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Background document for Session 4

World Health Organization

Protocol for estimating the prevalence of *pfhrp2/pfhrp3* gene deletions among symptomatic falciparum patients with false-negative RDT results

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Project summary

Study objectives

This study is intended to determine whether the local prevalence of mutations in the *P. falciparum hrp2/3* genes has reached a threshold that might require a local or national change in diagnostic strategy. The specific objectives are to:

1. Measure the prevalence of suspected false-negative HRP2 RDT results among symptomatic patients attending public health facilities with *P. falciparum* infection detected by microscopy or a pf-pLDH RDT;
2. Detect the parasite density and frequency of *pfhrp2/3* gene deletions in that cohort;
3. Determine the predictive value of false-negative HRP2 RDT results for *pfhrp2/3* gene deletions in different settings;
4. Identify provinces in which the prevalence of *pfhrp2/3* gene deletions is at or above 5%, warranting a change in RDTs.

Study site

Pre-selected public health facilities representing the spectrum of transmission and geographical diversity across the country

Target population

Individuals seeking care for febrile illness at health facilities with *P. falciparum* malaria confirmed by pf-pLDH RDT or microscopy, but with a negative HRP2 RDT result

Study design

Cross-sectional, multi-site study

Primary output measures

1. Prevalence of suspected false-negative HRP2 RDT results among patients with *P. falciparum* malaria
2. Prevalence of *pfhrp2/3* gene deletions among those with a suspected false-negative HRP2 RDT result

This document was prepared as a pre-read for the meeting of the Malaria Policy Advisory Committee and is not an official document of the World Health Organization.

Sample size

A sample size of 370 per sampling domain (37 per health facility) is recommended to quantify whether or not the prevalence of *pfhrp2* deletion is < or > 5%. Once the sample of 370 Pf cases have been enrolled then molecular confirmation of *pfhrp2* deletions amongst suspected false negative cases should ensue.

Sampling method

In at least 10 pre-selected health facilities in provinces at risk, a cross-sectional study will measure the suspected and confirmed prevalence of *pfhrp2/3* gene deletions causing false-negative HRP2 RDT results. 37 *P. falciparum* positive cases should be enrolled in each health facility

Data collection

1. Identify provinces to be included in the study.
2. Select at least 10 health facilities per province for testing (facility sample size may vary depending on logistical and budgetary constraints).
3. Test all individuals presenting with suspected malaria using both a WHO-recommended HRP2 RDT and a non-HRP2 method (e.g., pf-pLDH RDT (separate single or multiple test line RDT)) or microscopy.
4. Record all results for malaria cases identified using any method.
5. Obtain informed consent (or assent depending on the age of majority and federal guidelines in the country of enrolment), and enrol all patients with a suspected false-negative HRP2 RDT result in the study.
6. Obtain a minimum of two dried blood spots (DBSs) from consenting individuals.
7. Complete a brief questionnaire on each enroled participant.
8. After testing 370 individuals with *P. falciparum* malaria (ideally ~37/site across the 10 sites in the province), enrolment can stop, DBS and questionnaires can be compiled and shipped for molecular confirmation of *pfhrp2* deletions.

9. Supplemental data collection options are described in Appendix 1.

Statistical and analytic plan

The prevalence of suspected false-negative HRP2 RDT results and *pfhrp2/3* gene deletions will be established at the sampling domain (e.g. provincial level), with 95% confidence intervals (CI) estimated for all point estimates. If desired, point estimates and 95% CIs can be weighted according to relative facility size or patient flows. Differences between point estimates across sociodemographic characteristics and transmission levels, or other collected variables can be determined using X^2 and/or logistic regressions, as desired.

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1. Background and rationale

Rapid diagnostic test (RDT) kits offer great potential for the immediate diagnosis of malaria infections. Rapid diagnosis allows for prompt treatment, especially in rural settings. RDTs are lateral flow immunochromatographic tests that detect *Plasmodium* parasite antigens in blood [1]. Three antigens are detected by current RDTs: histidine rich protein 2 (HRP2), lactate dehydrogenase (LDH) and aldolase. HRP2 is an abundant protein expressed only by *P. falciparum* and is the target for the most commonly used RDTs. Although the antibodies on the test strip are designed to recognize the HRP2 antigen, they may also cross-react with another antigen of the HRP family, namely HRP3, due to strong similarities in the amino acid sequences [2]. HRP2-based RDTs tend to be more sensitive and heat-stable than RDTs that detect LDH or aldolase [3].

While HRP2 RDTs generally have the highest sensitivity of the RDTs for *P. falciparum* malaria [3], parasite strains have recently been identified that have deletions in the genes encoding HRP2 or the similar HRP3 protein. Strains with *pfhrp2* and *pfhrp3* gene deletions are undetectable by HRP2 RDTs [4]. HRP2 RDTs can sometimes still detect strains with only a *pfhrp2* deletion, particularly in high parasite density infections, due to antibody cross-reactivity with epitopes of HRP3 [4]. In 2010, Gamboa et al. [5] first reported the identification of *P. falciparum* parasites with *pfhrp2/3* gene deletions in the Amazon River basin in Peru. Subsequent retrospective analyses at different sites in the Loreto region of the Peruvian Amazon showed an increase in the prevalence of parasites with gene deletions between specimens collected from 1998 to 2001 (20.7%) and those collected from 2003 to 2005 (40.6%) [5]. The prevalence of parasites with *pfhrp2/3* gene deletions shows substantial local variability. Studies in other countries, such as India [6], Mali [7], Honduras [8], Ghana [9], Columbia [10, 11] Myanmar [12], Suriname [13], Guyana [8] and Senegal [14], have found much lower prevalence estimates, although the rigour of study design has been variable. In recent unpublished data from Eritrea, the prevalence of *pfhrp2* deletions was estimated to be very high (80%), suggesting that an HRP2-only testing strategy may not be valid in some settings [15]. There have been no reports of parasites failing to express LDH or aldolase, as these targets are essential enzymes for parasite metabolism and survival.

In settings where microscopy is either unavailable or infeasible due to time or resource constraints, it is imperative that malaria be treated based on RDT results. Monitoring the accuracy of the RDT results is thus critical. The main causes of false-negative RDT results are related to product quality and performance, transportation or storage conditions, operator error, or parasite density below the limit of detection; however, deletions of the genes encoding the target antigen must also be considered [4].

The purpose of this document is to present a standardized protocol that *P. falciparum*-endemic countries can use to identify the prevalence of parasites with *pfhrp*2/3 gene deletions causing false-negative HRP2 RDT results. The methods contained herein can be used to map the distribution of these deletions, estimate the predictive value of suspected false-negative HRP2 RDT results for gene deletion, and identify areas where diagnostic strategies may need to be changed.

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2. Research Objectives

This study is intended to determine whether the local prevalence of mutations in the *P. falciparum hrp*2/3 genes has reached a threshold that might require a national or subnational change in malaria RDTs. The specific objectives are to:

1. Measure the prevalence of suspected false-negative HRP2 RDT results among symptomatic patients attending public health facilities with *P. falciparum* infection detected by microscopy or a pf-pLDH RDT.

2. Detect the parasite density and frequency of *pfhrp2/3* gene deletions in that cohort.

3. Determine the predictive value of suspected false-negative HRP2 RDT results for *pfhrp2/3* gene deletions in different settings.

4. Identify provinces that are at or above a 5% prevalence threshold of *pfhrp2/3* gene deletions, as this indicates a need to switch from using exclusively HRP2-based RDTs for detecting *P. falciparum*.

3. Study site / target population

This study will focus on individuals seeking treatment for microscopically or RDT-confirmed *P. falciparum* malaria at public health facilities. Negative HRP2 RDT results but pf-positive results by pf-pLDH RDT or microscopy indicate the possibility of *pfhrp2/3* gene deletion as the reason for the false-negative result. Given the importance of HRP2 detection to the diagnostic strategy, WHO is urging at-risk countries to assess the prevalence of such *P. falciparum* gene deletions. Prioritized for study are areas (i) with a recognized discordance between HRP2 RDT and microscopy results, (ii) with non-representative or sporadic reports of *pfhrp2/3* deletions in the country, and (iii) that neighbour an area where frequent *pfhrp2/3* deletions have been identified. In such countries, public health facilities within all provinces with *P. falciparum* malaria transmission should be included. Facilities eligible for inclusion in the study should ideally represent the geographical spread of malaria transmission across the province.

4. Research Methods

4.1 Research design

A cross-sectional study design will be used to measure the two primary outputs. Health facilities will routinely test suspected malaria cases with an HRP2 RDT and an alternative method (i.e., pf-pLDH RDT or microscopy). The frequency of suspected false-negative HRP2 RDT results is primary output 1. Dried blood spots (DBSs) will be collected from individuals with suspected false-negative HRP2 RDT results. Molecular testing on the DBSs will determine the prevalence of *pfhrp2/3* gene deletions in the cohort (output 2).

4.2 Primary output indicators

The following indicators will serve as the primary study outputs for assessing the prevalence of *pfhrp2/3* gene deletions in the selected population:

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1. Proportion of all *P. falciparum* malaria patients with suspected false-negative HRP2 RDT results (i.e., a negative HRP2 RDT result but positive pf-pLDH or Pf microscopy result).

2. Proportion of all *P. falciparum* malaria patients with suspected false-negative HRP2 RDT results found to have *pfhrp2/3* gene deletions.

The proportion of patients with suspected false-negative HRP2 RDT results will be determined through routine diagnostic testing at health facilities using dual-method testing (HRP2 RDT plus microscopy or pf-LDH RDT[1]). Although this figure provides a preliminary indication of the potential level of prevalence of *pfhrp2/3* gene deletions, molecular testing is required to confirm the true prevalence. To save time and resources, molecular testing to identify *pfhrp*2/3 deletions will only be performed on DBSs collected from individuals with suspected false-negative HRP2 RDT results (see section 4.5). Discordant diagnostic results may be due to other factors, such as false-positive pf-pLDH test lines (possibly due to cross-reactivity with non-falciparum species) or low parasite densities at or below the limit of detection of the HRP2 and pf-pLDH RDTs. In addition, there are several situations in which this indicator could miss a true *pfhrp2/3* gene deletion (see Table 2 below): First, individuals will not be detected or enrolled if they have a low-density infection that is missed by pf-pLDH RDT and also by HRP2 RDT due to *pfhrp2/3* gene deletion. Second, HRP2 RDT may still detect some infections with a *pfhrp2* deletion due to cross-reactivity of test antibodies with HRP3; individuals will therefore not be enroled based on the criteria of suspected false-negative RDT results. Finally, the testing protocol will not detect *pfhrp2/3* deletions in patients coinfected with HRP2-expressing clones. For these reasons, this indicator represents the lower limit of the true prevalence of *pfhrp2/3* gene deletions.

4.3 Secondary output indicators

1. Parasite density, as measured by quantitative PCR and/or microscopy, in patients with suspected false-negative HRP2 RDT results.

4.4 Sample size

Sample size is based on the desire to obtain relatively precise estimates of false-negative HRP2 RDT results caused by *pfhrp2/3* gene deletions at the survey domain level (province, state or similar) within countries implementing this protocol. The sample size estimates are based on a proportion obtained from simple random sampling, with a sampling design effect (deft) = 1.5 (to account for observations correlated within clinics vis-à-vis *pfhrp2/3* gene deletions) and a probability of committing a type-1 error = 95%, such that the 905% confidence interval does not overlap with the threshold of 5%. Note that a design effect other than 1.5 can be used if data on *pfhrp2/3* (or other relevant output) exist and can be used to estimate a design effect.

$$n \geq deft\left[\frac{Z^2(P)(1-P)}{D^2}\right]$$

[1] RDTs should be used that contain pf-pLDH-specific test lines and not pan-pLDH test lines. This will ensure that only *P. falciparum* infections are detected and avoid the identification of non-falciparum species (Pv, Pm, Po) species, which would cause discordant results (HRP2 negative, pan-pLDH positive)

Sample size is based on estimating output indicators 1 and 2[2], where the upper bound of the 95% CI does not overlap with 5% for estimates in which the observed prevalence of false-negative HRP2 RDT results caused by *pfhrp2/3* gene deletions is below 5% (signalling that the observed level of *pfhrp2/3* gene deletion is below 5% with 95% confidence), and where the lower bound of the 95% CI does not overlap with 5% for estimates in which the observed prevalence is above 5% (signaling that the observed level of *pfhrp2/3* gene deletion is above 5% with 95% confidence).

During the statistical analysis, health facilities should be included as a random effect so that the prevalence estimates and the 95% CI are adjusted for the variability in the probability of finding a malaria case at a health facility.

To demonstrate that the prevalence of *pfhrp2/3* gene deletion is below or above 5%, a sample size based on an expected population prevalence of 3.2% (n=370) or 8.0% (n=318), respectively, per sampling domain would be adequate. As a minimum, therefore, a sample of 370 individuals with a *P. falciparum* infection per sampling domain (e.g. province) is recommended (37 per health facility, 10 health facilities per sampling domain) based on the formula above. Within the domain, health facilities should be selected on the basis of probability proportional to size depending on the fever or suspected malaria caseload.

As the prevalence of *pfhrp2* deletions gets closer to the 5% threshold, detecting if it is above or below the threshold requires an increasingly large sample size, up to a maximum sample size exceeding 30,000 *P. falciparum* malaria cases per domain when within +/- .4% from 5% prevalence based on the formula above.

Therefore, it is recommended to conduct the survey first with a sample of 370 per domain. Molecular analysis should then be undertaken on the samples suspected to have *pfhrp2*/3 deletions and a statistical analysis of the prevalence with 95% CI computed. The analysis will result in one of three outcomes per province:

Outcome 1: That the upper limit of the 95% CI does not overlap with 5%. In this case there is a high statistical confidence that *pfhrp2/3* deletion causing false negative RDT results is below 5%

Outcome 2: The lower limit of the 95% CI is above 5%. This result means that there is a high statistical confidence that *pfhrp2/3* deletion causing false negative RDT results is greater than 5%

Outcome 3: The statistical analysis shows that it is inconclusive (5% contained within the 95% CI) as to whether or not the prevalence of *pfhrp2/3* deletion causing false negative RDT results is greater than or less than 5%.

4.5 Sampling

In general, in each province, a systematic random sample of a minimum of 10 health facilities should be selected from a complete list of all facilities, stratified by facility type

[2] Proportion of all *P. falciparum* malaria patients who have suspected false-negative HRP2 RDT results (positive on pf-pLDH RDT or microscopy and negative on HRP2 tests); proportion of all *P. falciparum* malaria patients with suspected false-negative HRP2 RDT results found to have *pfhrp2/3* gene deletions

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and including a measure of facility size (e.g., number of fevers or suspected malaria outpatients seen at the health facility in an average month). For each province, the selection of facilities should be based on the relative size of each facility, so that sampling is based on probability proportional to size (PPS). Budget permitting, more than 10 health facilities per province can be used to recruit the required sample; this will increase estimate precision and decrease the length of time needed to meet enrolment targets.

4.6 Data collection and fieldwork

The following general steps for data collection should be followed. ***[Note, it will be necessary for each country to develop a specific standard operating procedure (SOP) in order to tailor these steps to their particular context and needs].***

1. The National Malaria Control Programme (NMCP) should identify provinces to be included in the study. This selection should be guided by an assessment of which provinces may be most at risk for *pfhrp2/3* gene deletions (see section 3). Provinces with moderate to high transmission (where the negative impact of false-negative RDT results will be greatest) should be considered, whereas provinces without malaria transmission should be omitted.

2. Select a specified number of public health facilities (minimum of 10) in each province of the country to be included in the survey.

    - The number of facilities per province to be included in the sample should take into account the expected mean number of suspected malaria patients seen in the facility each week and the mean test positivity rate in the target area in order to ascertain the expected number of positives each week. As a general rule, the aim is to finish the fieldwork and collect a minimum sample size of 370 positives within an 8-week period.

    - Health facilities for the sample should be selected from a complete list (sampling frame) of health facilities in each province, using systematic random sampling based on PPS [17] (and proportional to the size of the facility type strata in each domain). The sampling frame must include some estimate of facility size (fever or suspected malaria case load) and type (e.g., public, private, level, etc.).

    - *Note that if budgetary or logistical constraints preclude the selection and inclusion of facilities using random sampling, a purposeful (or convenience) sample of facilities can be used. However, it should then be noted that province-level estimates of pfhrp2/3 gene deletions will not be statistically representative of the province.*

3. Test all individuals presenting with suspected malaria using two separate RDTs, including the HRP2 RDT used in the programme and a pf-pLDH RDT, or the HRP2 RDT used in the programme and quality-assured microscopy.

    - The HRP2 RDT should meet WHO procurement criteria and be approved for use by the Ministry of Health. It should be the type typically used by the NMCP for malaria diagnosis, as per national guidelines.

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- A pf-pLDH RDT that meets WHO procurement criteria (Table 2) or microscopy can be used for the secondary diagnosis. At present, there is only one pf-pLDH RDT that meets the panel detection criteria for *P. falciparum* [16]; therefore, for the purposes of the survey, countries may exceptionally select pf-pLDH-based RDTs that (i) have been evaluated in WHO malaria RDT product testing; and (ii) have a PDS >90 at 2000p/µl, and false-positive and invalid rates <2% (see Table 1.

- If microscopy is to be used, prepare one thin and one thick blood film following national guidelines aligned with the WHO SOPs for malaria microscopy.[3]

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[3] http://www.wpro.who.int/mvp/lab_quality/mm_sop/en/

TABLE 1.

Non-HRP2-based RDT options that can be used for the survey and their corresponding performance characteristics

	Product	Product code	Manufacturer	Panel Detection Score[a]										Round
				200 parasites/µl					2000 parasites/µl					
				Pf samples[b]				Pv samples[c]	Pf samples[b]				Pv samples[c]	
				HRP2, pf-pLDH (dual antigen single test line)	HRP2 test line	pf-pLDH test line	pan-pLDH-only test		HRP2, pf-pLDH (dual antigen single test line)	HRP2 test line	pf-pLDH	pan-pLDH-only test		
	Pf only													
1	CareStart™ Malaria Pf (HRP2/pLDH) Ag Combo 3-Line[d]	RMSM-05071	Access Bio, Inc.	NA	94.0	38	NA	NA	NA	99.0	92.0	NA	NA	7
2	SD Bioline Malaria Ag P.f (HRP2/pLDH)[d, e]	05FK90	Standard Diagnostics, Inc.	NA	87.0	52.0	NA	NA	NA	100.0	97.0	NA	NA	6
	Pf and Pv													
3	BIOCREDIT Malaria Ag Pf/Pv (pLDH/pLDH)	C60RHA25	RapiGEN Inc.	NA	NA	75.0	NA	100.0	NA	NA	98.0	NA	100.0	7
	Pf, Pf and Pv													
4	SD Bioline Malaria Ag P.f/P.f/P.v[d]	05FK120	Standard Diagnostics, Inc.	NA	84	36.0	NA	91.4		100.0	98.0		100.0	6

Meets procurement criteria for case management	Does not meet procurement criteria for case management	Meets criteria only for use in surveys for *pfhrp2* deletions

Performance measure	Recommended WHO procurement criteria
Panel detection score for Pf and Pv 200p/µL samples	≥ 75%
False-positive rate against clean-negatives	<10%
Invalid rate	<5% of tests conducted

NA, not applicable; Pf, Plasmodium falciparum; Pv, Plasmodium vivax

a - According to methods of WHO malaria RDT product testing a sample is considered detected only if all RDTs from both lots read by the first technician, at minimum specified reading time, are positive

b- Round 1, n=79; Round 2, n=100; Round 3, n=99; Round 4, n=98; Round 5, n=100; Round 6, n=100; Round 7, n=100

c - Round 1, n=20; Round 2, n=40; Round 3, n=35; Round 4, n=34; Round 5, n=35; Round 6, n=35; Round 7, n=35

d - PDS presented in the table is based on a HRP2 test line and Pf-pLDH test line. The overall result at 200 p/µl based on positive HRP2 or pf-pLDH test line is 88 for 05FK90; 85 for 05FK120 and at 2000p/µl it is 99 for RMSM-05071; 100 for 05FK90; 100 for 05FK120

e- Indicates a WHO prequalified product

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4. Record results of all suspected malaria cases tested during the study.

    - On the facility tally sheet, record results of each test line of the diagnostic tests used (Appendix 2).

    - Inform patient of results and provide treatment for positive test results on the primary or secondary RDT, as per national guidelines.

5. Enrol participants in the study.

    - Once treatment has been prescribed to a positive individual, screen for eligibility using the following criteria:

    Inclusion criteria

        - Positive for *P. falciparum* infection by pf-pLDH test or microscopy AND negative by HRP2 RDT
        - At least 12 months old

    Exclusion criteria (for primary outputs)

        - Negative on both HRP2 diagnostic tests and the other tests
        - Positive by HRP2 RDT
        - Any signs or symptoms of severe illness

    - Invite individuals meeting the inclusion criteria to participate in the study.

    - Provide a clear explanation of the study in the local language and obtain informed consent (Appendix 3) or assent for those respondents below the legal age, according to national guidelines (Appendix 4).

6. Obtain a minimum of two DBSs (50µl per spot) for consenting individuals.

    - Among consenting individuals, perform an additional finger prick to obtain a DBS on study-provided filter paper or card.

    - Label the filter paper with a unique barcode (or patient ID) (if there is room on the paper for a label).

    - Place the filter paper in a clean, dry and protected area, and allow to dry for 24 hours.

    - Once dry, place the filter paper with the desiccant (from the RDT package) in a plastic study bag. (Label bag with the barcode or patient ID if it is not stuck directly

7. Complete a brief questionnaire for the enroled participant.

    - Complete the brief study questionnaire (Appendix 5).

    - Place the participant's barcode on the completed questionnaire

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8. After enroling a minimum of 37 individuals with a *P. falciparum* infection at each facility (n will be 370 across the 10 sites selected in the province), the study team supervisor should use the facility tally sheets to calculate the proportion of *P. falciparum* cases with false-negative HRP2 RDT results (indicating potential *pfhrp2/3* gene deletion) in the province, using the formula below (see step 2).

$$\text{Proportion of } \textit{P. falciparum} \text{ cases with false-negative HRP2 RDT results} = \frac{\text{\# HRP2 RDT negative results from a confirmed } \textit{P. falciparum} \text{ case (by pf-pLDH RDT or microscopy)}}{\text{\# confirmed } \textit{P. falciparum} \text{ cases (by either RDT or microscopy)}}$$

9. Once the desired sample size of infected individuals is obtained for the province, filter papers, tally sheets and accompanying questionnaires should be collected from all facilities and shipped for confirmatory testing and molecular analysis for *pfhrp2/3* deletions. Molecular-based confirmation of pfhrp2/3 deletions as the cause of false-negative RDTs is needed to ensure that discordant results are not due to reasons other than *pfhrp2/3* gene deletions. Such reasons include operator error, false-positive pf-pLDH test lines, or samples at the limit of detection of the RDTs, which may sometimes react sufficiently to generate a positive test line but other times may not. The contribution of these alternative causes of discordant results will vary.

10. Once the true number of cases of *pfhrp2/3* deletions causing false negative HRP2 RDTs is known, then, for each province, a statistical analysis of the prevalence with 95% CI can be computed. The analysis will result in one of three outcomes per province:

    a. **Outcome 1:** That the upper limit of the 95% CI does not overlap with 5%. In this case there is a high statistical confidence that *pfhrp2/3* deletion causing false negative RDT results is below 5%

    b. **Outcome 2:** The lower limit of the 95% CI is above 5%. This result means that there is a high statistical confidence that *pfhrp2/3* deletion causing false negative RDT results is greater than 5%

    c. **Outcome 3:** The statistical analysis shows that it is inconclusive (5% contained within the 95% CI) as to whether or not the prevalence of *pfhrp2/3* deletion causing false negative RDT results is greater than or less than 5%.

11. If outcome 3 is obtained, *pfhrp2* deletions are found to be prevalent (lower 95% confidence interval is > 5%) in any province, the country programmes should make a nationwide switch to RDTs that do not rely exclusively on HRP2 for detecting *P. falciparum,* prioritized on the basis of the highest to lowest prevalence of *pfhrp2* deletions across provinces.

    a. A threshold of 5% was selected because it somewhere around this point that the proportion of cases missed by HRP2 RDTs due to non-hrp2

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expression may be greater than the proportion of cases that would be missed by less-sensitive pLDH-based RDTs.

b. A nationwide change is suggested because mathematical models show parasites lacking *pfhrp2* genes will spread[4] under HRP2-only RDT pressure and because the use of multiple RDTs in a country can complicate procurement and training practices.

12. If outcome 1 is obtained in all provinces, this country is recommended to establish a monitoring mechanism whereby this study is repeated in two years.

13. If outcome 2 is obtained in one or more provinces, this country has a few options depending on available resources:

    a. establish a monitoring mechanism whereby this study is repeated in two years (same as Outcome 1) or

    b. repeat the study in one year or

    c. continue enrolling patients to achieve larger sample size.

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[4] Gatton ML, Dunn J, Chaudhry A, Ciketic S, Cunningham J, Cheng Q. Use of PfHRP2-only RDTs rapidly selects for PfHRP2-negative parasites, with serious implications for malaria case management and control. J Infect Dis. 2017. doi: 10.1093/infdis/jix094.

TABLE 2.

Summary of test result combinations as per study protocol and limitations of the study approach including only individuals with discordant HRP2 RDT results (positive by pf-pLDH or microscopy AND negative by HRP2 RDT)

HRP2 RDT	pf-pLDH RDT or microscopy	Diagnosis	Enroled in study	DBS collected	Questionnaire collected	Interpretation of results and limitations in detecting *pfhrp2/3* deletions
+	+	*P.falciparum*	No	No	No	• May be infection with *pfhrp2* deletion but HRP3 was detected by HRP2 RDT • May be multiclonal infection with parasites with and without *pfhrp2/3* deletion
+	-	*P.falciparum*	No	No	No	• False-positive HRP2 RDT (or persisting HRP2 after resolution of infection) • May be infection with *pfhrp2* deletion but HRP3 was detected by HRP2 RDT or may be multiclonal infection with parasites with and without *pfhrp2/3* deletion AND • Low parasite density at or below the limit of detection of pf-pLDH RDTs and/or microscopy
-	+	*P.falciparum*	√	√	√	• False-positive pf-pLDH RDT or microscopy • Low parasite density at limit of detection of RDTs (variable reactivity of test lines)
-	-	Negative for malaria	No	No	No	• Cannot exclude low-density infection missed by both RDTs, with undetected *pfhrp2/3* deletion

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4.7 Sample storage

DBSs collected on filter papers will be sent on for PCR +/- sequencing at a qualified participating laboratory (contact cunninghamj@who.int for links to specific laboratories). Barcodes or individual patient identification numbers should be used to link the filter paper to the participant questionnaire. The filter paper will be stored for transport in a plastic bag with the desiccant taken from the RDT package. Any unused blood or DNA remaining after molecular testing and immunoassays should ideally be frozen at -70°C for long-term storage.

4.8 Composition of supervision and field teams

It is recommended that there be a minimum of one supervisor per province where the study is to be undertaken. Within each selected study clinic, one to two study staff should be trained to record the results of malaria diagnostic testing for all suspected malaria cases, enrol study participants according to the protocol, collect DBSs on filter paper, conduct the questionnaire interviews, and properly store and package all samples/questionnaires for shipment to the laboratory. Either the supervisor or person with the requisite expertise should manage the data after collection, create indicator variables and analyse the data. Ideally, this should be done at the central level.

4.9 Data storage and management

All paper questionnaires, consents and assents will be kept in a locked area so that only investigators and data management staff have access. Data should be double entered both at a provincial- or central-level facility and at the participating laboratory facilities, using the data entry screens provided. Questionnaire and data entry templates may need to be tailored for each country; coding guides will be developed for all study variables and should be used across sites to ensure comparability.

Once double data entries have been compared and any errors reconciled, data will be cleaned on an ongoing basis. All data will be collected using unique identification numbers linking the epidemiological, clinical and laboratory data and maintained in secure, password-protected files. All paper records collected will be stored in a secure location under lock and key.

The data are broadly classified as individual patient data, malaria infection data, laboratory data, and consent data. Patient confidentiality will be ensured by archiving the consent forms at a central project location and ensuring that participant names are not entered into any databases or converted to electronically readable text. All study personnel will be required to use password-protected computers to access the project data. Encryption will be required for all tablets or electronic data capture devices used for data collection purposes. Permission will be required for data reuse. On-site data managers and their assistants will be trained in all data entry and management processes, and their training logs will be maintained and archived for data quality assurance checks

All study investigators and staff should participate in training on the conduct of human subject research prior to the study's data collection phase. Interviewers and data collection personnel will be trained in the importance of maintaining consistency in the patient recruitment and data collection protocols and procedures.

The quality assurance approach will focus on providing support for the selection of study subjects and study sites, data collection, and management procedures. Data verification techniques will include logic, range and consistency checks. Data validation will be implemented via electronic data entry mechanisms, such as input masks, conditional logic and validation rules. Study personnel will be trained on the rationale and importance of the data verification and validation processes, using specific examples to describe potential implications for the study results. Intermediate statistical analyses will serve as detective and corrective controls by identifying changes in enrolment rates, protocol deviations, duplication of data entry values, or incorrect data values. These results will be communicated to all key personnel on a weekly basis for as long as the cross-sectional data

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collection is underway. Keeping both paper and electronic data will also serve as a secondary check for the accuracy of data.

4.10 Laboratory analyses

4.10.1 Molecular characterization

Molecular characterization should be conducted at a laboratory that has experience in malaria molecular techniques and subscribes to the WHO external quality assessment (EQA) scheme for malaria nucleic acid amplification testing (NAAT) or other scheme for malaria molecular methods. Quantitative PCR is preferred over non-quantitative nucleic acid amplification methods, especially if parasite density is not measured by microscopy.

The methods proposed below are based on Cheng et al. [4].

4.10.2 DNA extraction and quality control

For verification of the DNA quality, an aliquot of the DNA should be used for amplification of the *msp1*, *msp2* and *glurp* genes, according to standard published protocols [18].

4.10.3 Molecular species diagnosis

Specific primer pairs should be used for four or five separate and specific amplification reactions of *P. falciparum*, *P. vivax*, *P. malariae, P. ovale* and *P. knowlesi*.

4.10.4 Characterization of pfhrp2 and pfhrp3 sequences and gene deletions in samples

Suggested primer sequences, PCR conditions and expected amplification product sizes have been published [4]. *Pfhrp2* and *pfhrp3* genes should be characterized by amplification of two gene segments. One segment extends from the end of exon 1 to the start of exon 2, including the intron of each gene. The other segment consists of the entire exon 2, which codes for the histidine-alanine-rich repeat region of each protein. PCR assays should include appropriate controls, including DNA from lab strains with known deletions, such as DD2 (*pfhrp2*-deleted) and (*pfhrp3*-deleted). If the *pfhrp2* gene can be amplified, the sequence of the exon 2 amplicon will be determined and translated into an amino-acid sequence. This will enable the classification of the Pf HRP2 protein as type A, type B or type C/borderline structural group, according to the multiplied number of type 2 and type 7 repeats (see above). If the *pfhrp2* and/or *pfhrp3* genes cannot be amplified despite good quality of DNA (see 4.9.2) and amplification of other single-copy gene sequences, it suggests that genes have been deleted. Optionally, in order to further confirm and characterize subtelomeric deletions, the following upstream and downstream flanking genes of *pfhrp2* and/or *pfhrp3* can be amplified: the HPC230 gene located ~5.5 kb upstream and HSP70 located ~6.5 kb downstream of *pfhrp2*; and the HPC475 gene located ~1.7 kb upstream and ACL located ~4.4 kb downstream of *pfhrp3*.

4.11 Data analysis procedures

Following double data entry (see section 4.8) and reconciliation of any errors, the prevalence of false-negative HRP2 RDT results (diagnostic-based) that are suspected to be caused by *pfhrp2/3* gene deletions (output indicator 1) will be determined at the provincial level, with 95% CIs estimated for all point estimates. This process should follow the tabulation format in the “dummy” table provided

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(Appendix 6). Provincial-level estimates of output 1 will then be disaggregated by age group, sex, village/home residence, and recent antimalarial treatment in order to see whether any patterns emerge. If desired, point estimates and 95% CIs can be weighted by relative facility size or patient flows. Differences between point estimates across sociodemographic or other collected variables can be investigated using X^2 and/or logistic regressions, as desired.

After completion of the laboratory analyses, the prevalence of *pfhrp2/3* gene deletions based on genotyping (output indicator 2) will be estimated at the provincial and national levels, with 95% CIs for all point estimates. This process should follow the tabulation format provided in the "dummy" table provided (Appendix 6). Output 2 will be disaggregated by province, age group, sex, village/home residence, and recent antimalarial treatment in order to see whether any patterns emerge. If desired, point estimates and 95% CIs can be weighted by relative facility size or patient flows, as well as by relative province size for national-level estimates. Differences between point estimates across sociodemographic or other collected variables can be investigated using X^2 and/or logistic regressions, as desired.

5. Study timeline

It should be noted that the amount of time it will take to enrol the desired number of study participants will depend on: 1) the number of suspected malaria cases seen each week at each facility; 2) the test positivity rate (i.e., number of positives per suspected malaria case) at each facility; and 3) the sample size needed to detect whether the observed prevalence of false-negative HRP2 RDT results caused by *pfhrp2/3* gene deletions is above or below the 5% threshold (see section 4.3). Prior to implementing this protocol, the NMCP and study teams should assess the expected time for enroling the desired number of respondents within each province and plan accordingly. An example timeline is presented in Table 5; note that the start month will depend on the local transmission context.

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TABLE 3.
Illustrative study timeline

	Year									
Activities	**J**	**F**	**M**	**A**	**M**	**J**	**J**	**A**	**S**	**O**
IRB approval										
Sampling frame development										
Selection of provinces and facilities										
Procurement of all study supplies										
Community engagement (as needed)										
Recruit and train data collectors										
Data collection at facilities										
Data entry										
Laboratory analysis										
Data analysis										
Presentation of findings										

6. Human Subjects

6.1 Overview

All investigators will be trained in the ethical conduct of human research, the study objectives, methods of effective communication with study participants, and collection of high-quality data. The importance of informed consent and how to administer consent forms will be emphasized, and the study team will receive additional training specific to the tasks they will perform (e.g., interview techniques, sample collection and data confidentiality).

Prior to fieldwork being conducted, the relevant ethics committees/institutional review boards will be presented with all of the necessary documentation, including questionnaires, proposed procedures to minimize risk in the process of data collection, and consent forms and data management plans to ensure the confidentiality and safety of data. All research participants will be asked to provide individual consent (or assent depending on the age of majority and federal guidelines in the country of enrolment) for their participation in the research portion of the study (i.e., genetic analysis of the infecting parasites).

Participation in the study is voluntary, and participants have the right to refuse or withdraw at any time. Informed consent should be obtained both verbally and in writing from all participants in the preferred local language (Appendix 3). As part of the consent process, the study will be explained and the consent form will be read to each person or given to participants to read themselves. Participants will be asked questions to ensure their

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comprehension. It should be emphasized that participation is voluntary, and that participants have the right to withdraw consent at any time and the right to refuse to answer any question. The consent form will detail the design of the study and analyses to be done, including a description of data storage. If participants agree, they will be asked to sign the consent or assent form or provide a thumbprint in conjunction with the signature of an independent witness, depending on national guidelines. Since no personal identifying information will be collected from patients, the data and specimens will remain anonymous.

For children under the legal age, consent will be obtained from at least one parent or guardian; this is sufficient given the minimal risk of the study. In addition, child assent (Appendix 4) will be obtained for children over 6 but under 16 years of age, in addition to the consent of a parent or guardian. Children providing assent will be asked to sign next to their name or provide a thumbprint accompanied by the signature of an independent witness on the assent form. In cases where subjects under 18 years of age are considered "mature minors" (sometimes defined as pregnant, married, or otherwise the head of their household, depending on the country-specific context) and are able to provide consent for themselves, assent will not be sought. Examples of consent and assent forms are included in Appendices 3 and 4. The reading level of the consent form should be no higher than primary school level 8. All interviewers will be trained extensively in the consent procedure, and each form will be co-signed (or verified by their mark) by a team member in order to ensure that all participants have consented. A copy of the consent form will be given to each subject. The names of the investigators will be included on all consent forms, with phone numbers and addresses for the participants to use if they have any questions.

6.2 Risks to human subjects

Parents/guardians and children will be informed of all potential risks at the time of the survey administration. This survey is of minimal risk to participants. The amount of blood collected is very small (~100–200µl), and participants may experience only a small bruise at the site from which blood is collected. The initial prick may lead to minor temporary discomfort or pain. Trained personnel will perform finger pricks in order to ensure that they are done in as safe a manner as possible. Precautions will be taken to avoid bleeding by applying cotton wool and pressure immediately to the prick site. Risk of infection will be minimized by cleaning the finger with an alcohol swab prior to pricking and using disposable lancets – one for each individual in order to avoid cross-contamination/transmission of infectious agents. Any concerns about potential risks will be mitigated as much as possible through community sensitization prior to the survey.

6.3 Protection against risk

The data collected as part of the questionnaire is not considered to be of a sensitive nature Therefore, there are minimal risks expected for the participant. Furthermore, steps will be taken to ensure that each study participant's personal information will be protected. All questionnaires will be labelled with the participant's name and personal ID number. Filter paper samples and other samples will be labelled using a personal ID barcode only.

The proposed strategy to reduce any risks includes:

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1. Explaining the physical procedures carefully to each participant so that they understand the potential pain associated with the collection of malaria data but also that the pain is most likely to be temporary.

2. Ensuring that health workers can answer commonly asked questions and understand the nature of the questions being asked.

3. Ensuring that health workers using RDTs in their routine work are observed for their competency in collecting and handling biological specimens and that all data entry personnel (these may also be the health workers) are trained in confidentiality, safety and informed consent procedures; all team members should be trained in universal precautions for handling biological specimens.

4. Training field supervisors in protocol management. Spot checks by the PI and Co-PI will provide further assessment of protocol management.

5. Using the most efficacious testing procedures available to ensure sterile and safe biological data collection and testing.

6. Assessing the practices for protecting against any blood-borne infections, including HIV, according to national guidelines, and corrective action plans should such infection occur from needle sticks during the collection of data. Training/retraining in the standard universal precautions (i.e., use of gloves and sterile equipment for all fluid transactions) will minimize the possibilities of transmission from participants to data collectors or vice versa. If a needle stick should occur, the recipient will immediately be offered appropriate counselling and treatment from the nearest relevant health facility according to national protocol.

7. Ensuring that the confidentiality procedures are designed to meet all contingencies in order to preserve the privacy of the participants.

6.4 Data monitoring and protection plan

Participants, parents and guardians will be informed that participating in a research study may involve a loss of privacy. All records will be kept as confidential as possible, and steps will be taken to ensure that each study participant's personal information will be protected. All long-term storage of questionnaire data will be labelled with the participant's personal ID. Filter paper samples will be labelled using only a personal unique ID number, or barcode, which will only be linkable by select study staff. After the questionnaires are completed, they will be maintained in a secure database by the data management staff. For the laboratory analyses, only key staff will possess the link between the laboratory samples and the participants' identifiable information. All data will be stored in locked cupboards and on password-protected computers accessible only to core study staff. After all data have been entered and verified, they will be stripped of personal identifiers. No individual identities will be used in any reports or publications resulting from the study.

6.5 Incentives

There will be no money or commodities offered as incentive for participation in the study.

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8. Appendices

Appendix 1: Options for supplemental data collection

Appendix 2: Facility tally sheet

Appendix 3: Informed consent form template

Appendix 4: Assent form template

Appendix 5: Participant questionnaire

Appendix 6: Tabulation plan for prevalence of *pfhrp2/3* deletions

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Appendix 1: Options for supplemental data collection to determine the prevalence of *pfhrp2/3* gene deletions

According to the study protocol, only samples from patients with suspected *pfhrp2* deletions are analysed for *pfhrp2/3* gene deletions. This approach reduces the number of patients that need to be enroled and in turn the number of samples that need to be collected, transported and analysed by PCR. However, as outlined in Table A1, there are limitations to this approach, as other malaria suspects with *pfhrp2/3* gene deletions will be missed. If additional human and financial resources are available, some limitations may be overcome. For example, by enroling a subset of RDT-negative individuals and collecting DBSs for PCR, the prevalence of *pfhrp2/3* gene deletions among PCR-confirmed low-density *P. falciparum* infections, missed by both RDTs or by RDT and microscopy, can be determined. By contrast, if the budget allows, all malaria suspects positive for a *P. falciparum* infection by any diagnostic can be enroled, consented and asked to provide a DBS (and questionnaire) for PCR analysis in order to confirm *pfhrp2/3* gene deletions among all positive cases. This approach will identify infections caused by *pfhrp2* negative but *pfhrp3* positive parasites that still react with HRP2-based RDTs due to cross-reactivity between HRP2 and HRP3, as well as multiclonal infections with parasites with and without *pfhrp2/3* deletion.

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TABLE A1

Supplemental data collection options and associated limitations in detecting *pfhrp2/3 gene* deletions

HRP2 RDT	pf-pLDH/ microscopy	Diagnosis	Enroled in study	DBS collected	Questionnaire collected	Interpretation of results and limitations in detecting *pfhrp2/3* deletions
+	+	*P.falciparum*	√	√	√	• May be infection with *pfhrp2* deletion but HRP3 was detected by HRP2 RDT • May be multiclonal infection with parasites with and without *pfhrp2/3* deletion
+	-	*P.falciparum*	√	√	√	• False-positive HRP2 RDT (or persisting HRP2 after resolution of infection) • May be infection with *pfhrp2* deletion but HRP3 was detected by HRP2 RDT • May be a low-density Pf infection that does not result in pf-pLDH reaction with RDT due to low antigen concentration • May be multiclonal infection with parasites with and without *pfhrp2/3* deletion
-	+	*P.falciparum*	√	√	√	• False-positive pf-pLDHRDT or microscopy • Low parasite density at limit of detection of RDTs causing variable RDT reactivity
-	-	Negative for malaria	√	√	√	• Cannot exclude low-density infection missed by both RDTs, with undetected *pfhrp2/3* deletion. Use PCR to exclude malaria infection.

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Appendix 2: *Pfhrp2/3* gene deletion survey facility tally sheet

This sheet is to be filled out by all facilities implementing the *pfhrp2/3* gene deletion survey. In each province, once 370 individuals with *P. falciparum* malaria have been enroled in the study (37at each of 10 enrolment sites per province), calculate the proportion of discordant diagnoses (i.e., pf-pLDH or microscopy positive AND HRP2 RDT negative) among all positive diagnoses. Then, consult section 4.6 points 10 and 11 to determine interpretation of results and associated actions after statistical analysis of the molecular confirmation data..

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A	B	C	D	E	F	G	H	I	J	K	L	M
Suspected malaria case I.D.	**Date of visit / test (DD/MM/YY)**	**Name**	**Gender**	**Age in years**	**HRP2 RDT[5] result**	**pf-pLDH RDT result**	**Microscopy result**	**Suspected false-neg HRP2 (pf-pLDH or microscopy positive AND HRP2 RDT negative)**	**Pf case confirmed by at least one diagnostic method (Yes, No)**	**Informed consent / assent completed**	**DBS obtained**	**Questionnaire completed**
1.			M / F		**+ / - /invalid /ND**	**+ / - / invalid/ND**	**+ / - NA**	Y / N	Y / N	Y / N / NA	Y / N/ NA	Y / N / NA
2.			M / F		**+ / - /invalid /ND**	**+ / - / invalid/ND**	**+ / - NA**	Y / N	Y / N	Y / N / NA	Y / N/ NA	Y / N / NA
3.			M / F		**+ / - /invalid /ND**	**+ / - / invalid/ND**	**+ / - NA**	Y / N	Y / N	Y / N / NA	Y / N/ NA	Y / N / NA
4.			M / F		**+ / - /invalid /ND**	**+ / - / invalid/ND**	**+ / - NA**	Y / N	Y / N	Y / N / NA	Y / N / NA	Y / N / NA
5.			M / F		**+ / - /invalid /ND**	**+ / - / invalid/ND**	**+ / - NA**	Y / N	Y / N	Y / N / NA	Y / N/ NA	Y / N / NA

** Expand rows as needed*

[5] In the case that a combination test that contains both HRP2 and pf-pLDH test lines on the same strip is being used, the results are for the individual test line rather than for the overall RDT result.

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Phase 1: Tally total after 370 *P. falciparum* cases detected.

Total malaria suspects tested (column A – sum) _____

P. falciparum cases detected (column J – sum of 'yes' responses)_____

Number of suspected false-negative HRP2 RDT results (column I – sum of 'yes' responses) (a) _____

Total positive *P. falciparum* diagnoses by any test (column J – sum of 'yes' responses) (b) _____

Percentage of all Pf cases with suspected false-negative HRP2 RDT results that need molecular analysis for pfhrp2/3 deletions (a / b)

_____

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Appendix 3: Informed consent form (template)

(*Note: the age of consent may differ between countries; as such, this form should be used in accordance with national guidelines)*

Determining whether histidine rich protein gene deletions causing false-negative HRP2 RDT results among symptomatic falciparum patients have reached a threshold requiring a change in malaria RDTs

Principal investigator: NAME

Co-investigators and other collaborators: NAMES

Study title: Determining whether histidine rich protein gene deletions causing false-negative HRP2 RDT results among symptomatic patients with confirmed *P. falciparum* malaria have reached a threshold requiring a change in malaria RDTs

Survey sites: NAMES

Sponsor: NAMES

Introduction

I am …….. and I work with the National Malaria Control Programme. You (or your child) are invited to participate in a research study to better understand how well rapid diagnostic test kits work in this country. You (or your child) are being asked to participate in this study because you may have malaria.

This study has been reviewed by Local Institutional Review Board. No research activity will be conducted until you have had an opportunity to review this consent form, ask any questions you may have, and provide consent. We encourage you to ask questions now and at any time. If you decide to participate, you will be asked to sign the consent form or to provide a thumbprint in conjunction with the signature of an independent witness. If you would like a copy of this form, one will be provided to you. You (or your child's) participation is completely voluntary and will in no way affect the treatment and care you receive for malaria.

Why is this survey being done?

We are conducting this research survey because we want to look at samples of blood from people who have malaria and then use the blood samples to see if the malaria tests we buy and use are working (give a positive result) or not working (give a negative result when they shouldn't). A false-negative result can mean that there is a problem with the test or that the malaria parasites have characteristics that can make them hard to detect.. We will ask you questions about malaria and perform some tests to determine if you have malaria.

What are the study procedures? What will I be asked to do?

If you agree to take part in the survey, we will ask you questions about the location of your house, your sex and age, and the medicines you have taken for malaria in the past. We will ask about what you do to prevent malaria. We will also take a few drops of blood by pricking your finger. We will take 1–2 drops of blood. The amount of blood taken will be very small and there is very little risk of harm to anyone who agrees to participate in the

survey. We will test the blood sample for evidence of malaria by putting a small drop of blood onto paper for further tests to confirm malaria. These tests will be carried out in XX location. Participating in this survey will take approximately 15 minutes. This is a one-time survey and there will be no follow-up visits.

What are the risks or inconveniences of the study?

There is very little risk of harm to anyone who agrees to participate in the survey. There may be a small bruise or temporary mild pain on the finger where the blood is taken. There is also a small chance of infection when blood is drawn. However, our careful procedures make this very unlikely. A possible inconvenience may be the time it takes to complete the questions (15 minutes). A second risk could be that someone outside the study team accesses your information; this is rare because we will not use your name (or your child's name) when labelling the blood that is sent to the laboratory.

What are the benefits of the study?

There will be no direct benefit to you for taking part in this study; if you agree to donate your blood, then your participation may result in public health programmes being sure that the malaria tests can accurately diagnose malaria. Your participation will help us understand if the malaria parasite is changing over time.

Are there costs to participate?

Participation is free of charge, but there is also no compensation to you or your child if you decide to take part in this study.

How will my personal information be protected?

We will be careful to keep your information private. We will make every effort to ensure that your information (or your child's information) is kept as confidential as possible. For example, we will not use your name or other identifying information on study documents or in any publications; we will replace it with an identification number. Only those treating you today will have the key to link the samples and information attached to your name. All data will be stored in locked cupboards and on password-protected computers.

Can I stop being in the study and what are my rights?

You do not have to participate in this study if you do not want to. You are free to choose for you and your child whether you want to take part in this survey. If you do not want to participate in this survey or do not want to complete the survey, there will be no penalty to you. You can choose to stop participating at any time without penalty.

Long-term storage of samples for future tests:

There may be some leftover blood after this project is over. We want to store any such leftover blood. We may use it only for malaria-related studies in the future. You or your child can still participate in this survey even if you do not want the blood stored for future use. Even if you do not allow us to store the blood for future use, you/your child will still receive the usual medical care.

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Who do I contact if I have questions about the study?

If you have any questions about this survey you can contact the principal investigator, NAME at TELEPHONE NUMBER. If you have any questions about your rights as a survey participant, or if you want to talk about the survey with someone who is not part of this research project, please contact NAME AND ADDRESS.

Today's survey: The consent form has been explained to me and I agree to take part in the survey. I understand that I am free to choose for me or my child to be in this survey and that saying "NO" will have no effect on me.	If you agree, circle "YES", if you do not agree, circle "NO".	
	YES	NO

Adult/mature minor providing consent for self or child	Name	Signature/print	Date___ / ___ / ___
Witness	Name	Signature	Date___ / ___ / ___

***A participant can sign or verbally state his/her consent and mark the form with a fingerprint in the presence of a witness who will then sign, in the event the subject cannot read the consent document. (Note: Date should be DD/MM/YY)**

Your signature below means that you voluntarily agree to allow us to store your filter paper blood sample for future studies:

Long-term storage and future studies: I agree to allow the study team to store my (or my child's) (filter paper) blood sample for future studies. I understand that I can change my mind to not have my filter paper blood sample stored and used for future research. I may also ask that my filter paper blood sample not be used for certain types of testing.	If you agree, circle "YES," if you do not agree, circle 'NO'.	
	YES	NO

Adult/mature minor providing consent for self or child	Name	Signature/print	Date___ / ___ / ____
Witness	Name	Signature	Date___ / ___ / ____

***A participant or parent can sign or verbally state his/her consent and mark the form with a fingerprint in the presence of a witness who will then sign, in the event the subject cannot not read the consent document. (Note: Date should be in DD/MM/YY).**

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Appendix 4: Assent form (template) for minors ages 7–15

(Note: the age of assent may differ between countries; as such, this form should be used in accordance with national guidelines)

Determining whether histidine rich protein gene deletions causing false-negative HRP2 RDT results among symptomatic falciparum patients have reached a threshold requiring a change in malaria RDTs

Principal investigator: NAME

Co-investigators and other collaborators: NAMES

Study title: Determining whether histidine rich protein gene deletions causing false-negative HRP2 RDT results among symptomatic falciparum patients have reached a threshold requiring a change in malaria RDTs

Performance sites: NAMES

Sponsor:

Introduction

I am …….. and I work with the National Malaria Control Programme. You are invited to participate in a research study to better understand how well rapid diagnostic test kits work in this country. Please keep in mind that your parent has already agreed to participation, but you are also free to decide for yourself.

Why is this study being done?

We are conducting this research survey because we want to look at samples of blood from people who have malaria and then use the blood samples to see if the malaria tests we buy and use are working (give a positive result) or not working (give a negative result when they shouldn't). We will ask you questions about malaria and perform some tests to determine if you have malaria.

What are the study procedures? What will I be asked to do?

We will take a few drops of blood by pricking your finger. The amount of blood taken will be very small and there is very little risk of harm to anyone who agrees to be in the survey. We will test the blood sample for evidence of malaria. Participating in this survey will take approximately 15 minutes. This is a one-time survey and there will be no follow-up visits.

What are the risks or inconveniences of the study?

There is very little risk of harm to anyone who agrees to be in the survey. There may be a small bruise or temporary mild pain on the finger where the blood is taken.

What are the benefits of the study?

There will be no direct benefit to you for taking part in this study.

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Are there costs to participate?

Participation is free of charge, but there is also no compensation to you if you decide to take part in this study.

Can I stop being in the study and what are my rights?

You do not have to be in this study if you do not want to. You are free to choose if you want to be part of this survey. You are free to talk to your parents or the study team.

Who do I contact if I have questions about the study?

If you have any questions about this survey you can contact the principal investigator, NAME at TELEPHONE NUMBER. If you have any questions about your rights as a survey participant, or if you want to talk about the survey with someone who is not part of this research project, please contact NAME AND ADDRESS.

Statement of permission

By signing or placing my thumbprint below, I agree that:

- I have read this form or it has been read to me;
- I have been able to ask questions about it. People answered my questions;
- I choose to be in this evaluation;
- I have been told that I can drop out from this evaluation at any time.

Child Assenting	Name	Signature/print	Date___ / ___ / ___
*Witness	Name	Signature	Date___ / ___ / ___

*** I have witnessed the informed permission and can attest that the child has been given details of the evaluation, told about the risks and benefits, and had the opportunity to have questions answered. (Note: Date should be DD/MM/YY)**

I have accurately read or witnessed the accurate reading of the permission form to the participant child and the individual has had the opportunity to ask questions. I confirm that the individual has given permission freely.

Print name of the study staff_____

Signature of the study staff _____

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Appendix 5: Participant questionnaire

Note: Form should be in duplicate – with barcode or patient identification number on each one (matching filter paper barcode or patient identification number). One copy stays with the NMCP for analysis of indicator 1, and the other goes to the lab with filter paper for analysis of indicator 2.

Forms should be pre-filled to indicate the health centre and RDT-specific information, i.e., name, product code, target antigens, etc., and sections that are not applicable (NA).

Target antigens (4 and 5) and results for each test band (4c) should be recorded as per the convention below. If there is only one test line (T1) or two test lines (T1, T2), record 'NA' in the other test line cells.



To be completed prior to participant interview		
1.	Barcode/Patient ID	*Place barcode sticker or write patient ID here*
2	Health centre	*Pre-entered for each health centre on printed form*
3.	Date of visit	Day____ Month___ Year____
4.	RDT 1 (must include HRP2)	*Pre-entered for each health centre on printed form* a. Name: b. Product code: c. Lot number: d. Expiry date: e. Target antigens: 1. T1: 2. T2: 3. T3:
5.	RDT 2	*Pre-entered for each health centre on printed form* a. Name: b. Product code: c. Lot number: d. Expiry date: e. Target antigens: 1. T1: 2. T2: 3. T3:
6.	Microscopy	Yes No

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7.

7a. RDT 1 Must include HRP2

Pre-fill this questionnaire with relevant NA according to the RDT used.

7b. RDT 2

Control line (C) (present, absent)	T3 (+ , -, NA)	T2 (+, -, NA)	T1 (+, -, NA)	Result *As per manufacturers' instructions:* Positive: Pf, non-Pf, Pv, mixed Pf and non-Pf , mixed Pf and Pv; negative, invalid	Initials of operator/reader

Control line (C) (present, absent)	T3 (+, - , NA)	T2 (+, -, NA)	T1 (+, -, NA)	Result *As per manufacturers' instructions:* Positive: Pf, non-Pf, Pv, mixed Pf and non-Pf, mixed Pf and Pv; negative, invalid	Initials of operator/reader

Microscopy	**Positive/Negative/NA**	**Species**	**Parasite count (parasites per microliter)**	**Initials of microscopist**
7c. Field health facility				
7d. National Laboratory crosscheck (read 1)				
7e. National Laboratory crosscheck (read 2)				

To be obtained from individual enroled in the study, or the parent/caregiver of those under 16

8.	Age in years	_____
9.	Sex	a. M b. F

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10.	Village where participant resides	_____ *From pre-populated list if possible*
11.	In the past 2 weeks, have you taken any medicine for malaria?	a. No → Go to question 13 b. Yes
12.	Antimalarial medicine taken	*From pre-populated list* a. ACT (whichever ACT is first-line drug in the country) b. Other ACTs (could be other names for first-line drug) c. Fansidar / SP / Sulfadoxine/pyrimethamine d. Quinine e. Panadol (antipyretics available in country) f. Other _____ g. Unknown h. NA
13.	Have you travelled outside your community in the past 30 days?	a. No → end b. Yes → go to question 14
14.	Where did you travel?	a. Country_____ b. Region _____ c. District_____ d. City/Village_____ e. NA Note: answers should be drop-down choices and place given for multiple entries
15.	Molecular analysis	a. single copy gene 1 – present/absent/not done b. single copy gene 2 – present/absent/not done c. single copy gene 3 – present/absent/not done d. HRP2 Exon1 – present/absent/not done e. HRP2 Exon 2 – present/absent/not done f. HRP2 flanking 230 – present/absent/not done g. HRP2 flanking 228 – present/absent/not done h. HRP3 Exon 1 present/absent/not done i. HRP3 Exon 2 – present/absent/not done j. HRP3 flanking 485 – present/absent/not done k. HRP3 flanking 475 - present/absent/not done l. HRP2 luminex signal – value/not done m. HRP2 ng/ml – value/not done

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Appendix 6

Tabulation plan for prevalence of *pfhrp2/3* deletions

Characteristic	Phase 1: Suspected false-negative HRP2 RDT prevalence[b] (n=XX) (95% CI)	*pfhrp2/3* deletion prevalence[c] (n=XX) (95% CI)
Age in years		
<2		
3–5		
6–9		
10–19		
20–29		
30–39		
40–49		
50–59		
≥60		
Sex		
Male		
Female		
Location		
Urban		
Rural		
Province (survey domain)		
Province 1		
Province 2		
Province 3		
Province 4		
Province 5		
Health facility (optional)		
Facility 1		
Facility 2		
Facility 3		
Facility 4		
Facility 5		
Facility 6		
Facility 7		
Facility 8		
Facility 9		
Facility 10		
Antimalarial treatment past 2 weeks		
Yes		
No		
Total		

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a – Tabulations are based on pfhrp2/3 deletion screening only in P. falciparum cases with discordant results. If all Pf cases or all suspects are screened for pfhrp2/3 deletions, then this form should be revised accordingly.
b – Suspected false-negative HRP2-RDT P.falciparum prevalence = # discordant results (HRP2 negative & pf-pLDH or microscopy positive) / all P. falciparum cases confirmed by any diagnostic.
c – pfhrp2/3 deletion prevalence = # Pf cases with pfhrp2/3 deletion causing false-negative HRP2 RDT results / total # P. falciparum cases

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