Plasmodium falciparum hrp2 and *hrp3* gene deletion status in Africa and South America by highly sensitive and specific digital PCR

Short title: ***hrp2/hrp3* deletion typing by digital PCR**

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Abstract

Background: The most commonly used *Plasmodium falciparum* rapid diagnostic tests target the Histidine-Rich Proteins 2 and 3 (HRP2, HRP3). An increasing number of countries report parasites that carry *hrp2* and/or *hrp3* gene deletions, resulting in false negative test results. Molecular surveillance of *hrp2* and *hrp3* deletions is crucial but adequate protocols have been lacking.

Methods and Findings: We have developed novel assays for deletion typing based on droplet digital PCR (ddPCR), targeting *hrp2* exon1, *hrp2* exon 2, and *hrp3*. In the ddPCR assay, *hrp2* or *hrp3* and a control gene were quantified with very high accuracy in a single tube. The theoretical limit of detection of the ddPCR assay was 0.33 parasites/uL, and thus well suited for typing of low-density asymptomatic infections. The deletion was reliably detected in mixed infections with wild-type and *hrp2*-deleted parasites when the proportion of parasites carrying the deletion was >40%. For a side-by-side comparison with the conventional nested PCR (nPCR) assay, 248 samples from asymptomatic individuals from western Kenya were screened in triplicate by ddPCR and nPCR. No deletions were observed by ddPCR, while by nPCR no band for *hrp2* was observed in 8% of samples. The ddPCR assay was applied to screen 830 samples from six countries in Africa and South America. No or very few deletions were observed in Kenya (n=241), Zanzibar/Tanzania (n=91), and Ghana (n=223). In southwestern Ethiopia, 1/47 (2.1%) samples carried *hrp2* deletion, and 35/47 (74.5%) *hrp3* deletions. In Brazil, 87/187 (46.5%) samples carried *hrp2* deletions, and 116/187 (62%) *hrp3* deletions. In Ecuador, no *hrp2* deletions were observed, but 22/41 (53.7%) samples carried *hrp3* deletions.

Conclusions: Compared to nPCR, the ddPCR assay minimizes the risk of false-negative results (i.e. *hrp2* deletion observed when the sample is wild type), increases sensitivity, and greatly reduces the number of reactions that need to be run. Pronounced differences in the prevalence of deletion were observed among sites, with more *hrp3* than *hrp2* deletions.

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Introduction

In 2019, over 200 million cases of malaria and over 400,000 deaths were recorded [1]. *Plasmodium falciparum* remains the primary cause of malaria in humans. Fast and accurate diagnosis and treatment of clinical episodes are key components of malaria control. Diagnosis is commonly performed either by light microscopy, or rapid diagnostic tests (RDTs). RDTs are lateral flow devices that detect parasite proteins in human blood through immunohistochemistry. Light microscopy requires basic lab infrastructure and skilled microscopists. In contrast, RDTs require minimal infrastructure and training, and results are available within approximately 15 minutes at a cost of less than 1 USD per test. RDTs are the only field-deployable diagnostic tool available at peripheral health centers and for community screening to diagnose asymptomatic infections, for example through reactive case detection [2]. In 2016, over 300 million RDTs were used by malaria control programs [3].

The most sensitive RDTs for *P. falciparum* rely on the detection of Histidine Rich Protein 2 (HRP2) [4]. HRP2 is a highly expressed secreted protein, and thus an ideal target for diagnosis. It is also the target for a new generation of ultra-sensitive RDTs with a limit of detection of <100 parasites/µL [5, 6]. While alternative RDTs detecting other proteins, e.g. parasite lactase dehydrogenase (pLDH), or aldolase, are available, they are generally less sensitive [7, 8]. HRP2-based RDTs can also detect HRP3, a structurally similar protein sharing multiple epitopes with HRP2.

In 2010, a report revealed that a large proportion of *P. falciparum* parasites in Peru did not carry the *hrp2* gene [9], and thus could not be detected by HRP2-based RDTs. Since then, an increasing number of reports from Latin America [10-14], Africa [15-22], and Asia [23, 24] found varying proportions of parasites with *hrp2*-deletion, reaching up to 80% of clinical cases in certain hospitals in Eritrea [18]. In addition, *hrp3* can

be deleted. The deletion of either *hrp2* or *hrp3*, or both genes, has no known impact on parasite fitness. RDTs can yield a positive result if *hrp2* is deleted but *hrp3* is expressed, but sensitivity of the RDT is lower in this case [25].

Molecular surveillance to assess the frequency of *hrp2* and *hrp3* deletion is crucial to decide whether HRP2-based RDTs can be used [26]. The WHO recommends to use alternative diagnostics if the prevalence of *hrp2* deletion is above 5% [27]. At this level, the number of false-negative tests due to *hrp2* deletion will exceed the number of false-negative tests because of lower sensitivity of the alternative diagnostics. The prevalence of *hrp2* deletion has been found to differ substantially within countries [12, 17], thus the choice of diagnostics might need to be adapted at sub-national level. Where low levels of deletions are present, HRP2-based RDTs remain a highly useful tool for diagnosis.

Deletion screening has been classically done using nested PCR (nPCR) followed by gel electrophoresis [26]. Absence of a band is interpreted as deletion. False-negative results could occur when PCR conditions are suboptimal, or when parasite density is low and amplification is stochastic. To overcome this limitation, 3-fold repetition of the nested *hrp2* PCR and a control PCR (e.g. *msp2*, or *glurp*) is recommended [26]. As a result, for each sample twelve PCRs need to be run. Deletion status might remain unresolved if results differ among replicates. As an additional problem, multiple clone *P. falciparum* infections are common in most endemic settings [28]. In case of a multiple clone infection with a wild-type parasite and a parasite carrying the deletion, the wild type parasite will result in a band on the gel when using the nPCR assay. Multiple clone infections can thus mask the presence of deletions, resulting in an underestimation of the frequency of deletion [29]. More recently, quantitative PCR (qPCR) protocols for *hrp2*/*hrp3* deletion typing were published [30], greatly enhancing throughput. However, when parasite densities are low,

106 considerable variation in quantification is observed between replicates [31]. As a result of the technical
107 challenges for accurate typing, maps of *hrp2* deletion frequency remain scattered and incomplete.
108
109 We have developed a novel method for typing of *hrp2* and *hrp3* deletions based on droplet digital PCR
110 (ddPCR). ddPCR yields highly accurate quantification of parasites [31]. In a ddPCR experiment, the reaction
111 volume is partitioned into approximately 15,000 microdroplets, which are then subject to end-point PCR.
112 Each droplet functions as an individual PCR reaction, with amplification occurring if the droplet contains
113 template DNA. The number of positive droplets corresponds to the number of template DNA copies. Using
114 two different probes, two targets can be quantified in a single reaction well, e.g. a control gene and a
115 target gene. As each droplet with template can be considered a 'within-well replicate', a single well offers
116 the sensitivity and specificity of a large number of replicates by nPCR or qPCR. The risk of false negative
117 results (i.e. no amplification when template is present) is thus minimal compared to nPCR or qPCR. The
118 novel assay greatly reduces the number of reactions to be run, increase sensitivity and accuracy, and can
119 detect the deletion in mixed infections. The assay was extensively validated using culture strains, and field
120 isolates from Kenya, Zanzibar/Tanzania, Ethiopia, Ghana, Brazil, and Ecuador.
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Methods

Ethics statement

Informed written consent was obtained from all study participants or their parents or legal guardians prior to sample collection. The study was approved by the University of Notre Dame Institutional Review Board (approvals 18-08-4803, 19-04-5321, 18-12-5029), the Institutional Scientific and Ethical Review boards of the Noguchi Memorial Institute of Medical Research, University of Ghana, the Committee on Human Research, Publication and Ethics, School of Medical Science, Kwame Nkrumah University of Science and Technology, Kumasi (CHRPE/AP/375/20), the Zanzibar Medical Research Ethics Committee (ZAMREC/0001/Feb/17), the Institutional Review Board of Tulane University (17-993573), the Institutional Review Board of the Ifakara Health Institute (003-2017), the Ethics Commission of North-western and Central Switzerland (Req-2017-00162), the Institutional Review Board of Institute of Health, Jimma University, Ethiopia (RPGC/486/06), Maseno University Ethics Review Committee (MUERC protocol number 00456), the Ethics Committee for Research in Human Beings of the Pontificia Universidad Católica del Ecuador (CEISH-571-2018), the Ministry of Public Health of Ecuador (MSP-DIS-2019-004-O), and the institutional review board of Oswaldo Cruz Foundation, Brazil (no. 022/2009).

Digital PCR assays

Four novel ddPCR assays were developed. One assay targets the conserved first 120 bp of *hrp2* exon 2, and thus is located directly adjacent to the histidine-rich repeats. Different breakpoint for the *hrp2* deletion have been described [26]. The novel primers for exon 2 are located in a region that is deleted in all known deletion variants, thus irrespective of the specific breakpoint, *hrp2* deletion will be detected. The second assay targets *hrp2* exon 1. While exon 1 does not contain antigens that are recognized by RDTs, the deletion of this exon 1 would prevent proper expression of the protein. The third assay targets

149 *hrp3*. Each assay was multiplexed with an assay targeting *serine-tRNA ligase* (PF3D7_0717700, herein
150 referred to as '*tRNA'*). *tRNA* is a conserved, essential single copy gene, that is frequently used as reference
151 for gene expression assays [32]. In wild type infections not carrying a deletion, the copy numbers of *tRNA*
152 and *hrp2* or *hrp3* are identical.
153
154 Novel primers and probes were developed for all assays (Table 1). Assay conditions are given in
155 Supplementary File S1. Across over >3000 genomes available through MalariaGen and PlasmoDB, no SNPs
156 were recorded in primer and probe sequences, thus the assay can be used for the screening of isolates of
157 global origin. Assay conditions were optimized to achieve maximal separation between positive and
158 negative droplets (Figure 1, Supplementary File S2, Figure S1).
159
160 **Table 1**: Primer and probe sequences
161

Assay		**Sequence 5'-3'**
	hrp2 exon 2 forward	CATTTTTAAATGCTTTTTTATTTTTATATAG
hrp2 exon 2	hrp2 exon 2 reverse	CTTGAGTTTCGTGTAATAATCTC
	hrp2 exon 2 probe	FAM-CGCATTTAATAATAACTTGTGTAGCAAAAATGC
	hrp2 exon 1 forward	ATATTTATACATTTTTGTTATTATTTCTTTTTC
hrp2 exon 1	hrp2 exon 1 reverse	CGTTATCTAACAAAAGTACGGAG
	hrp2 exon 1 probe	FAM-CAAAAACGGCAGCGGATAATACTT
	hrp3 forward	ATGCTAATCACGGATTTCATTTTA
hrp3	hrp3 reverse	ATCGTCATGGTGAGAATCATC
	hrp3 probe	FAM-CCTTCACGATAACAATTCCCATACTTTAC
	tRNA forward	CATCAAATGAAGATTTAACAAGAG
tRNA	tRNA reverse	CTTTTTGATTCTATAGTTTCATCTTTATG
	tRNA probe	HEX-CTACCTCAGAACAACCATTATGTGCT

3D7 and Dd2 parasite culture strain mixtures

In order to determine the ability to detect mixed clone infections with only one strain carrying the deletion, experimental mixtures were made from two well-characterized laboratory strains, 3D7 (no deletions), and Dd2 (carrying the *hrp2* deletion). Each strain was cultured separately, DNA extracted, and quantified by ddPCR using the *hrp2*/*tRNA* ligase assay. No *hrp2* was detected in Dd2. Mixtures were prepared with a concentration (of both strains combined) of 10, 50, 100, and 500 genomes/µL, and with a 3D7 to Dd2 ratio of 100:0, 80:20, 60:40, 40:60, 20:80, and 0:100. Each mixture was run in triplicate using the *hrp2* exon2/ *tRNA* ligase assay. The ratio of *hrp2* to *tRNA* copy numbers was compared to expected values.

Nested PCR assay for *hrp2*

In order to compare the ddPCR assay directly to the established nPCR assay, 248 samples from asymptomatic carriers in western Kenya were run by the *hrp2* exon 2 assay, and the *hrp2* nsPCR in triplicate. Assay conditions for the nPCR followed published protocols [33, 34] and are given in Supplementary File S1.

Field isolates

Field isolates were screened from Kenya, Zanzibar in the United Republic of Tanzania, Ethiopia, Ghana, Brazil, and Ecuador. Samples from Kenya (n=248) were from Chulaimbo and Homa Bay in western Kenya close to Lake Victoria. Samples were collected in a cross-sectional survey including individuals of all ages from January-August 2019. Finger-prick samples were collected in EDTA microtainers and infections detected by qPCR. Overall *P. falciparum* prevalence across both sites was 16% [35]. No diagnosis by RDT or microscopy was done.

Samples from Zanzibar (n=91) had been collected in the frame of a study on reactive case detection (RCD) from May 2017 to October 2018 [2]. Asymptomatic infections identified through RCD were typed. During RCD, infections were diagnosed by HRP2/pLDH-based RDT (SD BIOLINE Malaria Ag Pf HRP2/pLHD), a blood spot was collected on filter paper, and infections diagnosed by qPCR [2]. Prevalence (not including index cases) was 0.8% by RDT and 2.4% by qPCR. All samples with a density (by qPCR) of >100 parasites/µL were selected for *hrp2*/*hrp3* deletion typing, irrespective of RDT result.

Samples from Ethiopia (n=47) included clinical cases and asymptomatic individuals sampled in June to November 2016 from Jimma Zone, Oromia Region. *P. falciparum* prevalence was 4% by microscopy and 8.3% by qPCR [36]. No RDT screening was conducted.

From Ghana, 2 sets of samples were typed. The first set (n=11) was collected in June-September of 2017 from febrile school children aged 5-14 years [37]. The second set (n=212) was collected in Mankranso and Agona Hospitals in the Ashanti region from febrile patients from September to December 2020.

In Brazil, samples (n=187) were collected in Cruzeiro do Sul, Upper Juruá Valley, northwestern Brazil, in 2010-2013. This is the country's main malaria hotspot, which accounted for nearly 15% of all *P. falciparum* infections in Brazil at the time of the study. Samples were collected from clinical patients 4 to 73 years of age (mean, 26.6) enrolled for a drug efficacy trial [38]. Only baseline samples from patients with microscopy- and PCR-confirmed *P. falciparum* infection were included in this study. Even though these samples had been collected nearly a decade ago and might not reflect the current status of *hrp2*/*hrp3* deletion, they were included in light of the known high levels of *hrp2*/*hrp3* deletion in Latin America to

confirm the ability of the assays to reliably detect deletions in field isolates [39]. Because of little template volume available, Brazilian samples were not screened for *hrp2* exon 1 deletion.

In Ecuador, samples (n=41) were collected from clinical patients from March 2019 to April 2020. The samples were collected in Esmeraldas and Carchi Provinces in the north-west of the country, where most *P. falciparum* cases of Ecuador are reported. All infections were confirmed by microscopy and collected as blood spots in filter paper. Three samples were collected from travelers coming from the Pacific coast in Colombia but diagnosed in Ecuador.

Data analysis

For the analysis of the ddPCR data, the following criteria were applied: A minimum of 2 droplets positive for *tRNA* were required to include a sample in data analysis. Samples were repeated if a deletion was observed but ≤5 droplets were positive for *tRNA*. At a density of >5 droplets positive for *tRNA*, the probability of a false-negative result for *hrp2* or *hrp3* (i.e. no positive droplet in a wild-type infection) is less than 1:500. Data for all samples and assays are provided in Supplementary File S3.

Results

Assay development and validation

New primers and probes were developed for *hrp2* exon 1, *hrp2* exon 2, *hrp3*, and *tRNA* (Table 1). Upon optimization of assay conditions, clear separation between negative and positive droplets was obtained across a wide range of parasite densities (Figure 1, Supplementary Figure S1). No positive droplets for *hrp2* or *hrp3* were observed in case of deletion, while the separation between negative droplets and those positive for *tRNA* remained clear (Figure 1, Supplementary Figure S1).

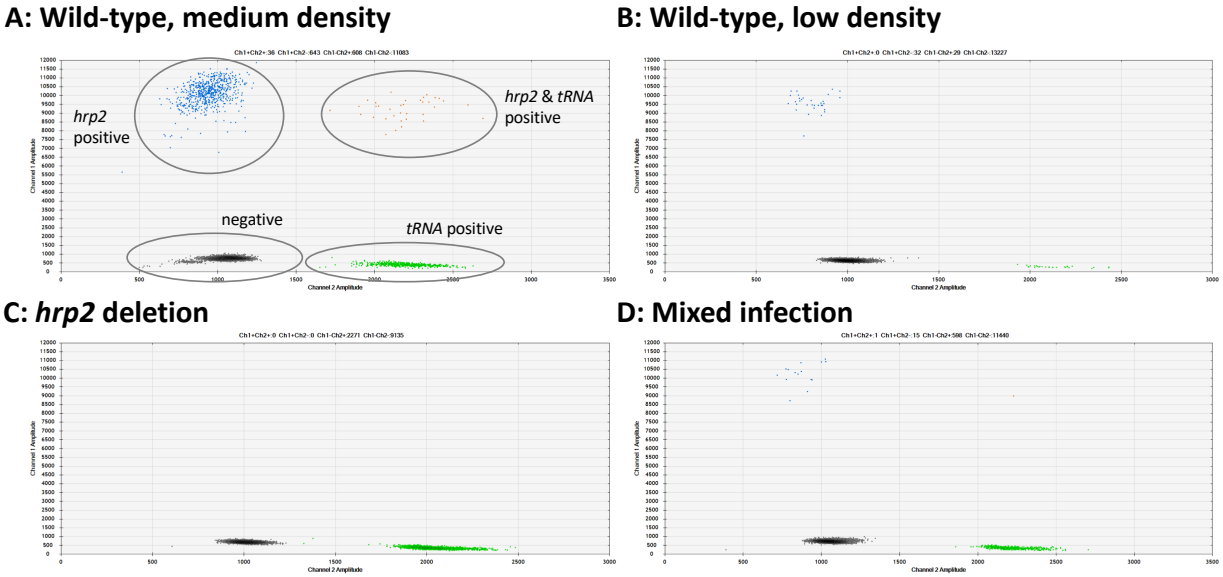
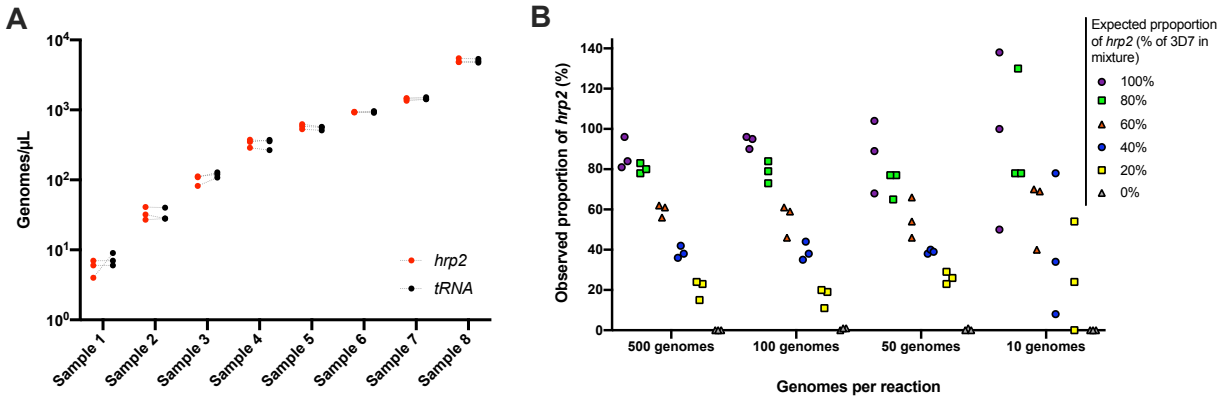


Figure 1: Examples of *hrp2* exon 2 deletion typing by ddPCR. Droplets positive for *hrp2* are shown in blue (top left of each panel). Droplets positive for *tRNA* are shown in green (bottom right). Droplets positive for *hrp2* and *tRNA* are shown in orange (top right). Negative droplets (for both *hrp2* and *tRNA*) are shown in gray (bottom left). A) Wild-type infection of medium density. Approximately 600 droplets are positive each for *hrp2* and *tRNA*, and 36 for both targets. B) Wild-type sample of low-density: 32 and 29 droplets are positive for *hrp2* and *tRNA*, respectively. C) *hrp2* deletion: Droplets are positive for *tRNA*, but no droplets are

243 positive for *hrp2*. D) Mixed infection with wild-type parasites and parasites carrying *hrp2* deletion. Only 15 droplets are positive
244 for *hrp2*, but 598 droplets are positive for *tRNA*.

245

246 To evaluate reproducibility and the limit of reliable detection, 248 isolates from asymptomatic carriers in
247 Kenya were typed for the *hrp2* exon 2 assay in triplicate. Geometric mean density was 95 genomes/µL,
248 and 47/248 samples were at densities <10 genomes/µL. By ddPCR, 235/248 (94.6%) of samples met
249 inclusion criteria of ≥2 droplets positive for *tRNA* in all 3 replicates. Highly similar quantification among
250 replicates was observed (Figure 2A). For each sample, the highest and lowest value of *hrp2* copies/µL was
251 recorded. Correlation among technical replicates was very high (R^2=0.990). Likewise, for each sample, the
252 highest and lowest value of *tRNA* copies were recorded, and correlation was very high (R^2=0.991).



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254

255 **Figure 2**: Validation of assay. A) Samples typed in triplicate for the *hrp2* exon 2/*tRNA* assay. Representative examples of different
256 parasite densities are shown. For each sample, the quantification of *hrp2* exon 2, and of *tRNA* is shown. Results from the same
257 run are connected by a dashed line. B) Mixtures of 3D7 (wild type) and Dd2 (*hrp2* deletion). Mixtures were run in triplicate at
258 densities of 10-500 genomes/reaction, and at ratios of 0-100% Dd2. The expected proportion of *hrp2* to *tRNA* copies corresponds
259 to the proportion of 3D7 in the mixture. The observed proportion reflects the expected proportion closely for all mixtures at 500
260 and 100 genome/reaction.

261

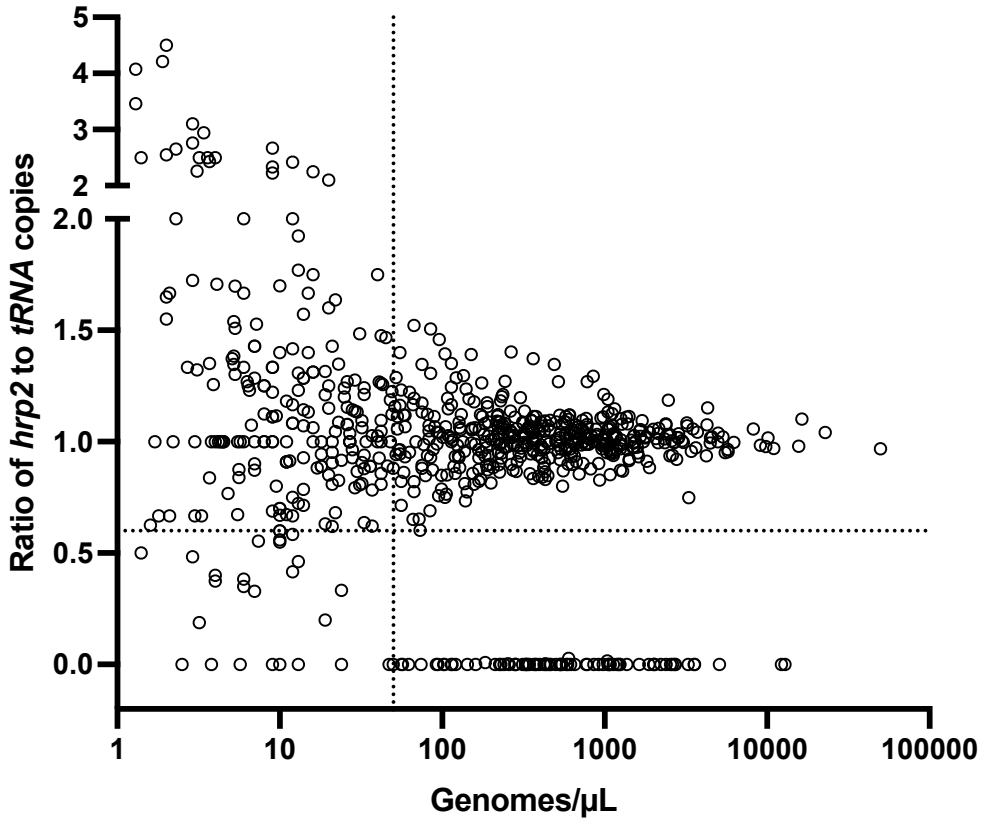
No deletions were detected in the 248 samples from western Kenya. I.e., no samples with droplets for *tRNA* but no droplets for *hrp2* were observed, even though density was low in many samples. In the ddPCR, approximately two-thirds of the reaction volume was transformed into droplets. Applying the threshold of 2 droplets positive for *tRNA*, three template genomes were required per reaction well to reach that threshold. Up to 9 µL of template DNA could be added to one reaction when primers and probes are kept at 100 µM. Thus, the theoretical limit of detection was 0.33 parasites/µL. If ≤5 droplets are positive for *tRNA* and a deletion is observed, it is recommended to repeat the sample.

Detection of mixed infections with *hrp2*-negative and wild type parasites

To test the ability of the assay to detect deletions when only part of all parasites in an isolate carry the deletion, experimental mixtures were prepared with DNA from parasite culture of 3D7 (wild-type), and Dd2 (*hrp2* deletion). Mixtures were prepared at ratios from 0% to 100% Dd2, and at densities of 10-500 genomes/reaction. At densities of 100 to 500 genomes/µL, the quantification by ddPCR represented the mixture ratio with high accuracy (Figure 2B). Whenever 40% or more of parasites carried the deletion, the observed ratio was clearly below 100%. In cases where only a small proportion of all parasites carried the deletion (20% Dd2 vs. 80% 3D7), the difference in quantification of *hrp2* and *tRNA* was too small to observe the deletion. Likewise, at densities <50 genomes/reaction the ratio did not accurately reflect the experimental mixture.

The results were corroborated by screening of 739 field samples from five countries. In wild type isolates, a very similar quantification of *hrp2* and *tRNA* was expected. Unless samples carried a clear deletion (i.e.

285 no or very little *hrp2* signal), in all samples with densities of >50 copies/µL, the ratio of *hrp2* to *tRNA* copies
286 was above 0.6 (Figure 3). With increasing parasite density, the ratio got closer to 1.
287



288

289 **Figure 3**: Ratios of *hrp2* to *tRNA* copies in 684 field isolates. With increasing parasite density (X-axis), the ratio becomes close to
290 1. Deletions (with no wild type parasites present) have a ratio of 0. Dashed lines show a ratio of *hrp2* to *tRNA* copies of 0.6, and
291 50 genomes/µL. Mixed infection can be reliably detected at densities >50 genomes/µL, and if >40% of parasites carry the deletion.
292

293 A lower ratio of *hrp2* to *tRNA* copies was observed in samples from Zanzibar, where the mean ratio of
294 *hrp2*/*tRNA* copies was 0.82 in absence of any samples that carried *hrp2* deletion (Supplementary Figure
295 S2). The reason is not known, but might be caused by sampling and storage procedures. Blood samples
296 from Zanzibar were collected on filter paper, and stored at ambient temperature for over three years

297 prior to extraction. Possibly, this could have resulted in DNA degradation, that affected *hrp2* more than
298 *tRNA*.
299
300
301 **Comparison of ddPCR to gel-based nested PCR**
302
303 The *hrp2* exon 2 ddPCR assay was compared to the classical nPCR assay with visualization of products on
304 agarose gel in 248 asymptomatic infections from western Kenya. The density of asymptomatic infections
305 is often low and thus amplification by PCR can be stochastic. All assays were run in triplicate, i.e. *hrp2*-
306 exon2/*tRNA* by ddPCR, *hrp2* nested PCR, and *msp2* nested PCR.
307
308 Samples were included in analysis if the PCR for the control gene was positive in all three replicates, i.e. if
309 >2 droplets were positive for *tRNA* in the ddPCR assay, or a band was detected for *msp2* in all three
310 replicates. For the gel-based assay, 85.5% (212/248) samples met inclusion criteria (Table 2). Among those
311 positive, for 144/212 samples a band for *hrp2* was observed in all three replicates. A band in two replicates
312 was observed for 34 samples, and a band in a single replicate for 17 samples. For 17 samples, despite
313 obtaining a band for *msp2* in all three replicates, no band for *hrp2* was observed. These 17 samples would
314 thus be classified as *hrp2* deletion, resulting in a prevalence of deletion of 8.0% (17/212). Figure 3 shows
315 representative examples of results of the nPCR and ddPCR assays.
316
317 **Table 2**: Comparison between nested PCR and ddPCR-based *hrp2* deletion typing
318

	nested PCR	**ddPCR**
Total samples	248	248

Met inclusion criteria	212 (85.5%)	235 (94.8%)
Deletion in 3/3 replicates	17	0
Deletion in 2/3 replicates	17	0
Deletion in 1/3 replicates	34	2
No deletion	144	233
Prevalence of deletion	8.0% (17/212)	0% (0/235)

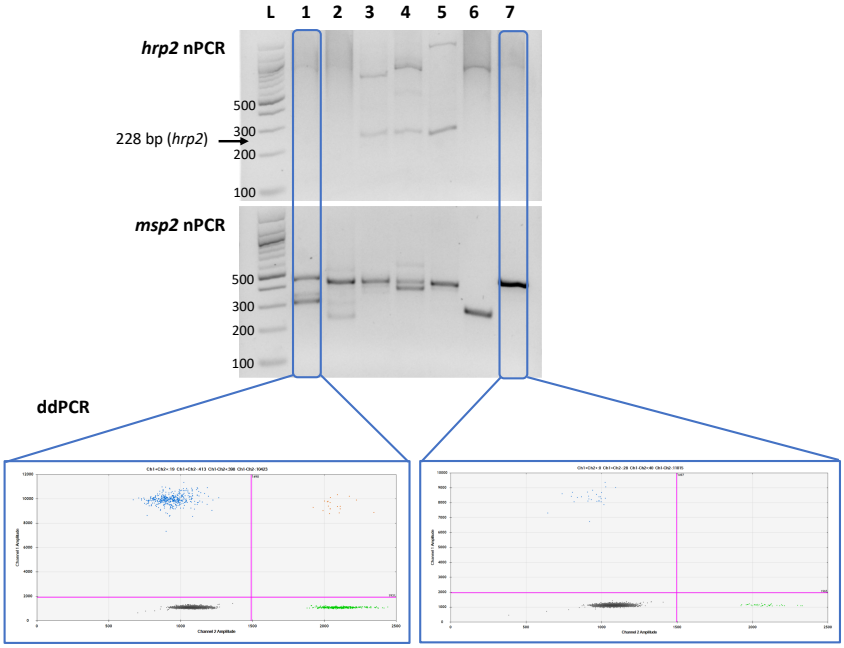


Figure 3: Comparison of nPCR and ddPCR for *hrp2* deletion typing. Representative examples of results obtained by *hrp2* and *msp2* nPCR, and by ddPCR. The expected size of the *hrp2* band is 228 bp. A band is visible in samples 3, 4, and 5, but no band is visible in samples 1, 2, 6, and 7. *msp2* was run as control for the nPCR assay. *msp2* is a size polymorphic gene with amplicons ranging from approximately 200 to 500 bp. Bands are observed for all samples, and multiple bands are observed in case of polyclonal infections. L = 100 bp DNA ladder (New England BioLabs). Samples were run in triplicate, and the same results was obtained all three times. By ddPCR, no deletions were observed in any samples. For samples 1 and 7, the *hrp2* exon 2/*tRNA* ddPCR plot is shown. Droplets are visible for both targets, thus no deletion is observed.

By ddPCR, the criteria for inclusion (≥2 droplets for *tRNA*) was met by 94.8% (235/248) of samples. Among them, ≥2 droplets for *hrp2* were detected in all three replicates in 233/235 samples. In 2 samples in only two of the three replicates ≥2 droplets were positive for *hrp2*. Both of these samples had one replicate with 1 positive droplet for *hrp2*, and 5 positive droplets for *tRNA*. In none of the samples all three or two out of three replicates were negative for *hrp2*. Thus, the prevalence of deletion by ddPCR was 0%.

***hrp2* and *hrp3* deletions in Africa and South America**

The new ddPCR assay was applied to screen for deletions in 830 samples from Kenya, Zanzibar, Ethiopia, Ghana, Brazil, and Ecuador. The frequency of deletions for all loci and sites is given in Table 3.

Table 3: *hrp2* and *hrp3* deletions in Africa and South America

		Deletions				
	N	*hrp2* exon 2	*hrp2* exon 1	*hrp3*	*hrp2+hrp3*[1]	Mixed[2]
Site						
Kenya	241	0% (0/241)	0% (0/241)	0% (0/241)	0% (0/241)	
Zanzibar	91	0% (0/91)	0% (0/91)	0 % (0/91)	0% (0/91)	
Ethiopia	47	2.1% (1/47)	2.1% (1/47)	74.5% (35/47)	2.1% (1/47)	1 x *hrp3*
Ghana	223	0% (0/226)	0% (0/223)	0.4% (1/223)	0% (0/170)	1 x *hrp2*, 3 x *hrp3*
Brazil	187	46.5% (87/187)	NA	62.0% (116/187)	46.0% (86/187)	2 x *hrp2*, 2 x *hrp3*
Ecuador	41	0% (0/41)	0% (0/41)	53.7% (22/41)	0% (0/39)	

[1] Isolates with deletions of *hrp2* and *hrp3*. Note that these isolates are also counted as deletions in the columns for *hrp2*, and for *hrp3* (e.g. in Brazil 87 isolates carried *hrp2* deletion, of which 86 also carried *hrp3* deletion).

[2] Isolates with only a proportion of all parasites carrying the deletion.

From Kenya, 241 samples were typed and no deletions of *hrp2* or *hrp3* were observed. In Zanzibar, no deletions were observed among 91 samples. Among 223 samples from Ghana, 1 *hrp2* deletion/wild type mixed sample was detected (ratio of *hrp2* to *tRNA* copies was <0.6, and parasite density >50 genomes/µL), 1 *hrp3* deletion, and 3 samples with *hrp3* deletion/wild type mixes.

In Ethiopia, 47 samples met inclusion criteria. One sample carried a deletion of *hrp2* exons 1 and 2, resulting in a frequency of deletion of 2.1%. *hrp3* deletion was observed in 35/47 (74.5%) samples, and one sample carried a mixed infection with wild type/*hrp3* deletion. The sample with *hrp2* deletion was among the samples with *hrp3* deletion, i.e. both genes were deleted.

From Brazil, 187 samples were screened. 87 samples carried a deletion of *hrp2*. Two additional samples carried mixed infections with wild type parasites and *hrp2* deletion. Deletion of *hrp3* was observed in 116/187 (62.0%) samples, and 86/187 (46.0%) samples carried deletions of *hrp2* and *hrp3*. No *hrp2* deletions were observed in Ecuador, but 22/41 (53.7%) samples carried *hrp3* deletions.

Discussion

Molecular surveillance of the extent of *hrp2* and *hrp3* deletions is a high priority task to select the optimal tools for *P. falciparum* diagnosis. The novel assay based on ddPCR yielded highly accurate results, and was able to detect mixed infections with wild-type parasites and parasites carrying *hrp2* deletion. The good performance of the assay was shown by typing of samples from six countries. The samples reflected a range of parasite densities, from low-density asymptomatic infections to high-density clinical infections, and sites represented a range in the frequency of *hrp2* and/or *hrp3* deletions. Across over 800 field samples screened, <10 samples needed to be repeated because of poor separation between negative and positive droplets, or failure of droplet generation.

High sensitivity is required for accurate typing of low-density infections. We validated a threshold of two droplets positive for *tRNA* as limit of detection. When 9 µL of DNA are used as template, this results in a theoretical limit of detection of 0.33 parasites/µL. Sensitivity can be further increased by concentrating the DNA prior to typing.

The ddPCR assay showed increased sensitivity and specificity to type low-density infections compared to nPCR. Out of 248 asymptomatic samples from western Kenya, 95% could be analyzed by ddPCR, but only 85% by nPCR. More importantly, results on deletion status differed substantially. By ddPCR, no deletions were observed. By nPCR, no band was observed for *hrp2* in 8% of samples that had a positive band for the control gene (*msp2*) in all three replicates. Thus, the frequency of deletion was above the 5% threshold, and it would be erroneously recommended to discontinue the use of HRP2-based RDTs. The frequency of deletion by nPCR was similar to a previous study conducted in a nearby site in western Kenya that found *hrp2* deletion in 8/89 samples using nPCR [40]. The direct comparison of ddPCR and nPCR in a large

number of samples points to a possible overestimation of *hrp2* deletion frequency by studies relying on nPCR.

In almost all transmission settings, polyclonal *P. falciparum* infections are frequent [28]. Using a gel-based assay for deletion typing, in a mixed infection with a wild type parasite and a parasite carrying a deletion, the wild type parasite will produce a band and mask the deletion. These infections can be detected by RDT, but depending on the proportion of polyclonal infections, they can result in pronounced underestimation of the true frequency of deletion [29]. The highly accurate quantification by ddPCR allows detection of mixed infections. Based on experimental mixtures of 3D7 and Dd2, and field samples, mixed infections were reliably detected when at least 40% of parasites carried the deletion, and at densities above 100 genomes per reaction. Using a well-working qPCR assay with an amplification efficacy of 100%, a similar difference between *hrp2* and *tRNA* copy numbers would result in less than half a cycle difference. This is within the normal variation of technical replicates [31, 41], and thus such mixed infections could not be detected by qPCR.

Twenty-nine isolates from Zanzibar and 16 from Ghana had tested negative by HRP2-based RDT despite high density of 100 to >10,000 parasites/µL [2], yet no *hrp2* deletions were observed in these populations. False-negative RDT results might be caused due to incorrect handling, prozone effect [42], or sequence variation without deletion of the *hrp2* gene [43]. The finding corroborates the importance of molecular typing. Studies comparing microscopy and RDT results can give important clues for the presence of deletions [44], but molecular typing is required for confirmation [18].

No *hrp2* deletions were found in Zanzibar, and Kenya, and one mixed infection in Ghana. The data from Ghana contrasts an earlier study that reported a frequency of deletion of >30% [15]. A single *hrp2* deletion

was found in southwestern Ethiopia. This is in stark contrast to very high levels of deletion in western Ethiopia [45], Eritrea [18], and Sudan [46]. The results corroborate the need for studies assessing *hrp2* deletion and selection of diagnostic tools at sub-national level.

Contrasting findings were obtained from the sample sets from South America, with very high levels of deletion in Brazil, and no deletions in Ecuador. Brazil and Ecuador share no borders, and the amount of human migration is limited. The results add to the heterogenous pattern of *hrp2*/*hrp3* deletion in South America, with high frequency of *hrp2* deletion in the Amazon [9, 12, 39], but low levels among the Pacific coast [11, 12]. An outbreak of parasites with *hrp2* deletion at the Peruvian Pacific coast was caused by infections imported from the Amazon [47]. Brazil is committed to *P. falciparum* elimination, and the samples typed originated from the main hotspot of transmission [48]. Rapid diagnostic tests (RDTs) remain relatively little used in Brazil. Microscopy remains the diagnostic method of choice, and RDTs are mostly used in remote areas, e.g., populations from Amerindian Reserves and some traditional riverine communities with no access to conventional microscopy. The frequency of *hrp2* deletion in Brazil clearly exceeds the 5% threshold, thus it is recommended that no HRP2-based RDTs are used.

To determine whether the frequency of the deletion exceeds the threshold of 5%, the WHO recommends that for each site a minimum of 370 is typed [49]. Limitations of the molecular assays available for typing have been a major hindrance to type that number of samples in many sites where *hrp2* or *hrp3* deletion is suspected. Even a low proportion of false-negative results could impact the decision to discontinue HRP2-based RDTs. As a result of the scarcity of field data, the spatiotemporal dynamics of *hrp2* deletion in parasite populations, drivers of the deletion, potential fitness costs, and clinical consequences remain poorly understood. Results from simulation studies suggest that the use of *hrp2*-based RDTs selects for

434 *hrp2*-negative parasites, in particular if transmission levels are low and a large proportion of all infections
435 turn clinical and result in treatment seeking [50, 51].
436
437 In conclusion, the novel, high-throughput, highly sensitive and specific ddPCR assay will facilitate
438 molecular surveillance for *hrp2* and *hrp3* deletion, and thus aid selection of diagnostic tests to accelerate
439 malaria control and elimination. Data obtained using this assay will help to understand the evolutionary
440 processes underlying the de-novo emergence and spread of the deletion.
441
442
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447
448
449 **Supplementary Files**
450 Supplementary File S1: Assay conditions
451 Supplementary File S2: Supplementary Figures S1, S2
452 Supplementary File S3: Database
453
454
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461 **Competing interests**

462 The authors state that no competing interests exist.

463

464 **References**

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