Table 2. RDT profile by district for individuals *P. falciparum*-positive by RDT (n=2,714). Abbreviations: Pf, *P. falciparum*; HRP2, histidine-rich protein 2; Pf-LDH, *P. falciparum*-specific lactate dehydrogenase; RDT, rapid diagnostic test.

Region / District	Total Pf+, n	RDT results		
		2 HRP2+, 1 PfLDH+	2 HRP2-, 1 PfLDH+	1 HRP2+, 1 HRP2-
Tigray	689	460 (67%)	140 (20%)	85 (12%)
Ahferom	117	83 (71%)	18 (15%)	16 (14%)
Atseged Tsimbila	160	86 (54%)	48 (30%)	26 (16%)
Gulomekeda	21	14 (67%)	5 (24%)	2 (10%)
K. Humera	176	115 (65%)	39 (22%)	21 (12%)
L. Adiabo	145	111 (77%)	22 (15%)	11 (8%)
T. Adiabo	69	51 (74%)	10 (14%)	6 (9%)
Amhara	1,342	997 (74%)	211 (16%)	131 (10%)
Metema	233	178 (76%)	30 (13%)	24 (10%)
Quara	516	435 (84%)	39 (8%)	42 (8%)
Tegede	296	197 (67%)	65 (22%)	33 (11%)
West Armachiho	295	187 (63%)	75 (25%)	32 (11%)
Gambella	683	658 (96%)	10 (1%)	15 (2%)
Itang	151	144 (95%)	2 (1%)	5 (3%)
Kule Refugee Camp	472	461 (98%)	4 (1%)	7 (1%)

Supplementary Table 3. Prevalence estimate sensitivity analysis. Abbreviations: CI, confidence interval; RDT, rapid diagnostic test; Pf-Pos, *P. falciparum*-positive by RDT.

Approach #1 (primary): Ci.impt function within asbio package, which calculates 95% CI of product of 2 proportions

	Pf-Pos, n	Discordant RDTs, n*	Discordant RDT prevalence, % (95% CI)	<i>pfhrp2</i> - PCR concordance (overall), %*	ci.impt prevalence estimate, % (95% CI)
Amhara	1342	211	15.8 (13.9-17.8)	72.7	11.5 (9.8-13.4)
Tigray	689	140	20.4 (17.5-23.7)	72.7	14.9 (12.5-17.7)
Gambella	683	10	1.5 (0.7-2.8)	72.7	1.1 (0.6-2.0)

Approach #2: Bootstrapping (1000 iterations)

	Pf-Pos, n	Discordant RDTs, n*	Discordant RDT prevalence, % (95% CI)	<i>pfhrp2</i> - PCR concordance (overall), %*	Estimated N with discordant RDT and <i>pfhrp2</i> - PCR result	Prevalence estimate, %	Bootstrap 95% CI
Amhara	1342	211	15.8 (13.9-17.8)	72.7	153.397	11.5	9.7-13.2
Tigray	689	140	20.4 (17.5-23.7)	72.7	101.78	14.9	12.2-17.4
Gambella	683	10	1.5 (0.7-2.8)	72.7	7.27	1.1	0.2-1.8

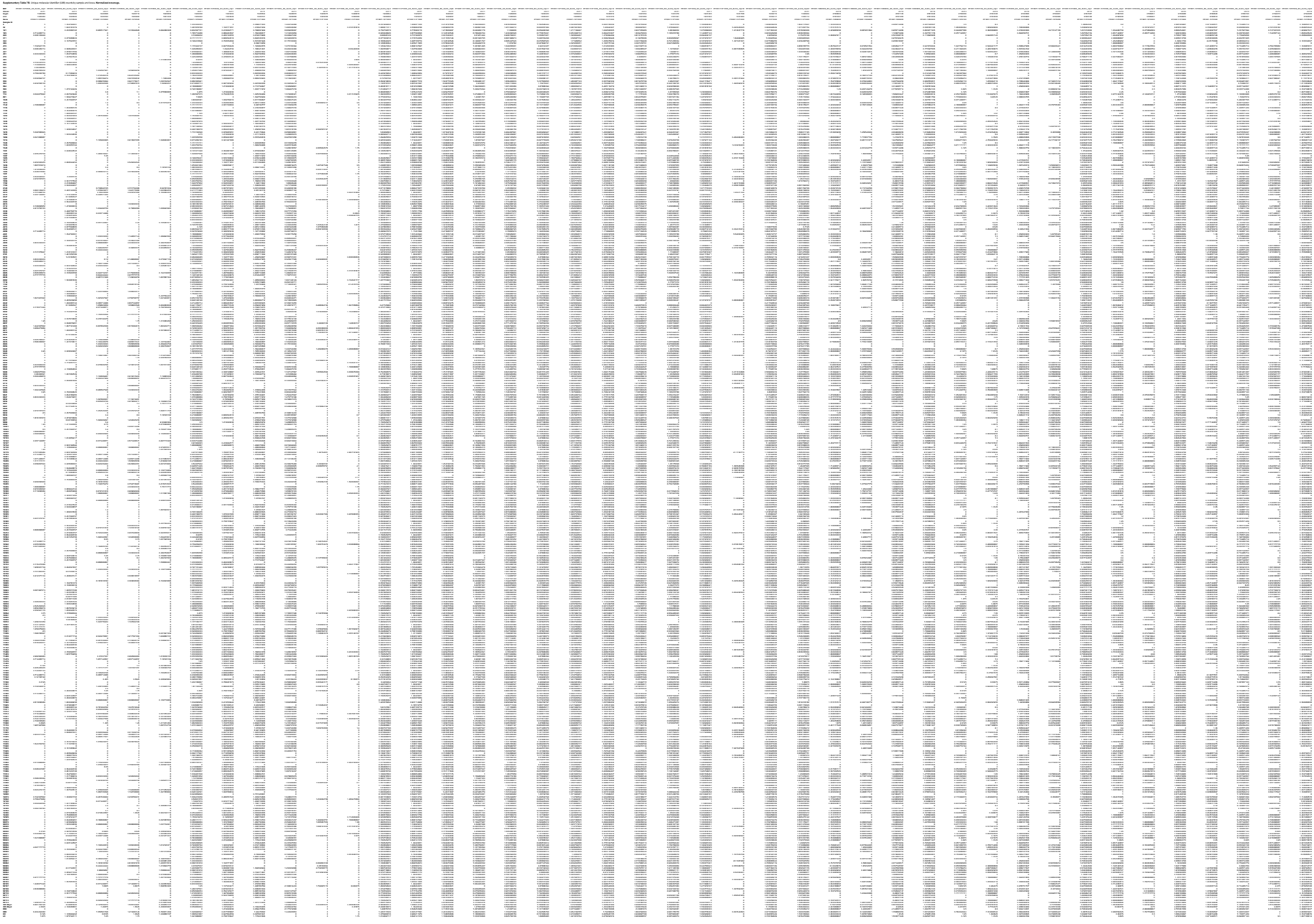
* Cases Pf-LDH-positive but HRP2-negative on both RDTs were considered potential candidates for *pfhrp2/3* gene deletion and defined as 'discordant.'

** Overall proportion of 'discordant RDT profile' infections with *pfhrp2* deletion calls by PCR.

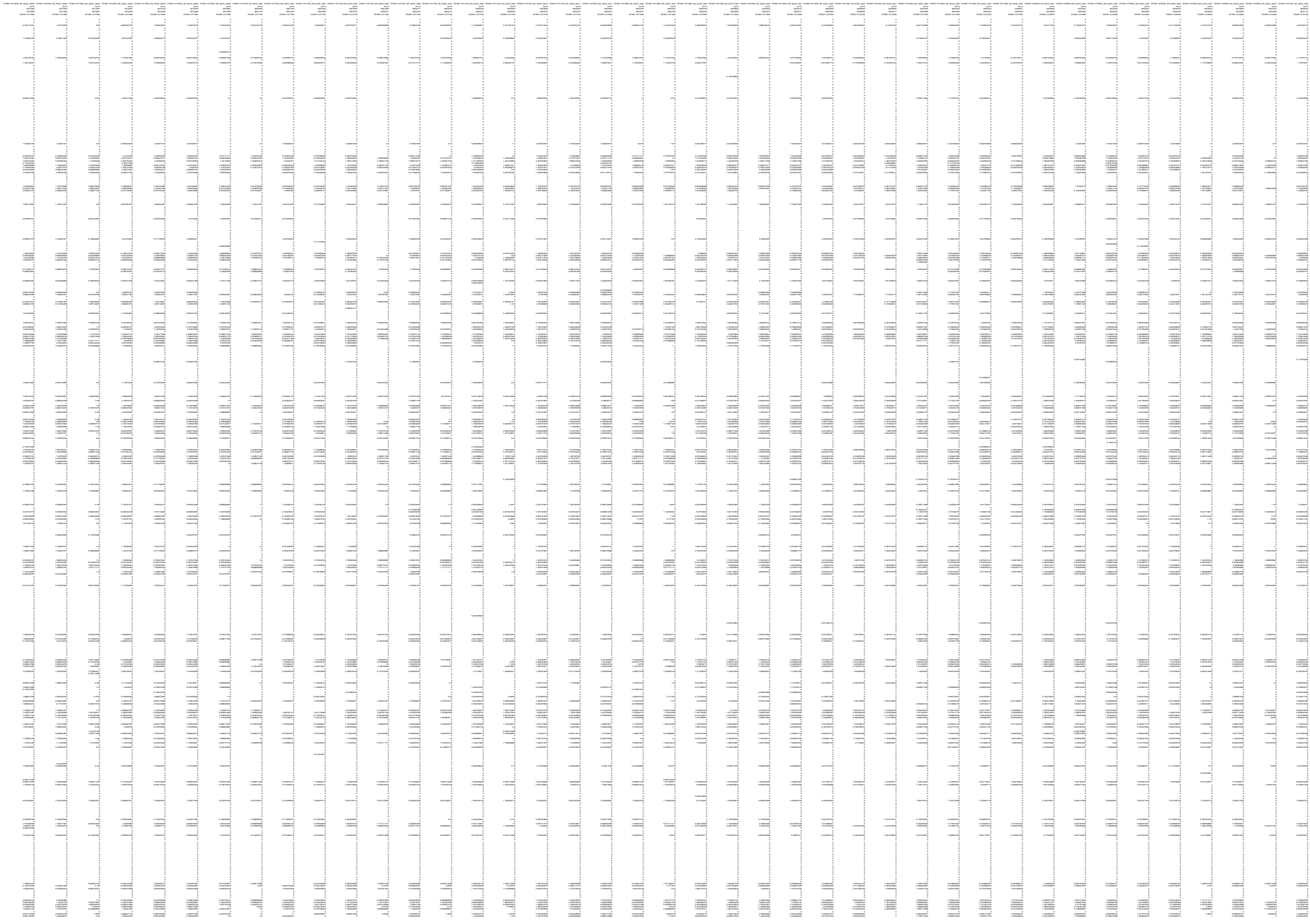
Supplementary Table 4. MIP subtelomeric structural profiles by region for 367 samples with both MIP calls and PCR data.

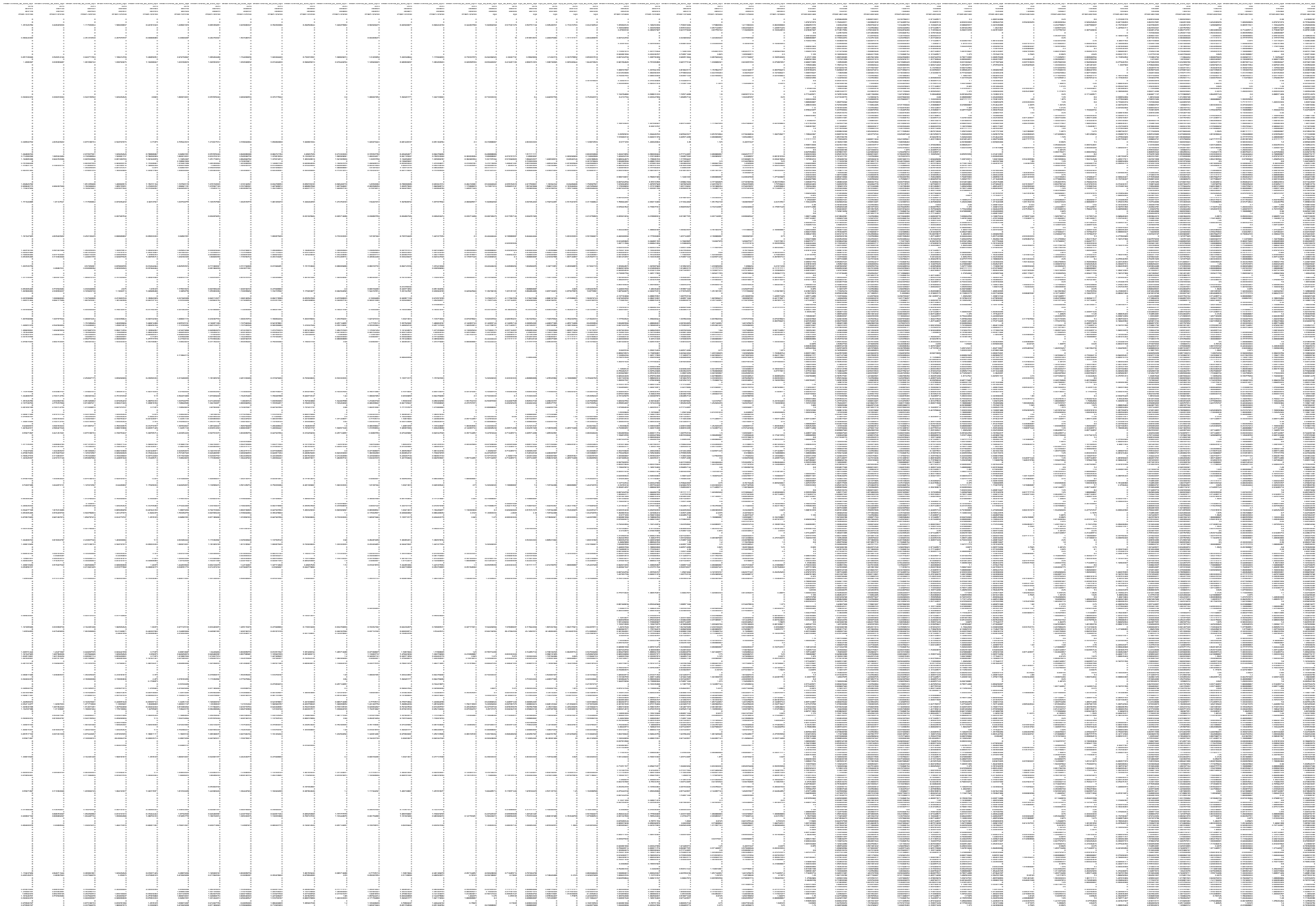
MIP call	Region		
	Tigray (n=128)	Amhara (n=208)	Gambella (n=31)
<i>pfhrp2+</i>	79	143	19
chr8-P1	40 (51%)	84 (59%)	13 (68%)
chr8-P2	18 (23%)	27 (19%)	3 (16%)
chr8-P3	21 (27%)	32 (22%)	3 (16%)
<i>pfhrp2-</i>	49	65	12
chr8-P4	49 (100%)	65 (100%)	12 (100%)
<i>pfhrp3+</i>	31	59	13
chr13-P1	31 (100%)	59 (100%)	13 (100%)
<i>pfhrp3-</i>	97	149	18
chr13-P3	41 (42%)	70 (47%)	7 (39%)
chr13-P4	56 (58%)	79 (53%)	11 (61%)

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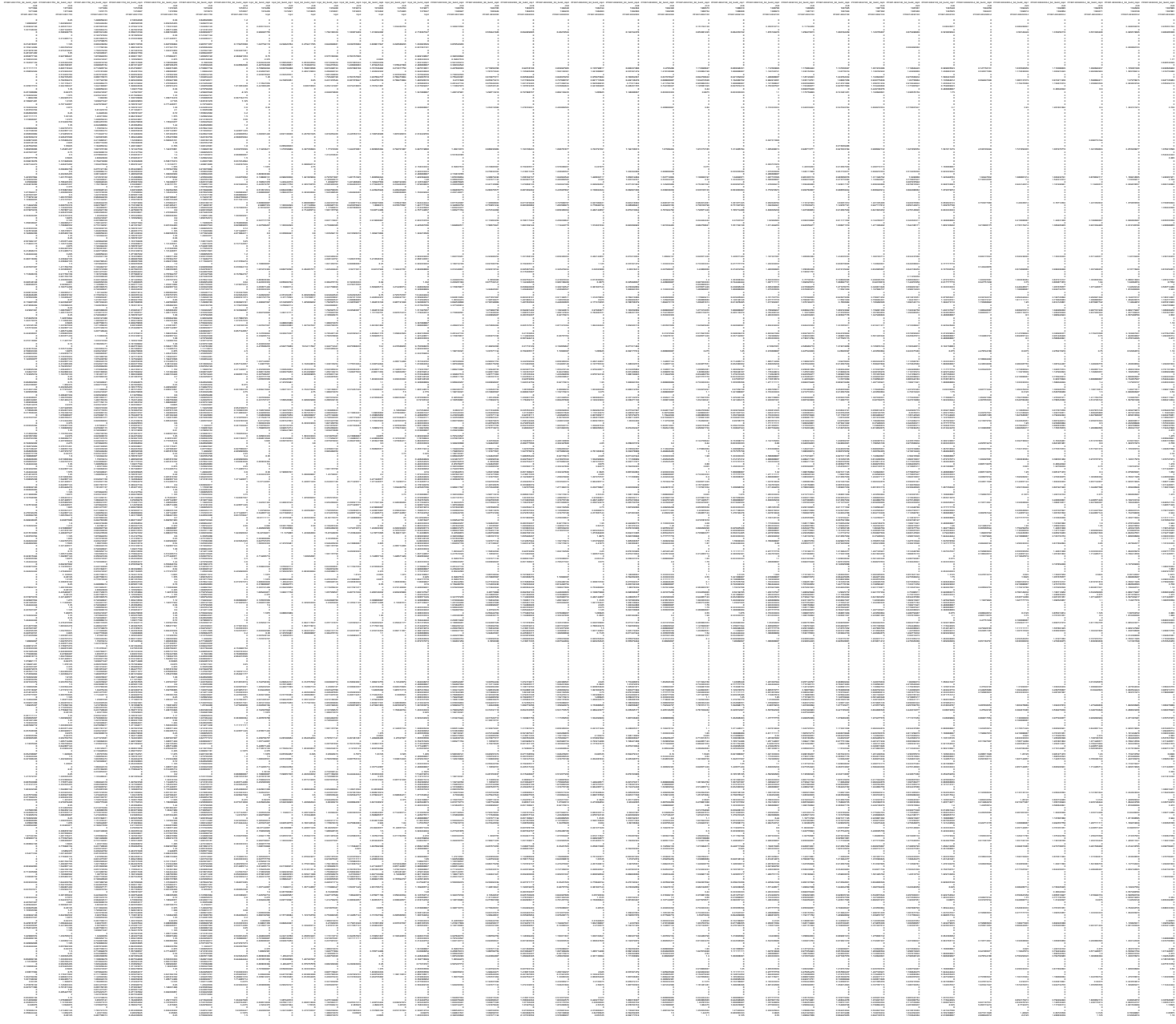


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Supplementary Table 9. ENA accession numbers of previously published WGS data.

Sample	ENA
QS0056-C	ERR1035493
QS0087-C	ERR1035520
QS0104-C	ERR1035536
QS0109-C	ERR1045266
QS0110-C	ERR1045267
QS0116-C	ERR1045271
QS0126-C	ERR1045280
QS0127-C	ERR1045281
QS0128-C	ERR1045282
QS0129-C	ERR1045283
QS0132-C	ERR1045286
QS0133-C	ERR1045287
QS0135-C	ERR1045288
QS0144-C	ERR1045295
QS0154-C	ERR1106575
QS0155-C	ERR1106606
QS0156-C	ERR1106576
QS0157-C	ERR1106577
QS0159-C	ERR1106579
QS0162-C	ERR1106581
QS0163-C	ERR1106582
QS0166-C	ERR1106584
QS0168-C	ERR1106586
QS0169-C	ERR1106587
QS0170-C	ERR1106590
HB3	ERR012788
DD2	ERR012840
3D7	ERR019061

Supplementary Table 10. Summary statistics of WGS coverage for samples sequenced in this study. Abbreviations: % Coverage, percent of the *P. falciparum* 3D7 (v3) genome with at least 1, 5, or 10 aligned reads per locus.

lab_ID	% Coverage 1X	% Coverage 5X	% Coverage 10X	median coverage value
1156	77.0	46.9	32.5	4
1194	88.1	69.0	53.2	11
1197	91.2	79.3	66.8	20
1208	92.3	84.1	74.7	30
1220	89.4	73.0	58.5	14
1225	79.0	46.6	30.4	4
1268	92.7	87.0	79.3	33
1284	92.2	83.0	72.8	26
1314	90.5	76.8	63.5	17
1333	85.7	60.2	42.2	7
5338	92.4	83.9	73.4	24
9592	93.5	89.2	82.9	36
9821	88.8	70.5	55.4	12
10809	76.3	46.4	33.2	4
10839	46.1	7.9	2.4	0
10885	91.2	78.3	64.1	16
10901	93.3	88.1	81.4	38
10905	91.1	79.4	67.7	21
10924	93.2	88.6	82.0	36
10937	93.0	85.7	76.6	30
10941	87.4	68.0	54.2	12
10957	91.7	80.5	68.5	21
12129	87.5	68.9	55.3	12
99112	92.9	86.7	78.6	31

Supplementary Table 11. RDT results from 12,572 enrolled subjects.

CareStart	SD Bioline					
	HRP2 and Pf-LDH	HRP2 only	Pf-LDH only	Negative	Missing	Invalid
HRP2 and Pv-LDH	536	31	7	18	0	1
HRP2 only	1000	549	49	123	1	3
Pv-LDH only	5	1	39	540	5	0
Negative	6	22	322	9297	0	7
Missing	0	0	0	0	1	0
Invalid	0	1	1	1	0	6

P. falciparum Beta tubulin (PfBTubulin)

Adapted from: Primers: Price RN et al. Lancet 2004. 364:438-447.
 Flap: Afonina I et al. Biotechniques 2007. 770-774.
 Combined assay: Parr JB et al. Malar J 2018. 17:137.

Forward Primer (5'→3') AATAAATCATAATGATGTGCGCAAGTGATCC
 Reverse Primer (5'→3') AATAAATCATAATCCTTTGTGGACATCTTCCTC
 Probe (5'→3')

Cycling conditions:	Temp	Duration	No. Cycles
	50C	2 min	x1
	95C	10 min	x1
	95C	15 sec	x45
	60C	1 min	

Reaction conditions: SYBR Green MM

Fwd primer	200 nM
Rev primer	200 nM
Template	2 µl
Total volume	12 µl

Pfhrp2 - exon 2

Adapted from: Baker J et al. J Infect Dis 2005. 192:870-877.

Forward Primer (5'→3') CAAAAGGACTTAATTTAATAAGAG
 Reverse Primer (5'→3') AATAAATTTAATGGCGTAGGCA

Cycling conditions:	Temp	Duration	No. Cycles
	94C	10 min	x1
	94C	50 sec	
	55C	50 sec	x45
	70C	60 sec	
	72C	7 min	x1

Reaction conditions: AmpliTaq Gold 360 Master Mix

Fwd primer	200 nM
Rev primer	200 nM
Template	10 µl
Total volume	25 µl

P. falciparum-specific pfl dh

Pickard AL et al. Antimicrob Agents Chemo 2003. 47(8):2418-2423.

ACGATTTGGCTGGAGCAGAT
 TCTCTATTCCATTCTTTGTCACTCTTC
 FAM/ AGTAATAGTAACAGCTGGATTTACCAAGGCCCA/TAMRA

Temp	Duration	No. Cycles
50C	2 min	x1
95C	10 min	x1
95C	15 sec	x40
60C	1 min	

Roche FastStart Universal Probe Master (Rox)

Fwd primer	200 nM
Rev primer	200 nM
Probe	200 nM
Template	2 µl
Total volume	12 µl

Pfhrp3 - exon 2 (single-step)

Baker J et al. J Infect Dis 2005. 192:870-877.

AATGCAAAGGACTTAATTC
 TGGTGTAAGTGATGCGTAGT

Temp	Duration	No. Cycles
94C	10 min	x1
94C	50 sec	
55C	50 sec	x45
70C	60 sec	
72C	7 min	x1

AmpliAq Gold 360 Master Mix

Fwd primer	200 nM
Rev primer	200 nM
Template	10 µl
Total volume	25 µl