Master protocol for surveillance of *pfhrp2/3* deletions and biobanking to support future research

Second edition

World Health Organization

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Contents

Acronyms

CrI	credible interval
DBS	dried blood spot
HRP2	histidine-rich protein 2
HRP3	histidine-rich protein 3
LDH	lactate dehydrogenase
PCR	polymerase chain reaction
PDS	panel detection score
pfhrp2	*Plasmodium falciparum histidine-rich protein 2*
pfhrp3	*Plasmodium falciparum histidine-rich protein 3*
RDT	rapid diagnostic test
WHO	World Health Organization

Disclaimers

1. This recommended master protocol has been developed by the World Health Organization (WHO) to guide surveillance and biobanking for *pfhrp2/3* gene deletions in malaria-endemic countries; however, WHO cannot accept any responsibility or liability for the conduct of studies by third parties that follow the protocol.
2. Studies conducted by third parties pursuant to the protocol cannot be considered "WHO studies". Appropriate approval(s) at local and/or national level should be obtained prior to the start of any such study.

1. Project summary

Title	
Study site(s)	Site 1: Name, city, district and province Site 2: Name, city, district and province Site 3: Name, city, district and province *(Add more sites as needed, or include just counties and add an annex with a list of health facilities)*
Protocol submission date	dd/mm/yyyy
Protocol number	Unique protocol number/version number
Principal investigator	Name: Degree: Institution: Address: street, city, postal code, country Tel: Email:
Co-investigators (insert additional name(s) if needed)	Name: Degree: Institution: Address: street, city, postal code, country Tel: Email:
Participating institutions (insert additional institution(s) if needed)	Name: Complete postal address: street, city, postal code, country Tel: Email:
Planned study dates	From mm/yyyy to mm/yyyy
Sponsor	Ministry of Health, country Complete postal address: street, city, postal code, country Tel: Email:

Objectives	This survey is intended to determine whether the local prevalence of false-negative rapid diagnostic test (RDT) results caused by *Plasmodium falciparum* histidine-rich protein 2/3 (*pfhrp2/3*) gene deletions has reached a threshold that would require a local or national change in diagnostic strategy. The specific objectives are to: 1. measure the prevalence of false-negative HRP2 RDT results caused by *pfhrp2/3* gene deletions among all symptomatic P. falciparum confirmed cases 2. 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 *P. falciparum* lactate dehydrogenase (Pf-LDH) RDT; 3. identify domains (e.g. provinces, states or districts) in which the prevalence of false-negative HRP2 RDTs results caused by *pfhrp2/3* gene deletions is above 5%, warranting a change of RDTs. 4. determine the predictive value of false-negative HRP2 RDT results for *pfhrp2/3* gene deletions in different settings; and 5. The biobanking activity is intended to support future malaria epidemiological research and the development of new and/or improved health products, particularly those targeting pfhrp2/3-deleted parasites.
Surveillance site	Pre-selected public health facilities representing the spectrum of transmission and geographical diversity across the country
Target population	Individuals meeting the case definition for suspected malaria case
Survey type	Cross-sectional, multi-site
Primary output measures	1. Prevalence of false-negative HRP2 RDT results caused by *pfhrp2/3* gene deletions among all symptomatic P. falciparum confirmed cases 2. Prevalence of suspected false-negative HRP2 RDT results among symptomatic patients with *P. falciparum* malaria 3. Prevalence of *pfhrp2/3* gene deletions among symptomatic falciparum patients with a false-negative HRP2 RDT result
Secondary output measures (optional)	1. Parasite density, as measured by quantitative PCR and/or microscopy, in patients with suspected false-negative HRP2 RDT results 2. Improved understanding of *pfhrp2/3* gene deletions, e.g. genetic relatedness and/or drug resistance and fitness, and new and/or improved diagnostic tools for detecting *pfhrp2/3*-deleted parasites 3. Prevalence of parasites with *pfhrp2/3* deletions among all symptomatic *P. falciparum* confirmed cases 4. Estimates of the intra-cluster correlation in the prevalence of *pfhrp2/3* deletions within sampling domains (e.g. provinces or districts), or other measures of heterogeneity than can help guide future studies
Sample size	A sample size of 300 confirmed *P. falciparum* cases per sampling domain (30 cases in each of 10 health facilities) is a default proposition to quantify whether or not the prevalence of false-negative RDT results caused by *pfhrp2* deletions is above 5%. A sampling domain can be a province, district, state or something else, depending on the administrative boundaries of the country. Once the sample of 300 *P. falciparum* cases has been enrolled, then molecular confirmation of *pfhrp2/3* deletions among suspected false-negative *P. falciparum* cases should ensue. While 300 confirmed *P. falciparum* cases is the default sample size, a sample size table is provided to allow for some flexibility. A tool has also been created for customized sample size estimates informed by baseline knowledge and desired power.

Sampling method	A cross-sectional survey will measure the suspected and confirmed prevalence of false-negative HRP2 RDT results caused by *pfhrp2/3* gene deletions in multiple pre-selected health facilities per sampling domain, e.g. provinces at risk.
Data collection	1. Identify domains (e.g. provinces, districts) to be included in the study. Factors to consider include: (i) capturing a range of transmission strata, (ii) ensuring broad geographical representation, and (iii) optionally, weighting based on population at risk. 2. Select 10 health facilities per domain for testing (if this is not possible, a smaller number of health facilities may be viable; see Table 2 for variations in the number of health facilities and recommended sample numbers). Any facility in which RDTs are being used is eligible; microscopy services are not a requirement. Selection of health facilities may be through randomization or deliberate choice (e.g. sentinel surveillance), and must ensure balanced representation of the target population. 3. In the target population (suspected malaria cases), conduct routine case management procedures and obtain informed consent (or assent, depending on the age of majority and federal guidelines in the country of enrolment) to perform an additional RDT and collect at least two dried blood spots (DBSs) for laboratory analysis and for biobanking/long-term storage to support future malaria epidemiological and diagnostic research. 4. Take clinical history, including questions regarding age, sex, recent malaria diagnostic testing, antimalarial therapy and travel. 5. Test all consenting suspected malaria cases simultaneously using both a WHO-recommended HRP2 RDT and a non-HRP2-based method (e.g. Pf-LDH RDT (separate single or multiple test line RDT)) or quality-assured microscopy in the health facility, and collect a minimum of two DBSs on filter paper or protein saver cards. 6. Record demographic and clinical history details and all test results on the survey report form. 7. Administer antimalarial therapy based on results from (either) RDT and/or microscopy, according to national guidelines. 8. Retain used RDTs for quality control and a minimum of two DBSs from all consenting *P. falciparum* patients for molecular +/- serological analysis. 9. Enrolment can stop once 30 individuals with confirmed *P. falciparum* malaria from each of 10 health facilities have been recorded in the survey tally sheet as having *P. falciparum* (a different number of confirmed individuals is required per health facility if using other than 10 clusters; see Table 2). 10. Ship all consent forms, tally sheets, survey report forms and patient samples to the central coordinating centre. 11. Central laboratory staff review survey report forms, identify the suspected *pfhrp2/3* deletion cases and prioritize these DBSs for molecular +/- serological analysis. 12. Proceed with supplemental data analysis according to prioritization and resources available, i.e. HRP2-positive samples, and HRP2- and Pf-LDH-negative samples; options are described in Annex 1. 13. Discard all RDTs, microscopy slides and DBSs after survey results have been finalized and reported, unless consent for long-term storage of DBSs has been obtained.

2. 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 of 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-based RDTs generally have the highest sensitivity of the RDTs for detecting *P. falciparum* malaria *(3)*, parasite strains have recently been identified that have deletions in the genes encoding the HRP2 or similar HRP3 protein. Strains with both *pfhrp2* and *pfhrp3* gene deletions are undetectable by HRP2-based RDTs *(4)*. HRP2-based 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 from specimens collected in 1998–2001 (20.7%) to those collected in 2003–2005 (40.6%) *(6)*. The prevalence of parasites with *pfhrp2/3* gene deletions shows substantial local variability. Studies in other countries, such as Colombia *(7,8)*, Ghana *(9)*, Guyana *(10)*, Honduras *(11)*, India *(12)*, Mali *(13)*, Myanmar *(14)*, Senegal *(15)* and Suriname *(10)*, have found very different prevalence estimates, although the rigour of study design has been variable. In Africa, published data from Eritrea showed the prevalence of dual *pfhrp2* and *pfhrp3* deletions to be very high (80%), requiring an urgent response and policy change away from a testing strategy relying solely on HRP2 *(16)*. There have been no reports of parasites failing to express LDH or aldolase, as these targets are essential enzymes for parasite metabolism and survival. Subsequently, other countries in the Horn of Africa, such as Djibouti *(17)* and Ethiopia *(18–20)*, also reported a high prevalence of *pfhrp2* and *pfhrp3* deletions, which led to the adoption of non-HRP2-based RDTs for diagnosis.

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)*. To avert a crisis like the one that emerged in Eritrea in 2016, WHO recommends that countries where *pfhrp2/3* deletions have been reported, as well as neighbouring countries, conduct surveillance for *pfhrp2/3* deletions, particularly among symptomatic patients *(3)*.

The purpose of WHO-recommended surveillance for *pfhrp2/3* deletions is to identify areas where the prevalence of false-negative RDT results caused by *pfhrp2/3* gene deletions is more than 5%, as this is the recommended threshold for switching to non-HRP2-based RDTs. In this context, it is important to point out that the prevalence of *pfhrp2* deletions in a population may be much higher than 5% to cause > 5% false-negative RDT results, as a number of biological factors can influence this outcome. These factors include expression of the *pfhrp3* gene in *pfhrp2*-deleted parasites (with the HRP3 antigen causing positive RDT results due to its cross-reactivity with HRP2 RDTs) and the prevalence of polyclonal infections (with pfhrp2-deleted and non-deleted parasites

coexisting in the same host) in a population *(21)*. The prevalence of deletions may also need to be higher than 5% in order to confidently conclude that the threshold has been exceeded, based on the statistical analysis.

The purpose of this document is to present a standardized protocol that *P. falciparum*-endemic countries can use to identify the prevalence of false-negative HRP2 RDT results caused by *pfhrp2/3* gene deletions among symptomatic falciparum patients. 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 deletions, and identify areas where diagnostic strategies may need to be changed.

3. Survey and research objectives

This survey is primarily intended to determine whether the local prevalence of false-negative HRP2 RDT results caused by *pfhrp2/3* gene deletions among symptomatic falciparum patients has reached a threshold that should trigger a national or subnational change of malaria RDTs. The specific objectives are as follows:

1. Measure the prevalence of false-negative HRP2 RDT results caused by *pfhrp2/3* gene deletions among all symptomatic *P. falciparum* confirmed cases
2. Measure the prevalence of suspected *pfhrp2/3* gene deletions among symptomatic falciparum patients attending public health facilities.
3. Identify domains that have greater than 5% false-negative RDT results caused by *pfhrp2/3* gene deletions in these patients, as this indicates a need to switch from using exclusively HRP2-based RDTs for detecting *P. falciparum*.
4. Determine the predictive value of suspected false-negative HRP2 RDT results for *pfhrp2/3* gene deletions in different settings.
5. Support future malaria epidemiological research and product research and development for malaria.

4. Survey site(s)/target population

This surveillance activity will focus on suspected malaria cases seeking care at public health facilities. Test results that are negative for *P. falciparum* by HRP2 RDT but positive by Pf-LDH RDT or microscopy indicate that *pfhrp2/3* gene deletions may be the reason for the false-negative HRP2 result. Given the importance of HRP2 detection to the diagnostic strategy, the World Health Organization (WHO) is urging at-risk countries to assess the prevalence of such false-negative HRP2 RDT results caused by *P. falciparum* gene deletions. Prioritized for surveillance 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 are neighbouring an area where frequent *pfhrp2/3* deletions have been identified. In such countries, public health facilities in all areas of *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 sampling domain.

4.1 Inclusion criteria

- Participants should meet the case definition for suspected malaria case

4.2 Exclusion criteria

- Participants should be excluded if they have been previously enrolled in the survey

5. Survey methods

5.1 Design

A cross-sectional survey design will be used to measure the primary outputs. Health facilities will systematically test suspected malaria cases with an HRP2-based RDT and an alternative method (i.e. Pf-LDH RDT or microscopy) and collect a minimum of two dried blood spots (DBSs). Although this approach is the direct and preferable method for this type of survey, an alternative approach using high-throughput immunoassays may be considered when RDT screening data are not available. In addition, immunoassays are useful for confirming the presence or absence of HRP2/3 proteins, especially when RDT and polymerase chain reaction (PCR) results for HRP2 status do not match. The details of this approach are provided in Annex 2.

The prevalence of suspected false-negative HRP2 RDT results among symptomatic patients with *P. falciparum* malaria is a primary output (output 2). Molecular testing of the DBSs from cases with suspected false-negative HRP2 RDT results will determine the prevalence of false-negative HRP2 RDT results caused by *pfhrp2/3* gene deletions all symptomatic *P. falciparum* confirmed cases enrolled in the survey (output 1) and among false-negative HRP2 RDT patients (output 3).

5.2 Primary output indicators

The following indicators will serve as the primary survey outputs:

1. prevalence of false-negative HRP2 RDT results caused by *pfhrp2/3* gene deletions among all symptomatic *P. falciparum* confirmed cases;

2. prevalence of suspected false-negative HRP2 RDT results (i.e. a negative HRP2 RDT result but a positive result by Pf-LDH RDT or *P. falciparum* microscopy, or HRP2-negative but Pf-LDH-positive in a serological test) among symptomatic patients with *P. falciparum* malaria; and

3. prevalence of *pfhrp2/3* gene deletions among symptomatic falciparum patients with a false-negative HRP2 RDT result.

The survey will identify the proportion of patients with suspected false-negative HRP2 RDT results through diagnostic testing at health facilities, using dual-method testing (HRP2 RDT plus microscopy or Pf-LDH RDT[1]). To save time and resources, molecular +/- serological testing to confirm *P. falciparum* infection and identify *pfhrp2/3* deletions will prioritize DBSs collected from individuals with suspected false-negative HRP2 RDT results. Discordant diagnostic results may be due to other factors, such as false-positive Pf-LDH test lines (possibly due to cross-reactivity with non-falciparum species) or low parasite densities at

1 RDTs should be used that contain Pf-LDH-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 (*P. vivax, P. malariae, P. ovale*), which would cause discordant results (HRP2-negative, pan-pLDH-positive).

or below the limit of detection of the HRP2 and Pf-LDH RDTs. In addition, there are several situations in which this indicator could miss a true *pfhrp2/3* gene deletion (see Table 1). First, individuals will not be detected if they have a low-density infection that is missed by Pf-LDH RDT or microscopy and also missed by HRP2 RDT because of *pfhrp2/3* gene deletion. Second, HRP2 RDTs may still detect some infections with a *pfhrp2* deletion due to cross-reactivity of test antibodies with HRP3. Finally, the testing protocol will not detect *pfhrp2/3* deletions in patients coinfected with HRP2-expressing clones, unless certain techniques are used, such as quantitative PCR *(22–24)*, deep sequencing *(25,26)* and digital PCR *(27)*. For these reasons, this indicator represents the lower limit of the true prevalence of *pfhrp2/3* gene deletions but should detect all clinically significant *pfhrp2/3* deletions.

Table 1. Summary of test result combinations and limitations of the approach, including only individuals with discordant HRP2 RDT results (positive by Pf-LDH or microscopy AND negative by HRP2 RDT)

HRP2 RDT	Pf-LDH RDT or microscopy	Molecular analysis performed[a]	Interpretation of results and limitations in detecting *pfhrp2/3* deletions
+	+	No	• May be infection with *pfhrp2* deletions but HRP3 was detected by HRP2 RDT • May be multiclonal infection with parasites with and without *pfhrp2/3* deletions
+	-	No	• False-positive HRP2 RDT (or persisting HRP2 after resolution of infection) • May be infection with *pfhrp2* deletions but HRP3 was detected by HRP2 RDT, or may be multiclonal infection with parasites with and without *pfhrp2/3* deletions **and** • Low parasite density at or below the limit of detection of Pf-LDH RDTs and/or microscopy
-	+	Yes	• False-positive Pf-LDH RDT or microscopy • Low parasite density at limit of detection of RDTs (variable reactivity of test lines)
-	-	No	• Cannot exclude low-density infection missed by both RDTs and microscopy, with undetected *pfhrp2/3* deletions

[a] While all HRP2-negative and Pf-LDH/microscopy-positive samples should undergo molecular analysis, it is recommended that a subset of the other categories also be analysed.

5.3 Secondary output indicators (optional)

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

2. improved understanding of *pfhrp2/3* gene deletions, e.g. genetic relatedness and/or drug resistance and fitness, and new and/or improved diagnostic tools for detecting *pfhrp2/3*-deleted parasites;

3. prevalence of parasites with *pfhrp2/3* deletions among all symptomatic *P. falciparum* confirmed cases; and

4. estimates of the intra-cluster correlation in the prevalence of *pfhrp2/3* deletions within sampling domains (e.g. provinces or districts), or other measures of heterogeneity than can help guide future studies.

5.4 Statistical analysis plan and sample size

The aim of the statistical analysis is to identify survey domains (provinces, districts, states or similar) where there is a high probability that the prevalence of false-negative HRP2 RDT results caused by *pfhrp2* gene deletions is above 5%. Unlike previous versions of the protocol, here, the focus is on identifying areas that are above the threshold (one-tailed test), rather than above or below the threshold (two-tailed test).

Raw data consist of counts of the number of confirmed false-negative HRP2 RDT results caused by *pfhrp2* gene deletions (numerator) and the number of confirmed and enrolled *P. falciparum* cases (denominator) in each health facility. These can be fed into the software package DRpower, accessible via an interactive web app (https://shiny.dide.ic.ac.uk/DRpower-app/). This software uses a Bayesian hierarchical model to estimate the prevalence of false-negative HRP2 RDT results caused by *pfhrp2* gene deletions, while accounting for correlations within clusters (for more information on the model, see Annex 3). The software returns a point estimate of the prevalence along with a 95% credible interval (CrI). This information should be presented when summarizing results, but should not be used directly to decide whether a domain is above the threshold. That decision is based on another output of the software: the probability that the prevalence of false-negative HRP2 RDT results caused by *pfhrp2/3* deletions is above 5%. This value is used to categorize study domains as follows:

> **Outcome 1:** The posterior probability of being above the 5% threshold is less than 0.95. In this case, there is not sufficient evidence to conclude that the proportion of false-negative HRP2 RDT results caused by *pfhrp2/3* deletions among symptomatic *P. falciparum* patients is greater than 5%.
>
> **Outcome 2:** The posterior probability of being above the 5% threshold is greater than or equal to 0.95. In this case, there is high statistical confidence that the proportion of false-negative HRP2 RDT results caused by *pfhrp2/3* deletions among symptomatic *P. falciparum* patients is greater than 5%.

Programmatic decisions can be made on the basis of these outcomes. Note that it is possible for the CrI to span the 5% threshold when the probability of being above 5% is greater than 0.95 (because the CrI is two-tailed but the statistical test is one-tailed).

Sample sizes are chosen to ensure a high probability (80% or more) of finding outcome 2 when the true prevalence of false-negative HRP2 RDT results caused by *pfhrp2/3* gene deletions is above 5%. As the prevalence gets closer to the 5% threshold, it becomes increasingly difficult to prove that prevalence is above this threshold, leading to large sample sizes. Therefore, 10% is used as representative of a high-prevalence setting, striking a balance between sensitivity and the need for sample sizes that are logistically feasible to collect. Consequently, in areas where outcome 2 is found, the prevalence of false-negative HRP2 RDT results caused by *pfhrp2/3* gene deletions will typically be significantly higher than 5%.

Table 2 gives the sample size required to achieve 80% power using the analysis approach proposed above. This table gives the total number of confirmed *P. falciparum* positive cases required (minimum required) for inclusion in the study. A key point to highlight in this example is that the random selection of at least 10 sites per domain will require enrolment of 300 *P. falciparum* confirmed cases, while a reduction in the number of sites will increase the number of samples required to achieve statistical power (i.e. selection of only six sites will require 678 confirmed malaria cases).

Table 2. Sample size required to achieve 80% power using the proposed analysis

Number of clusters (health facilities)	Sample size per cluster	Total sample size
1	--	--
2	--	--
3	--	--
4	--	--
5	496	2 480
6	113	678
7	68	476
8	51	408
9	37	333
10	30	300

Not all suspected malaria patients test positive for falciparum malaria. Therefore, to calculate the number of suspected *P. falciparum* cases to enroll, these numbers should be divided by the expected positivity rate. For example, if 40% of suspected *P. falciparum* cases are expected to come back positive, then the sample of 30 positive cases per cluster increases to 75 (30/0.4) suspected cases to enroll. This number should be further buffered to account for potential drop-out, e.g. due to sequencing failure or diagnostic accuracy. Finally, sample sizes may differ from one health facility to the next; the power of study designs with variable cluster sizes can be explored in the DRpower software and interactive web app (https://shiny.dide.ic.ac.uk/DRpower-app/).

5.5 Sampling

There is evidence that the prevalence of *pfhrp2/3* deletions may vary due to transmission intensity, and so countries are advised to establish transmission strata prior to selecting domains. Multiple domains may then be selected per stratum, aiming for good geographical spread. Depending on surveillance goals, it may be desirable to sample more domains from areas with greater population density.

Sampling in each domain can be via cluster randomization or sentinel surveillance. In the cluster randomized approach, a complete list of health facilities in the domain (e.g. province/district) should be compiled, and 10 health facilities should be sampled from this list using a random procedure. In the sentinel surveillance approach, 10 health facilities can be chosen deliberately, e.g. based on logistical feasibility, while striving to achieve a representative sample of the population.

Once health facilities have been selected, historical incidence trends can be used to establish the time frame over which the requisite samples can be obtained. This may lead to a variable sample size per health facility, which is valid as long as the overall study power is still 80% or higher (see interactive web app: https://shiny.dide.ic.ac.uk/DRpower-app/).

5.6 Data collection and fieldwork

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

The national malaria programme should identify the domains to be surveyed. A sampling domain can be a province, district, state or other unit, depending on the administrative boundaries of the country. This selection should be guided by an assessment of which domains may be most at risk for *pfhrp2/3* gene deletions; otherwise, domains can be randomly selected based on epidemiological zones. Domains with low, moderate and high transmission should be considered, whereas domains without malaria transmission should be omitted. The sample sizes may be reached more quickly in moderate to high transmission areas; however, the expected higher prevalence of multiclonal infections may mask the presence of *pfhrp2/3*-deleted parasites. This issue is less likely in low transmission areas and therefore these zones should be included.

1. Select a specified number of public health facilities (aiming for 10) in each domain of the country routinely using malaria RDTs to be included in the survey.

    - The number of facilities per domain to be included in the sample should take into account the expected mean number of suspected *P. falciparum* cases seen in the facility each week and the mean test positivity rate in the target area in order to ascertain the expected number of positive cases each week. As a general rule, the aim is to finish the fieldwork and collect a minimum sample size of 300 positive cases within an eight-week period (assuming 10 health facilities are selected; if the number of health facilities is reduced, a higher sample size will be required, as explained in section 5.4).

    - Health facilities for the sample should be selected from a complete list (sampling frame) of health facilities in each domain, using systematic random sampling based on probability proportional to size *(28)* (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:* 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 domain-level estimates of *pfhrp2/3* gene deletions may not be statistically representative of the domain. If convenience sampling is conducted, efforts should be made to ensure that the sites are representative of the domain as a whole; otherwise, results will not have good external validity.

2. Survey procedures and analysis of data

    - Patients are triaged according to normal procedures. Any patient considered by the routine health provider, e.g. physician or nurse, to be a suspected malaria case according to national guidelines will be asked for consent/assent (Annexes 4 and 5).

        - Only consenting individuals will sign their name and be given a copy of the information sheet with their survey/sample ID in case they change their mind and wish to not participate in the future or wish to have samples removed from storage.

- Consenting patients will be asked a series of questions relevant to their illness and tested simultaneously according to the manufacturers' instructions for use by two separate diagnostics: the HRP2-based RDT used in the national control programme and either a Pf-LDH RDT or microscopy. A minimum of two DBSs will be collected on filter paper/protein saver cards.

- Non-consenting patients will receive routine care, and no record of their name or identity will appear on any survey documents. The blank non-consent form will be assigned a unique ID to allow for monitoring of the total number of consenting and non-consenting suspected malaria cases attending the facility on the survey tally sheet (Annex 6). All informed consent forms will be kept in a secure location under lock and key.

- The HRP2 and Pf-LDH RDTs will meet WHO procurement criteria and have approval for use by the Ministry of Health.

    - A Pf-LDH RDT that meets WHO procurement criteria (Table 3) or microscopy can be used for the secondary diagnosis. Currently, there are no RDTs that meet the WHO performance criteria for detection of *P. falciparum* based on their Pf-LDH test line alone *(29)*; therefore, for the purposes of screening patients in the survey, countries may exceptionally select Pf-LDH-based RDTs that (i) have been evaluated in WHO malaria RDT product testing; ii) meet performance criteria for detecting HRP2-expressing *P. falciparum*; and (iii) have a panel detection score (PDS) > 90 at 2000 parasites/µL, and false-positive and invalid rates < 2% (see Table 4).

    - If microscopy is to be used, one thin and one thick blood film should be prepared following national guidelines and aligned with the WHO standard operating procedures for malaria microscopy *(30)*.

Table 3. WHO-recommended malaria RDT options for detecting both HRP2-expressing and non-expressing *P. falciparum* malaria for *pfhrp2/3* gene deletion surveillance

Performance criteria	A: *P. falciparum* PDS[a] ≥ 75% at 200 parasites/µL
	B: *P. vivax* PDS[a] ≥ 75% at 200 parasites/µL
	C: false-positive (FP) rate against clean negatives < 10%
	D: invalid rate (IR) < 5%
	E: *pfhrp2*-negative *P. falciparum* PDS > 75% at 200 parasites/µL (in areas where *pfhrp2* deletions are prevalent)

Table 4. Available non-WHO-prequalified tests meeting critical criteria[a]

Product name	Product code	Manufacturer name
Biocredit Malaria Ag Pf (pLDH)	C14RHG25, C14RHH25	Rapigen Inc.
Biocredit Malaria Ag Pf (pLDH/HRP2)	C13RHG25, C13RHH25	Rapigen Inc.
Biocredit Malaria Ag Pf/Pv (pLDH/pLDH)	C61RHG25, C61RHH25	Rapigen Inc.
CareStart™ Malaria Pf (HRP2/pLDH) Ag RDT	RMPM-02571	Access Bio Inc.
CareStart™ Malaria PAN (pLDH) Ag RDT	RMNM-02571	Access Bio Inc.

[a] Valid ISO 13485:2003, submission of application for WHO prequalification, and acceptable diagnostic performance against both HRP2-expressing and non-HRP2-expressing at 200 parasites/µL (*pfhrp2/3* single or double deletions), based on the most recent WHO laboratory assessment performed at the United States Centers for Disease Control and Prevention.

- For each consenting suspected malaria case, the health provider will attach a unique survey ID, chronologically ordered, to a survey case report form (Annex 7) and to the survey tally sheet (Annex 6). On the survey case report form, the health worker will record answers to questions regarding age, sex, and recent history of malaria diagnostic testing, treatment and travel.

- Next, using the labels attached to the survey case report form (with the same ID number as the form), the health worker (treating clinician or laboratory worker) will label two different RDTs or one RDT and microscopy slide and a filter paper or protein saver card for DBS. The health worker will perform the tests, record the results on the survey case report form (Annex 7, sections 5–7), and either inform the patient of the results directly (clinician) or refer (laboratory worker) the patient back to the treating clinician for treatment in the case of positive test results on the primary or secondary RDT, or microscopy as per national guidelines. Results from either RDT should be acted upon, as both are WHO-prequalified. Microscopy is also considered an acceptable alternative to malaria RDTs. Negative RDT results should be managed as per national guidelines.

3. All used RDTs and, if applicable, microscopy slides from each consenting suspected malaria case will be stored until the survey is complete in a dry and protected area for survey quality control purposes.

4. A minimum of two DBSs (50 µL per spot) on filter paper or protein saver card will be placed in a clean, dry and protected area and allowed to dry for 3–4 hours. Once dry, the filter paper/cards will be placed with the desiccant (from the RDT package) in a gas-impermeable plastic bag labelled with the survey ID if it is not stuck directly on the filter paper.

5. Once the desired sample size of infected consenting individuals has been obtained at each facility, the survey case report forms and corresponding RDTs and DBSs should be compiled and sent to the central coordinating centre. No names or other unique identifying information should be contained on the forms, tally sheets, RDTs or DBSs. The only link between patient name and survey ID number should be on the consent form of the consenting individuals.

6. Upon receipt of the forms, RDTs and DBSs, the survey team supervisor must review the RDT results section of the report form (Annex 7, section 5–7, S1), and determine which DBSs to prioritize for molecular +/- serological analysis (specifically, those that are HRP2 RDT negative and Pf-LDH or microscopy positive, and depending on resources, also a subset of HRP2 positive and negatives; see Annex 1) and which to discard.

   Based on the number of discordant RDT or RDT-microscopy results, one can calculate the proportion of *P. falciparum* cases with suspected false-negative HRP2 RDT results (indicating potential *pfhrp2/3* gene deletions) in the health facility or domain (primary output indicator 2), using the formula below.

$$\text{Proportion of } \textit{P. falciparum} \text{ cases with suspected } \textit{pfhrp2/3} \text{ deletions} = \frac{\#\text{ of } \textit{P. falciparum} \text{ cases with discordant RDT (HRP2-/Pf-LDH+) or discordant RDT-microscopy (HRP2-/microscopy+) results}}{\#\text{ of confirmed } \textit{P. falciparum} \text{ cases (positive by Pf-LDH RDT or microscopy)}}$$

7. The DBSs (minimum of two) will be packaged and shipped for *P. falciparum* confirmatory testing and molecular +/- serological analysis for *pfhrp2/3* gene deletions to a WHO collaborating reference laboratory. Samples should be shipped under a material transfer agreement. Ideally, if more than two DBSs were collected, one DBS will remain in the survey country's reference laboratory at all times.

    - Molecular-based confirmation of *pfhrp2/3* deletions is needed to ensure that discordant results are caused by *pfhrp2/3* gene deletions and not by other factors, such as operator error, false-positive Pf-LDH test lines, false-positive microscopy results, or samples at the limit of detection of the RDTs, which may not always react sufficiently to generate a positive test line. The contribution of these alternative causes of discordant results will vary.

    - Serological confirmation of *pfhrp2/3* gene deletions may also be performed using immunoassays, especially on samples where there is lack of agreement between RDT and PCR results.

8. Once the true number of cases with false-negative HRP2 RDT results caused by *pfhrp2* gene deletions is known, the prevalence at the domain level can be calculated for each sampling domain (e.g. province, state), using the DRpower software or interactive web app (https://shiny.dide.ic.ac.uk/DRpower-app/). This software also calculates a 95% CrI around this estimate (see Annex 3). The raw calculation is based on the formula:

$$\text{Proportion of } \textit{P. falciparum} \text{ cases with false-negative HRP2 RDT results due to } \textit{pfhrp2/3} \text{ deletions} = \frac{\#\text{ of confirmed } \textit{P. falciparum} \text{ cases with } \textit{pfhrp2/3} \text{ gene deletions and HRP2-RDT negative results}}{\#\text{ of confirmed } \textit{P. falciparum} \text{ cases (positive by either Pf-LDH RDT or microscopy)}}$$

9. Statistical analysis in DRpower will also result in an estimate of the probability that the prevalence of false-negative HRP2 RDT results caused by *pfhrp2/3* gene deletions is above 5%. This will result in one of two outcomes per domain:

    Outcome 1: The posterior probability of being above the 5% threshold is less than 0.95. In this case, there is not sufficient evidence to conclude that the proportion of false-negative RDT results caused by *pfhrp2/3* gene deletions among symptomatic *P. falciparum* patients is greater than 5%.

    Outcome 2: The posterior probability of being above the 5% threshold is greater than or equal to 0.95. In this case, there is high statistical confidence that the proportion of false-negative HRP2 RDT results caused by *pfhrp2/3* gene deletions among symptomatic *P. falciparum* patients is greater than 5%.

10. If outcome 2 is obtained in any domain, the country programme should make a nationwide switch to RDTs that do not rely solely on HRP2 for detecting *P. falciparum*, prioritized on the basis of the prevalence of false-negative HRP2 RDT results caused by *pfhrp2/3* deletions across domains.

    - A threshold of 5% was selected because it is somewhere around this point that the proportion of cases missed by HRP2 RDTs due to non-HRP2-expressing parasites may be greater than the proportion of cases that would be missed by less-sensitive pLDH-based RDTs.

    - A nationwide change is suggested because mathematical models show that parasites lacking *pfhrp2* genes will spread *(31)* under HRP2-only RDT pressure and because the use of multiple RDTs in a country can complicate procurement and training practices.

11. If outcome 1 is obtained in all domains, it is recommended that the country establish a monitoring mechanism whereby this survey is repeated in two years (or sooner).

12. Once the results of the survey have been finalized and reported, only the DBSs from consenting individuals (denoted on the DBS itself), the plastic bag and case report form should be kept for long-term storage. All other materials associated with the survey (RDTs, microscopy slides and DBSs) should be discarded. Consent forms linking patient names to survey IDs should be maintained by the Ministry of Health. The biobank holder should not possess the information linking patient IDs to samples.

13. All activities should be carried out under the supervision of the principal investigator. A dedicated study coordinator should be identified. It is recommended that there be a minimum of one supervisor per domain where the survey is to be undertaken. One to two survey staff should be trained in each selected survey health facility to record the results of malaria diagnostic testing for all suspected malaria cases, enrol survey participants for long-term sample storage according to the protocol, collect RDTs and/or microscopy slides, collect DBSs on filter paper, conduct the questionnaire interviews, and properly store and package all samples/report forms 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.

5.7 Sample storage

5.7.1 Storage of DBSs, RDTs and microscopy slides

All RDTs and microscopy slides from consenting suspected malaria cases, labelled only with survey ID, should be stored in gas-impermeable plastic bags until the survey and data analysis are complete. This is in case they are needed to resolve data inconsistencies or for additional DNA material. **Once the survey report has been completed, these items should be discarded.**

The DBSs on filter paper, labelled with survey ID and tick box or label (No LTS (long-term storage) or No BB (biobanking)), should be stored in a gas-impermeable plastic bag with the same label and with the desiccant taken from the RDT package at ambient temperature in the health facility until they are transported to the central reference laboratory. There, they will be sorted for onward molecular (including DNA sequencing) +/- serological analysis at a qualified participating laboratory (contact malaria_rdt@who.int for links to specific laboratories). After the survey results have been analysed and reported, any unused DBSs or blood or DNA remaining on DBSs after molecular testing and immunoassays should be discarded, **unless patient consent/assent was granted**. For long-term storage, DBSs should ideally be frozen at -20 °C to -70 °C.

5.7.2 Long-term storage of DBSs: biobanking

As *pfhrp2/3* gene deletions are an emerging issue, little is known about the etiology, trends and associated genetic mutations that may confer survival advantages or disadvantages. New tools are needed to detect these deleted parasites, and the availability of *pfhrp2/3*-deleted parasite material could accelerate product development and evaluation. For these reasons, the biological materials collected during the survey could be used to advance future research and therefore consent for long-term storage/biobanking of leftover biological materials is requested.

At the time of consent, all patients will be given an information sheet that is labelled with a unique survey ID and contact information for the study principal investigator and the

national malaria reference laboratory. They can contact these individuals to request removal of their materials from the biobank. If they lose their information sheet and unique survey ID, their samples can be traced only by the principal investigator, who maintains password-protected access to the signed consent forms and corresponding survey IDs.

Long-term storage requires that those coordinating the survey indicate how they envisage the materials being used in the future; to whom the materials will be entrusted (e.g. the Ministry of Health, research institute) and how confidentiality will be maintained; how the quality of the materials will be controlled; how the donor's authorization can be retracted; and under what circumstances donors may need to be re-contacted. These requirements are laid out in Guideline 11 of the *International ethical guidelines for health-related research involving humans (32)*.

5.8 Data storage and management

Survey case report forms and survey tally sheets do not include any unique/identifiable information. Patient names are only included on consent and assent forms and will be kept in a locked area during and after the study; only the enrolling clinician, study coordinator and principal investigator will ever have access. Electronic data should be password-protected and double-entered at a provincial- or central-level facility. Software will be used for data management and analysis, using coding guides for all study variables.

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 and laboratory data and maintained in secure, password-protected files. During the survey, and at the central coordinating centre, all paper records collected should 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. Other than on the consent form, there should be no possibility to link survey IDs to patient names or any unique identifying characteristics. 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 should be trained in all data entry and management processes, and their training logs should be maintained and archived for data quality assurance checks.

All health provider staff at survey sites should participate in training on the conduct of survey data collection. Personnel should 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 survey subjects and survey 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. Surveillance personnel should 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 should be communicated to all key personnel on a weekly basis for as long as the cross-sectional data collection is under way. Keeping both paper and electronic data will also serve as a secondary check for the accuracy of data.

5.9 Laboratory analyses

5.9.1 RDTs

RDTs should be performed according to the manufacturers' instructions for use.

5.9.2 Molecular characterization

Molecular characterization should be conducted at a laboratory that has experience in malaria molecular and/or serological techniques and subscribes to the WHO external quality assessment scheme for malaria nucleic acid amplification testing (WHO malaria NAAT EQA scheme) *(33)* or other scheme for malaria molecular methods. Quantitative PCR is preferred over non-quantitative nucleic acid amplification methods, especially if parasite density is not being measured by microscopy.

The methods proposed by Cheng *et al. (4)* have been used in many previous studies, but there are other options depending on the reference laboratory.

5.9.2.1 DNA extraction and quality control

Verification of the DNA quality is an important aspect of quality control for detecting gene deletions. If conventional PCR methods are used, an aliquot of the DNA should be used for amplification of single-copy genes, such as *msp1*, *msp2* and *glurp* genes, according to standard published protocols *(34)*. For multiplex quantitative PCR users, amplifications of a single-copy parasite gene and a human gene occur simultaneously with amplifications of *pfhrp2* and *pfhrp3* in one assay as internal controls for DNA quality and DNA extraction efficiency; therefore, no additional controls are required.

5.9.2.2 Molecular species diagnosis

Species-specific PCR, either in separate reactions or in multiplex reactions, should be conducted to detect and confirm Plasmodium species, including *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale* and *P. knowlesi*.

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

Suggested primer sequences, PCR conditions and expected amplification product sizes for conventional PCR have been published by Cheng *et al. (4)*. One of the limitations of this method is that it is a qualitative method and does not detect gene deletions when *pfhrp2*-deleted parasites are mixed with wild-type parasites in the same sample (especially when it is not the predominant strain). A recent review on methods related to *pfhrp2* deletion surveillance discussed the availability of several new molecular methods and their advantages and limitations (see Table 5 below), including various control requirements for reliable identification of gene deletions *(35)*. Among these new methods, multiplex real-time PCR *(22–24)* and digital droplet PCR *(27)* are relevant for obtaining quantitative data and identifying deletions within mixed infections. Ideally, *pfhrp2* and *pfhrp3* genes should be characterized by amplification of two gene segments, namely exon 1 (including the intervening sequences between two exons) and exon 2 (including the intronic region between them). However, there are variations in the methods targeting different parts of the genes. Exon 1 codes for a signal peptide and exon 2 codes for the histidine-alanine-rich repeat region of each protein. PCR assays should include appropriate controls, including DNA from laboratory strains with known deletions, such as DD2 (*pfhrp2*-deleted) and HB3 (*pfhrp3*-deleted). If the *pfhrp2* and/or *pfhrp3* genes cannot be amplified despite good quality of DNA (see section 5.9.2.1), demonstrated by the amplification of other single-copy gene sequences, it suggests that the 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*.

Optional extra: If the *pfhrp2* gene can be amplified, the sequence of the exon 2 amplicon can be determined and translated into an amino-acid sequence. This will enable the classification of the 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.

Table 5. Different PCR assays for detecting *pfhrp2* and *pfhrp3* genes with advantages and limitations (adapted from *(35)*)

Advantages	Limitations
Conventional PCR	
• Can be performed in most molecular laboratories and is widely used in many countries • Can detect deletions of both exons • Can identify deletions of flanking genes	• Time consuming at > 2 hours per reaction and qualitative method requiring visualization of PCR products on agarose gels • Requires multiple different PCR reactions; > 6 reactions per sample for control and *pfhrp2/3* genes and high volume of DNA needed • Nested PCR and higher chances of contamination • Cannot detect gene deletions when gene-deleted and non-deleted parasites are mixed in the same sample
Multiplex real-rime PCR	
• Streamlined workflow with short turnaround time • Quantitative read-out • Can detect mixed infections with gene-deleted and non-deleted strains • Different target genes are multiplexed in a single reaction, requiring less volume of DNA	• Requires multichannel real-time PCR machine • Training is required for proper interpretation of results • Careful optimization required for individual laboratories • May not detect some partial gene deletions involving one exon, as most assays target one exon only
Digital droplet PCR	
• Higher confidence deletion calls than with other molecular methods • Can clearly detect mixed infections with gene-deleted and non-deleted strains	• Requires specialized equipment that is not widely available • Requires advanced laboratory and analysis expertise and training • More expensive than conventional approaches
Sequencing approaches	
• The exact chromosomal location and fragment size of deletions, as well as sequence changes that change codons or affect HRP2/3 expressions, can be mapped • Amplicon-based and whole-genome-based next-generation sequencing methods can be used for parasite population structure and relatedness • Complexity of infection and evolutionary analysis are possible when appropriately sampled data are generated	• More suitable for research studies than for routine programmatic use, as it is expensive and requires advanced laboratory infrastructure and bioinformatics analysis support • Current approaches are not well suited for initial deletion identification, especially in lower parasite density samples

5.9.3 Serology

From DBSs, ultra-sensitive HRP2 and pLDH detection by multiplex bead immunoassay or enzyme-linked immunosorbent assay (ELISA) may be used to support genotyping results and particularly to resolve discordance between RDT and PCR results.

5.10 Data analysis procedures

Following double data entry (see section 5.8) 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 2) will be determined at the domain/provincial level, with 95% CIs estimated for all point estimates.

Domain-level estimates of output 2 will then be disaggregated by age group, sex, village and recent antimalarial treatment to see whether any patterns emerge. Differences between point estimates across sociodemographic or other collected variables can be investigated using X_2 and/or logistic regressions, as desired. This process should follow the tabulation format in the "dummy" table provided (Annex 8).

After completing the laboratory analyses, once the true number of cases of false-negative HRP2 immunoassay results caused by *pfhrp2/3* deletions is known based on molecular analysis, the prevalence of false-negative HRP2-based RDT results due to *pfhrp2/3* gene deletions based on genotyping +/- serology will be estimated among the confirmed *P. falciparum* cases (main output indicator 1), with 95% CrIs for all point estimates, using software package DRpower, accessible via an interactive web app (https://shiny.dide.ic.ac.uk/DRpower-app/). This is based on the formula:

$$\text{Proportion of } \textit{P. falciparum} \text{ cases with false-negative HRP2 RDT results due to } \textit{pfhrp2/3} \text{ deletions} = \frac{\#\text{ of confirmed } \textit{P. falciparum} \text{ cases with } \textit{pfhrp2/3} \text{ gene deletions and HRP2-RDT negative results}}{\#\text{ of confirmed } \textit{P. falciparum} \text{ cases (positive by either Pf-LDH RDT or microscopy)}}$$

The main output indicator 1 can be disaggregated by domain, age group, sex, village and recent antimalarial treatment to see whether any patterns emerge. Differences between point estimates across sociodemographic or other collected variables can be investigated using X2 and/or logistic regressions, as desired. This process should follow the tabulation format in the "dummy" table provided (Annex 8).

In addition, the prevalence of *pfhrp2/3* gene deletions based on genotyping +/- serology will be estimated in the cases with suspected *pfhrp2/3* deletions (main output indicator 3) and can be calculated as follows:

$$\text{Proportion of confirmed } \textit{P. falciparum} \text{ cases with } \textit{pfhrp2/3} \text{ gene deletions among cases with suspected } \textit{pfhrp2/3} \text{ deletions} = \frac{\#\text{ of confirmed } \textit{P. falciparum} \text{ cases with } \textit{pfhrp2/3} \text{ gene deletions and HRP2-RDT negative results}}{\#\text{ of } \textit{P. falciparum} \text{ cases with discordant RDT (HRP2-/ Pf-LDH+) or RDT-microscopy (HRP2-/microscopy+) results}}$$

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

In addition, the final analysis could include:

- the total number of suspected malaria cases screened;
- the RDT positivity rate per health facility;
- the comparative performance of RDTs and RDT test lines for the detection of *P. falciparum*; and
- a parasite density analysis. This can be used for quality control to ensure that false-negative RDT results were not due to low parasite densities, and to ensure that gene-deleted samples had a high enough parasite density to confirm deletions. Parasite densities can also be used to make comparisons between discordant and concordant samples, and gene-deleted and non-deleted samples.

5.11 Dissemination of results

At the end of the study, the principal investigator will submit a report on the study and its main outcomes. This report will be shared with the national malaria control programme and the Ministry of Health and will enable recommendations to be formulated and the Ministry of Health to make informed decisions about whether the current guidelines should be updated. The data will also be shared with the WHO Global Malaria Programme so that they can be included in the Malaria Threats Map (36) and the World Malaria Report. The study report should:

- indicate if the study will be presented during a scientific meeting or published;
- indicate how the results will be disseminated to the study patients; and
- if the study is community-based, mention how the community will be informed and how community participation will be maintained.

6. Study timeline

It should be noted that the amount of time it will take to enrol the desired number of survey participants will depend on: (i) the number of suspected malaria cases seen each week at each facility; (ii) the test positivity rate (i.e. the number of positives per suspected malaria case) at each facility; and (iii) the sample size needed to detect whether the observed prevalence of false-negative HRP2 RDT results caused by *pfhrp2/3* gene deletions is above the 5% threshold (see section 5.4). Prior to implementing this protocol, the national malaria control programme and surveillance teams should assess the expected time needed to enrol the desired number of respondents within each domain and plan accordingly. An example timeline is presented in Table 6; note that the start month will depend on the local transmission context.

Table 6. Illustrative survey timeline

Activities	Year									
	J	F	M	A	M	J	J	A	S	O
Institutional review board approval										
Sampling frame development										
Selection of domains and facilities										
Procurement of all survey supplies										
Community engagement (as needed)										
Recruitment and training of data collectors										
Data collection at facilities										
Data entry										
Laboratory analysis										
Data analysis										
Presentation of findings										

7. Human subjects

7.1 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 should 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 report forms, 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. Consent for long-term storage/biobanking is separate from consent to participate in the survey. In all cases, consent is voluntary, and participants have the right to refuse or withdraw at any time.

Informed consent will be obtained both verbally and in writing from all participants in the preferred local language (Annex 4). As part of the consent process, the survey and biobanking 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 comprehension. It will 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 questions. The consent form will detail the design of the survey and the biobanking 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, if illiterate, provide a thumbprint in conjunction with the signature of an independent witness, depending on national guidelines.

For children under the legal age, consent will be obtained from at least one parent or guardian; this is sufficient given the minimal risk posed. In addition, assent (Annex 5) will be obtained from children, depending on the age of majority and federal guidelines in the country of enrolment, 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 Annexes 4 and 5. 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 to ensure that all participants have consented. A copy of the consent information sheet will be given to each subject and the certificate of consent maintained by the survey team. 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 or if they wish to withdraw their samples from the biobank in the future.

7.2 Risks to human subjects

This survey and biobanking activity is of minimal risk to participants. The amount of blood collected is very small (~100–200 μL), 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 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 to avoid cross-contamination/transmission of infectious agents. Any concerns about potential risks will be mitigated as much as possible, e.g. through community sensitization prior to the survey.

7.3 Protection against risk

The survey and biobanking data collected are not considered to be of a sensitive nature. Therefore, there are minimal risks expected for the participant. Concerning confidentiality, only consenting patients will write their name on the consent form, but this will not appear in any other registries/tally sheets, forms or diagnostic specimens associated with the survey or biobanking process. Steps will be taken to ensure that each study participant's name is protected. There is no linkage between clinic registries (which contain personal information) and survey report forms. DBS/filter paper samples and other samples will be labelled using a survey ID only and consent for biobanking will be indicated directly on the DBS.

The proposed strategy to reduce any risks includes the following:

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

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

3. Ensure 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. Train field supervisors in protocol management. Spot checks by the supervisory staff will provide further assessment of protocol management.

5. Use the most efficacious testing procedures available to ensure sterile and safe biological data collection and testing. The blood for RDTs/microscopy and DBSs will be collected simultaneously.

6. Assess 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/re-training 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 the national protocol.

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

7.4 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 survey/biobanking participant's personal information is protected. All long-term storage of personal data will be labelled with the participant's survey ID. Filter paper samples will be labelled using only a unique survey participant ID number, or barcode, which will only be linkable through the consent form (for consenting individuals), initially by the enrolling clinician and then later by the study coordinator and principal investigator. For the laboratory analyses, there will be no link between the laboratory samples and the participant's identifiable information. All consent forms with survey IDs will be stored in locked cupboards and on password-protected computers accessible only by the study coordinator and the principal investigator. No individual identities will be used in any reports or publications resulting from the study.

7.5 Incentives

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

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Annex 1. Supplemental data analysis to determine the prevalence of *pfhrp2/3* gene deletions

To achieve the survey protocol primary output measures, only samples from patients with suspected *pfhrp2* deletions are prioritized for analysis for *pfhrp2/3* gene deletions. This approach reduces the number of patient samples that need to be transported and analysed by PCR and/or serology. However, as outlined in Table A1.1, there are limitations to this approach, as other malaria suspects with *pfhrp2/3* gene deletions will be missed. If after assessing the resources available to analyse the suspected *pfhrp2/3* deletions, there are funds available, additional analysis can be conducted on a subset of DBSs from HRP2 RDT positive and negative patients (missed by both RDTs or by RDT and microscopy). The former 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* deletions. The latter will determine the presence of *pfhrp2/3* deletions in very low-density infections.

Table A1.1. Supplemental DBS analysis options and associated limitations in detecting *pfhrp2/3* gene deletions

HRP2 RDT	Pf-LDH/ microscopy	Diagnosis	Order of priority for DBS analysis	Interpretation of results and limitations in detecting *pfhrp2/3* deletions
+	+	*P. falciparum*	2	• May be infection with *pfhrp2* deletions but HRP3 was detected by HRP2 RDT • May be multiclonal infection with parasites with and without *pfhrp2/3* deletions
+	–	*P. falciparum*	3	• False-positive HRP2 RDT (or persisting HRP2 after resolution of infection) • May be infection with *pfhrp2* deletions but HRP3 was detected by HRP2 RDT • May be a low-density *P. falciparum* infection that does not result in Pf-LDH reaction with RDT due to low antigen concentration • May be multiclonal infection with parasites with and without *pfhrp2/3* deletions
–	+	*P. falciparum*	1	• False-positive Pf-LDH RDT or microscopy • Low parasite density at limit of detection of RDTs causing variable RDT reactivity
–	–	Negative for malaria	4	• Cannot exclude low-density infection missed by both RDTs, with undetected *pfhrp2/3* deletions. Use PCR to exclude malaria infection.

Annex 2. Alternative *pfhrp2/3* deletion surveys involving immunoassay-based initial screening and molecular confirmation of deletion status: study design, data analysis and reporting considerations

Background

In order to mitigate the threat of *pfhrp2/3* deletions on the programmatic use of HRP2 RDTs, WHO recommended a survey protocol in 2019 to monitor the prevalence of *pfhrp2/3* deletions among symptomatic *Plasmodium falciparum*-infected patients in endemic countries *(1)*. The survey is intended to determine whether the local prevalence of false-negative HRP2 RDT results caused by *pfhrp2/3* gene deletions among symptomatic falciparum patients has reached a threshold (5% or more) that requires a national or subnational change of malaria RDTs *(1)*. This survey method recommended testing suspected malaria cases with an HRP2 RDT and an alternative method (Pf-LDH RDT or microscopy) so that patient samples giving false-negative HRP2 RDT results can be readily identified and prioritized for investigation of potential *pfhrp2/3* gene deletions. Contrary to this recommendation, several studies have attempted to use alternative strategies for identifying pfhrp2/3 deletions. Some studies have utilized immunoassays such as multiplex bead-based assay (MBA) instead of RDTs for identifying samples that are negative or low for HRP2, using a biomarker profile of plasmodial antigens in blood spots collected from other surveys (e.g. health facility-based surveys or therapeutic efficacy studies). Samples that are negative for HRP2/3 but positive for another *P. falciparum* biomarker are identified for molecular investigation of deletions *(2–4)*. Although such surveys may be informative for detecting *pfhrp2/3* deletions and assessing the prevalence of those deletions, the reporting of results from such alternative surveys needs to clearly correlate with false-negative RDT outcomes, which is a critical parameter for assessing the threshold of RDT failure (≥ 5% failure). Another consideration for such alternative surveys must be to include adequate geographical representation in sampling and sufficient sample numbers to cover a domain (refer to sections 4.4 and 4.5 of the master protocol). Here, additional guidance is provided to make use of alternative immunoassay screening methods to collect and report programmatically relevant *pfhrp2/3* deletion survey outcomes.

1. Study design

1.1 Survey design considerations

This alternative survey method is recommended **only when the survey design outlined in the standard protocol cannot be implemented and specimens from other surveys are available for exploratory analysis of *pfhrp2/3* gene deletion** status in a population. The specimens for this alternative survey plan may come from different types of surveys. However, investigators need to make sure that such specimens will be relevant to address the primary outputs proposed in the original protocol. Some important considerations must include the following:

- In the alternative survey, data from quality-assured microscopy or at least one WHO-approved Pf-LDH RDT must be available so that only clinically relevant specimens (microscopy or Pf-LDH positive specimens) from **symptomatic patients** are selected for this survey.
- The sample size and multiple study site requirements for appropriate geographical distribution of samples must be consistent with the WHO guidance in the master protocol (sections 4.4 and 4.5).

- Choose all specimens from symptomatic *P. falciparum* cases included in the study that have microscopy or Pf-LDH RDT data for immunoscreening.

Since the frequency of suspected false-negative HRP2 RDT results among symptomatic patients with *P. falciparum* malaria is a primary output of the protocol, HRP2-negative specimens in the immunoscreening assay will become surrogates for the HRP2 RDT results in the data analysis. Molecular testing on the DBSs from HRP2 immunoassay-negative specimens will determine the prevalence of false-negative HRP2 immunoassay results due to *pfhrp2/3* gene deletions among all symptomatic *P. falciparum* confirmed cases (output 1), and the prevalence of *pfhrp2/3* deletions among symptomatic falciparum patients with a false-negative HRP2 immunoassay result (output 3), using the immunoscreening assay as a surrogate for HRP2 RDT negative results.

1.2 Primary output indicators

The following indicators will serve as the primary survey outputs:

1. Prevalence of false-negative HRP2 immunoassay results caused by *pfhrp2/3* gene deletions (HRP2 negative results in the **immunoassay** will serve as surrogates for HRP2 RDT negative results) among all symptomatic *P. falciparum* confirmed cases.

2. Prevalence of suspected false-negative HRP2 immunoassay results (negative HRP2 **immunoassay result** but positive Pf-LDH or Pf microscopy result) among symptomatic patients with *P. falciparum* malaria.

3. Prevalence of *pfhrp2/3* gene deletions among symptomatic falciparum patients with a false-negative HRP2 immunoassay result (HRP2 negative results in the **immunoassay** will serve as surrogates for HRP2 RDT negative results).

The survey will identify the proportion of patients with suspected false-negative HRP2 **immunoassay** results (**these are surrogates for false-negative HRP2 RDT results**) through immunoassay screening in a host country laboratory or WHO network laboratory. The choice of immunoassay platform will determine what additional biomarker data besides HRP2/3 data can be collected. The sensitivity for detection of biomarkers may vary between assay platforms *(1–8)*, but must be comparable to RDT sensitivity.

Molecular testing to confirm *P. falciparum* infection and identify *pfhrp2/3* deletions will be performed on all specimens that show negative HRP2 immunoassay results. A subset of specimens with different levels of HRP2 expression can be included as controls to confirm *pfhrp2* gene presence. In addition, specimens with low HRP2/3 signals (compared to other biomarkers) can be a sign of potential *pfhrp2/3* deletion and such specimens should also be selected for molecular analysis. If there are discordant diagnostic results (HRP2-/Pf-LDH+) due to false-positive Pf-LDH test lines, this will be identified based on the Pf-LDH data obtained from the multiplex antigen assay. If any specimen is included due to false-positive microscopy data (HRP2-/microscopy+), then this specimen is expected to be negative for all *P. falciparum* biomarkers in immunoassay; this will be confirmed in molecular analysis as well. Other caveats associated with certain interpretations, as explained in section 5.2 of the master protocol, are also applicable here. The advantages and limitations of different molecular assays of choice are also explained in the master protocol (Table 4).

1.3 Secondary output indicators (optional)

1. parasite density, as measured by quantitative PCR and/or microscopy, in patients with false-negative HRP2 immunoassay results (surrogates for HRP2 RDT negatives);

2. improved understanding of *pfhrp2/3* gene deletions, e.g. genetic relatedness and/or drug resistance and fitness, and new and/or improved diagnostic tools for detecting *pfhrp2/3*-deleted parasites; and

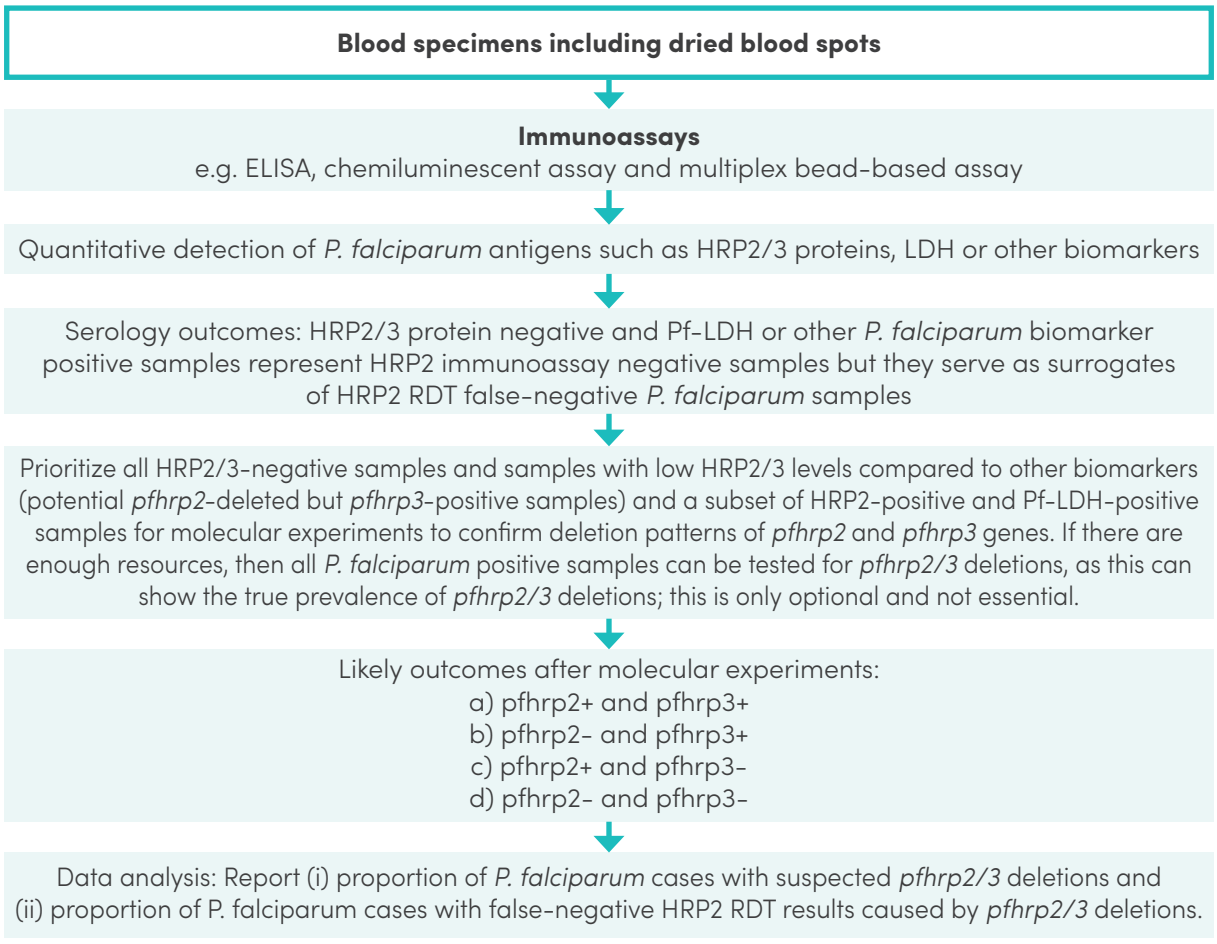
3. prevalence of parasites with *pfhrp2/3* deletions among all symptomatic *P. falciparum* confirmed cases.

The true prevalence of *pfhrp2/3* deletions can be determined only if all specimens included in the surveys are tested using molecular methods. When only HRP2-signal-negative specimens and a subset of HRP2-positive specimens are chosen for molecular analysis, the true prevalence of *pfhrp2* deletions is likely to be underestimated. This is because cross-reactivity between HRP2 and HRP3 will prevent some specimens lacking the *pfhrp2* gene from being selected for testing if only HRP2/3-negative specimens are chosen for molecular analysis.

The rest of the details for data analysis need to be followed as explained in the original protocol. The flow chart below (Fig. A2.1) summarizes important steps for using this alternative immunoassay-based screening to calculate (i) proportion of *P. falciparum* cases with suspected *pfhrp2/3* deletions and (ii) proportion of *P. falciparum* cases with false-negative HRP2 RDT results due to *pfhrp2/3* deletions.

Fig. A2.1. Flow chart

Immunoassay-based initial screening and molecular confirmation of *pfhrp2/3* gene deletion status



2. Immunoassays

Different immunoassays, such as ELISA, MBA and chemiluminescent assay (CLA), have been used for measuring malaria parasite protein biomarkers *(6,9–16)*. Commercial ELISA kits are available for detection of HRP2/3 and pan-pLDH separately. MBA and CLA have the advantage of measuring multiple biomarkers simultaneously, and CLA (Q-Plex™) is commercially produced. MBA is a laboratory-specific assay developed by individual laboratories, and commercial kits are not available for performing this assay. MBA has been widely used by one of the WHO HRP2 surveillance network laboratories for measuring multiple plasmodial biomarkers, including HRP2/3 and Pf-LDH (HRP2 and pLDH are commercially available for use in this assay). These immunoassays can also be used as a screening method in WHO-recommended surveys when the RDT screening method is not an option.

Dried blood spots are extensively used for plasmodial biomarker detection. This is a convenient sample type for storage and transportation. However, different assays may require serum/plasma specimens and investigators need to carefully review what assays will be practical and cost-effective. With MBA or CLA, it is important to measure Pf-LDH in addition to HRP2/3 for confirming the total number of *P. falciparum* infections. In immunoassays, it is important to determine quantitative levels of the antigens.

3. Molecular assays

Prioritize all HRP2/3-negative samples and samples with low HRP2/3 levels compared to other biomarkers (potential *pfhrp2*-deleted but *pfhrp3*-positive samples) and a subset of HRP2-positive and Pf-LDH-positive samples for molecular experiments to confirm deletion patterns of *pfhrp2* and *pfhrp3* genes. Although it is not a requirement, if resources are available, then all *P. falciparum*-positive samples can be tested for *pfhrp2/3* deletions. This will enable determination of the true prevalence of *pfhrp2/3* deletions in the sampling domain.

Different choices of molecular assays and relevant details are provided in the master protocol (section 5.9), and users can follow these guidelines.

4. Data analysis

Assay cut-offs for defining a lack of HRP2/3 proteins must be clearly established. It is important to define in advance criteria for determining the total number of *P. falciparum* cases (e.g. all microscopy positive or Pf-LDH RDT positive cases) and false-negative cases.

There are situations in which the molecular data would conflate the *pfhrp2* deletion estimate:

- Samples infected with more than one strain, wherein the minor clone is wild-type but the major clone is pfhrp2-deleted, may show a positive reaction with an HRP2 RDT and immunoassay.
- If there is residual HRP2 from a previous infection that was cleared, but the current new infection is due to pfhrp2-deleted parasites, the immunoassay and HRP2 RDT can be positive.

In these two scenarios, having Pf-LDH data and/or other parasite biomarker data with molecular data will help to confirm *pfhrp2* deletion status.

- *pfhrp2*-negative but *pfhrp3*-positive samples can be positive in immunoassay and HRP2 RDT testing and *pfhrp2* deletions can only be determined if such specimens are subjected to molecular testing (based on selecting specimens for molecular testing with low HRP2/3 signal relative to other biomarkers).

The following data from the final analysis are critical to report:

1. Based on the number of discordant immunoassay results (HRP2/3-negative, Pf-LDH-positive or other Pf-biomarker-positive) or immunoassay-microscopy discordant results (if only microscopy data are available and no other Pf biomarker data are available), one can calculate the proportion of *P. falciparum* cases with false-negative HRP2 immunoassay results (indicating potential *pfhrp2/3* gene deletions as **surrogate data for HRP2 RDT results**) in the health facility or domain, using the formula below.

$$\text{Proportion of } \textit{P. falciparum} \text{ caseswith suspected } \textit{pfhrp2/3} \text{ deletions} = \frac{\#\text{ of } \textit{P. falciparum} \text{ cases with discordant immunoassay (HRP2-/Pf-LDH+) or discordant immunoassay-microscopy (HRP2-/microscopy+) results}}{\#\text{ of confirmed } \textit{P. falciparum} \text{ cases (positive by either Pf-LDH RDT or Pf-LDH immunoassay results or microscopy)}}$$

2. Once the true number of cases of false-negative HRP2 immunoassay results caused by *pfhrp2/3* deletions is known based on molecular analysis, then, for each sampling domain e.g. province, the primary study outcome can be calculated:

$$\text{Proportion of } \textit{P. falciparum} \text{ cases with false-negative HRP2 immunoassay results due to } \textit{pfhrp2/3} \text{ deletions} = \frac{\#\text{ of confirmed } \textit{P. falciparum} \text{ cases with } \textit{pfhrp2/3} \text{ gene deletions and HRP2 immunoassay negative results}}{\#\text{ of confirmed } \textit{P. falciparum} \text{ cases (positive by either Pf-LDH RDT or immunoassay or microscopy)}}$$

It is important to recognize that the main caveats of using immunoassay detection methods is that they may not accurately reflect the thresholds calculated using HRP2 RDTs in the field, as immunoassays may vary in their sensitivity and specificity relative to RDT sensitivity and specificity. Nevertheless, the outcomes of this alternative approach using immunoassays for screening instead of HRP2 RDTs will be valuable to programmes in understanding the impact of *pfhrp2* deletions with respect to the performance characteristics of HRP2 RDTs.

5. Status of immunoassay availability in endemic countries

Many malaria-endemic countries have capacity to perform ELISA. However, some of the high-throughput immunoassays are commonly used in developed countries, including in the WHO HRP2 surveillance network laboratories. There are ongoing efforts to build capacity and training for rolling out high-throughput immunoassays in malaria-endemic countries. This includes support from Bill & Melinda Gates Foundation for laboratory capacity for genomics surveillance and *pfhrp2/3* deletion

surveillance and from the U.S. President's Malaria Initiative and the U.S. Centers for Disease Control and Prevention for training and laboratory capacity development.

Commercial kits are available for performing ELISA and CLA. However, high-throughput immunoassays such as MBA are custom-developed in research laboratories and occasionally shared with their partnering laboratories in endemic countries. In order to make these methods widely usable for programmes, it is important to have commercial kits available. Alternatively, endemic country staff need to be trained to make the necessary reagents for their use, which will require long-term training, capacity-building and quality assurance support.

6. Source of reagents

Most of the high-throughput immunoassay reagents are commercially available. Details are provided in Jang et al. 2022 *(5)*, Jang et al. 2020 *(13)* and Rogier et al. 2020 *(17)*.

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Annex 3. Mathematical details of the DRpower model used for sample size estimate

Estimating if the prevalence of false-negative HRP2 RDT results caused by *pfhrp2/3* deletions is above a given threshold

The DRpower software implements a Bayesian model of *pfhrp2/3* deletion prevalence. The Bayesian framework has several advantages, including incorporating prior information and propagating uncertainty in a principled way. The model itself is a random effects model *(1)* in which cluster-level prevalence is assumed to vary around the global prevalence (p) according to a beta distribution. The spread around this global prevalence is dictated by the intra-cluster correlation coefficient (ICC), denoted by r. A large value of r implies a wide spread of values because clusters have high correlation within them leading to overdispersion.

Mathematically, we can write:

$$X_i \sim Beta(\alpha,\beta), \qquad \text{(A1)}$$

$$\text{where } \alpha = p(\tfrac{1}{r} - 1),\ \beta = (1 - p)(\tfrac{1}{r} - 1),$$

where X_i is the prevalence in cluster i. The raw counts of the number of deletions in each cluster (k_i) are modelled as binomial, with the number of trials given by the total sample size in that cluster (n_i), and with the probability of success given by the cluster-level prevalence ($X_i = x_i$). The combination of the beta random effects distribution with the binomial distribution means that counts follow an implied beta-binomial distribution. The probability mass function of this distribution can be written as:

$$\Pr(k_i|n_i,p,r) = (\tfrac{1}{r})\ \frac{B(k_i+\alpha,n_i-k_i+\beta)}{B(\alpha,\beta)}, \qquad \text{(A2)}$$

where $B(x,y)$ is the beta function. The likelihood over all c clusters is the independent product of (A2):

$$\Pr(\boldsymbol{k}|\boldsymbol{n},p,r) = \prod_{i=1}^{C} \Pr(k_i|n_i,p,r). \qquad \text{(A3)}$$

Next, priors are defined on p and r. For the prevalence of deletions, p, a uniform prior is assumed over the range [0,1] so as to not preempt the possibility of it being any particular value. For the ICC, r, a *Beta*(1,9) prior is assumed, which places more probability density on small values, typically in the range [0, 0.3]. This prior is based on an analysis of historical *pfhrp2/3* studies, in which the ICC was commonly estimated to be in this range. The marginal posterior distribution of the prevalence is obtained by multiplying the likelihood in (A3) by these priors and integrating out the ICC:

$$\Pr(p|\boldsymbol{k},\boldsymbol{n}) \propto \int_0^1 \Pr(\boldsymbol{k}|\boldsymbol{n},p,r)\ \Pr(p)\Pr(r)dr. \qquad \text{(A4)}$$

This integration is performed numerically in DRpower via a quadrature-based method. The posterior distribution in (A4) can be summarized in various ways, for example in terms of the maximum a posteriori (MAP) estimate and the 95% CrI. To establish whether prevalence is above a given threshold μ (typically 5%), the posterior distribution over all values of p greater than μ can be integrated:

$$\Pr(P > \mu|\boldsymbol{k},\boldsymbol{n}) = \int_\mu^1 \Pr(p|\boldsymbol{k},\boldsymbol{n})dp. \qquad \text{(A5)}$$

If this probability is greater than a predesignated value (0.95 by default), we conclude that prevalence is above μ; otherwise, we conclude that prevalence is below μ. This is a Bayesian hypothesis test, meaning that it directly compares two competing hypotheses rather than trying to disprove a single null hypothesis.

Power and sample size calculation

Unfortunately, there is no simple formula for statistical power under this model. Instead, a simulation is undertaken as follows:

1. Define the global prevalence of deletions (p), the ICC (r) and the number of clusters (c).
2. Define the per-cluster sample size (n) to be the same in all clusters ($n_i = n$).
3. Simulate a data set of deletion counts ($\boldsymbol{k}$) from the beta-binomial model described above using these values of p, r, c, n.
4. Analyze this simulated data set using the Bayesian framework above. Determine if the correct conclusion is reached, i.e. that $p > \mu$ if this is indeed the case.
5. Repeat steps 3–4 many times (typically 1000 or more) to establish the proportion of times the correct conclusion is reached. This is referred to as the **empirical power**.
6. Repeat steps 2–5 over a range of values of n. Construct an empirical power curve and find the value of n at which power crosses the target level, typically 80%. This is the minimum sample size n_{opt} for this parameter combination.
7. Repeat steps 1–6 for a range of parameter combinations to produce sample size tables.

Table 2 in the protocol was produced using this method, assuming a fixed value of $r = 0.05$. This value is based on an analysis of historical *pfhrp2/3* deletion studies, in which the mean estimate of the ICC is around this level. Note that the sample size tables produced using this method are only valid if the intention is to analyze data using the DRpower Bayesian model. If the plan is to use a different analysis approach, then a different power and sample size analysis is required.

Reference

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Annex 4. 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 and should be translated into the local language)

Name of proposal and version: _____

Survey ID: Label placed here

This informed consent form is for adults over [age of majority] years who attend [name of site], who have been invited to participate in a survey for *pfhrp2/3* deletions and biobanking for future research

Name of principal investigator: _____

Name of organization: _____

Name of sponsor: _____

Survey sites: _____

This informed consent form has two parts:

I. Information sheet (to share information about the study with you)

II. Certificate of consent (for signatures if you agree to take part). You will be given a copy of the information sheet

Part I: Information sheet

I am _____ and I work with the National Malaria Control Programme. Today, you (or your child) are invited to participate in a research study to better understand if the malaria parasite is changing over time and affecting how well rapid diagnostic tests are working in this country. You (or your child) are being asked to participate in this study because you have presented with symptoms suggesting that 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. A copy of this form will be provided to you. Your (or your child's) participation is completely voluntary and will in no way affect the treatment and care you receive for malaria or any other condition.

Why is this survey being done?

We are conducting this research survey because we want to look at samples of blood from people who we suspect could have malaria and then use the blood samples to see if the malaria parasite is changing over time and affecting the way malaria tests are working. A false-negative test result can mean that there is a problem with the test or that the malaria parasites have changed in a way that can make them hard to detect. We will ask you a few questions and perform some additional tests to determine if you have malaria. We will ask you if we can store your samples for future malaria research.

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 basic questions such as your age, what village you live in, and the tests and medicines you have taken for malaria in the past few weeks. When we prick your finger to do the routine malaria rapid diagnostic test, we will also take a few extra drops to do an additional malaria test today and put drops onto paper for further testing to see whether the malaria parasites have changed in ways that make them hard to find. These tests will be carried out in a central reference laboratory either in country or overseas in one of the World Health Organization reference laboratories. There may be some leftover blood after we conclude the routine testing and survey. Instead of discarding it, we would like your permission to store any such leftover blood at [name of laboratory] for [number of years]; afterwards it will be destroyed. We may use it only for malaria-related studies in the future, particularly those that support the development of new diagnostic tests for malaria. Your materials will not be sold, and use of them will only be authorized by a national and/or institutional research ethics committee.

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 additional 10–15 minutes added to your visit today to complete the questions. This is a one-time survey and there will be no follow-up visits.

A second risk could be that someone outside the study team accesses your information; this is rare because we will not record your name (or your child's name) on any survey forms or your samples that are sent to the laboratory(s).

What are the benefits of the study?

The benefit of taking part in this study is that today we will do an extra test for malaria and this will improve the chances of finding malaria if it is in you. Also, if you agree to donate your leftover blood, then your participation may result in public health programmes having better tests for malaria in the future and understanding 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 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, blood samples or in any publications; we will replace it with an identification number. Only those taking your consent today and the principal survey investigator will be able to link your name to your survey identification number. The consent forms bearing your name and signature will be kept stored in locked cupboards and on password-protected computers. The people responsible for the long-term storage of your (or your child's) sample will not have your name.

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

You do not have to participate in this survey, nor do you need to permit us to store your leftover samples for future research. If you do not want to, there will be no penalty to you. You can withdraw yourself (or your child) from participating at any time without penalty, and you (or your child at age of majority) can also request that any leftover blood samples be withdrawn from long-term storage.

For parents providing consent on behalf of children: If you do consent for long-term storage of your child's leftover blood samples, we advise that, when your child reaches maturity, you give him/her the certificate of consent, which includes his/her survey ID number and contact information for the principal investigator, in case he/she wishes to have those materials removed from storage and use.

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

If you have any questions, you can contact the principal investigator: [name] at [telephone number]. If you have any questions about your rights, or if you want to talk with someone who is not part of this research project, please contact [name] and [address].

Survey ID: Label here

Part II: Certificate of consent

I have been invited to participate in a study that aims to better understand if the malaria parasite is changing over time and affecting how well rapid diagnostic tests are working in this country.

I have read the above information, or it has been read to me. I have had the opportunity to ask questions, and any questions that I have asked have been answered to my satisfaction. I consent voluntarily to participate in this study.

Print name of participant: _____

Signature of participant: _____

Date: _____
dd/mm/yyyy

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 on malaria. I understand that I can change my mind to not have my filter paper blood sample stored and used for future research.	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's signature: A witness's signature and the patient's thumbprint are required only if the patient is illiterate. In this case, a literate witness must sign. If possible, this person should be selected by the participant and should have no connection with the study team.

I have witnessed the accurate reading of the consent form to the potential participant, who has had the opportunity to ask questions. I confirm that the participant has given consent freely.

Print name of witness: _____ and thumbprint of participant:

Signature of witness: _____

Date: _____
dd/mm/yyyy

Long-term storage and future studies: I have witnessed the accurate reading of the request for long-term storage of samples for future studies on malaria. I understand that the participant can change his/her mind to not have the filter paper blood sample stored and used for future research. The potential participant has had the opportunity to ask questions. I confirm that the participant agrees:	If you agree, circle "YES," if you do not agree, circle "NO".	
	YES	NO

Investigator's signature: I have accurately read or witnessed the accurate reading of the consent form to the potential participant, who has had the opportunity to ask questions. I confirm that the participant has given consent freely.

Print name of investigator: _____

Signature of investigator: _____

Date: _____
dd/mm/yyyy

A copy of the information sheet with the survey ID affixed has been given to the patient. _____
(initials of the principal investigator or assistant)

Annex 5. Informed assent form (template) for children between 7 and 15 years who are invited to participate in a survey for *pfhrp2/3* deletions and biobanking for future research

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

Name of proposal and version: _____

Survey ID: Label placed here

Name of principal investigator: _____

Name of organization: _____

Name of sponsor: _____

Survey sites: _____

This informed consent form has two parts:

I. Information sheet (to share information about the study with you)

II. Certificate of assent (for signatures if you agree to take part)

Part 1: Information sheet introduction

My name is _____ and my job is to do research to see if the malaria parasite is changing over time and affecting the way malaria tests are working.

I am going to give you information and invite you to be part of a research study. You can choose whether or not you want to participate. We have discussed this research with your parent(s)/guardian and they know that we are also asking for your agreement. If you are going to participate in the research, your parent(s)/guardian also have to agree. But, if you do not wish to take part in the research, you do not have to, even if your parent(s)/guardian agreed.

You may discuss anything in this form with your parents or friends or anyone else you feel comfortable talking to. You can decide whether to participate or not after you have talked it over. You do not have to decide immediately.

There may be some words you don't understand or things that you want me to explain more about because you are interested or concerned. Please ask me to stop at any time and I will take time to explain.

Purpose: Why are you doing this research?

Sometimes malaria parasites can change over time and we want to be sure that the malaria tests we use are working well – giving a positive result when malaria is present and a negative result when malaria is not present. In order to find out, we have to test people's blood using different kinds of malaria tests.

We also want your permission to save and store any of the leftover blood to use it to do more research to help us to make better tests for malaria and to understand how malaria is changing over time.

Choice of participants: Why are you asking me?

Children, like you, get sick more often than adults from malaria. Therefore, it is really important to include children in this research.

Participation is voluntary: Do I have to do this?

You don't have to be in this research if you don't want to be. It's up to you. If you decide not to be in the research, it's okay and nothing changes. This is still your clinic; everything stays the same as before. Even if you say "yes" now, you can change your mind later and it's still okay.

I have checked with the child and he/she understands that participation is voluntary _____ (initial)

Procedures: What is going to happen to me?

Today, we are going to test you for malaria just the same way as we do normally. However, we will collect a few extra drops of blood to do additional tests for malaria today and store some on a little piece of paper to do other tests later. We would then like to store any leftover blood to use it for research in the future.

I have checked with the child and he/she understands the procedures _____ (initial)

Discomforts: Will it hurt?

There will be a bit of discomfort when we prick your finger for blood, but it will only last a few moments.

I have checked with the child and he/she understands the risks and discomforts _____ (initial)

Benefits: Is there anything good that happens to me?

By doing more than one test for malaria, we have a better chance of finding it and getting you on the treatment. Also, if you agree to donate your leftover blood, then your participation may someday result in your community having better tests for malaria in the future and understanding if the malaria parasite is changing over time.

I have checked with the child and he/she understands the benefits _____ (initial)

Reimbursements: Do I get anything for being in the research?

We do not offer any money or gifts for participating.

Confidentiality: Is everybody going to know about this?

We will not tell other people that you are in this research and we won't share information about you to anyone who does not work in the research study.

Any information about you will have a number on it instead of your name. Only the researchers will know what your number is and we will lock that information up with a lock and key.

Sharing the findings: Will you tell me the results?

As part of the research, we are doing two tests for malaria on your blood at the same time today, so we will let you and your parent(s)/guardian know the results of both of these tests immediately. The other tests to look at the parasite will be done elsewhere in the country or abroad, and these results will be shared with the government and other people like scientists to help them make decisions about the best test to use for malaria.

Right to refuse or withdraw: Can I choose not to be in the research? Can I change my mind?

You do not have to be in this research, or you can agree to be part of this research but not allow us to store your leftover blood for future research. No one will be mad or disappointed with you if you say no. It's your choice. You can say "yes" now and change your mind later and it will still be okay.

Who to contact: Who can I talk to or ask questions to?

You can ask me questions now or later. You can ask the nurse questions. I have written a number and address where you can reach us or, if you are nearby, you can come and see us. If you want to talk to someone else that you know, like your teacher or doctor or auntie, that's okay too.

If you choose to be part of this research, I will also give you a copy of this paper to keep for yourself. You can ask your parents to look after it if you want.

You can ask me any more questions about any part of the research study, if you wish to. Do you have any questions?

Part 2: Certificate of assent

I understand this research is to understand if the malaria parasite is changing over time and affecting the way malaria tests work. I understand that I will get a finger prick for two malaria tests today and then a few drops on paper will be taken to use for other malaria research in the future.

I have read this information (or had the information read to me). I have had my questions answered and know that I can ask questions later if I have them.

I agree to take part in the research.

OR

I do not wish to take part in the research and I have not signed the assent below. _____ (initialled by child/minor)

<u>Only if child assents:</u>

Print name of child _____
Signature of child: _____
Date: _____
dd/mm/yyyy

Long-term storage

Long-term storage and future studies: I agree to allow the study team to store my blood sample for future studies on malaria. I understand that I can change my mind to not have my filter paper blood sample stored and used for future research.	If you agree, circle "YES," if you do not agree, circle "NO".	
	YES	NO

Only if child assents:

Print name of child _____
Signature of child: _____
Date: _____
Day/month/year

If illiterate:

A literate witness must sign (if possible, this person should be selected by the participant, not be a parent, and should have no connection to the research team). Participants who are illiterate should include their thumbprint as well.

I have witnessed the accurate reading of the assent form to the child, and the individual has had the opportunity to ask questions. I confirm that the individual has given consent freely.

Print name of witness (not a parent) _____ ***AND thumbprint of participant***
Signature of witness _____
Date _____
Day/month/year

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

Print name of researcher _____
Signature of researcher _____
Date _____
Day/month/year

Statement by the researcher/person taking consent

I have accurately read out the information sheet to the potential participant and to the best of my ability made sure that the child understands that the following will be done:

1. **finger prick for malaria test and blood spot on filter paper**
2. **long-term storage of the filter paper for future research**

I confirm that the child was given an opportunity to ask questions about the study, and all the questions asked by him/her have been answered correctly and to the best of my ability. I confirm that the individual has not been coerced into giving consent, and the consent has been given freely and voluntarily.

A copy of this assent form has been provided to the participant.

Print name of researcher/person taking the assent _____

Signature of researcher/person taking the assent _____ Date _____
Day/month/year

Copy provided to the participant _____ (initialled by researcher/assistant)

Parent/guardian has signed an informed consent _____ Yes _____ No _____ (initialled by researcher/assistant)

Annex 6. *pfhrp2/3* gene deletion survey facility tally sheet

This sheet will be filled out by all facilities. In each domain, once 300 individuals[1] with *P. falciparum* malaria have been seen (30 at each of 10 enrolment sites per domain), calculate the proportion of discordant diagnoses (i.e. Pf-LDH- or microscopy-positive AND HRP2 RDT-negative) among all positive *P. falciparum* diagnoses, i.e. by Pf-LDH RDT or microscopy. When centrally compiled, section 5.6 points 6 and 8 can be used to interpret the results and determine the associated actions after statistical analysis of the molecular +/- serological confirmation data.

A	B	C	D	E	F	G
	Patient ID	**Date of visit / test (DD/MM/YY)**	**Informed consent/ assent**	**Pf case confirmed by Pf-LDH RDT/ microscopy**	**Cumulative number of consenting Pf positive cases (from column E)**	**Suspected false-neg HRP2 (Pf-LDH or microscopy positive AND HRP2 RDT negative)**
1			Y / N	Y / N/ NA		Y / N/ NA
2			Y / N	Y / N/ NA		Y / N/ NA
3			Y / N	Y / N/ NA		Y / N/ NA
4			Y / N	Y / N/ NA		Y / N/ NA
5			Y / N	Y / N/ NA		Y / N/ NA

* Expand rows as needed

Total suspected malaria cases tested (column A) _____

Tally total of *P. falciparum* cases detected and enrolled per facility (column F equals total confirmed *P. falciparum* cases enrolled).

Number of suspected false-negative HRP2 RDT results (column G – sum of "yes" responses) **(a)**

Total number of positive *P. falciparum* diagnoses by Pf-LDH RDT or microscopy (it is important to establish which method will be used for counting Pf cases before the study is undertaken, as this is an important criteria for defining the total number of Pf cases enrolled in the study) (last entry column F) **(b)**

Percentage of all *P. falciparum* cases with suspected false-negative HRP2 RDT results that need molecular +/- serological analysis for *pfhrp2/3* deletions = **(a / b)**

Target: 30 *P. falciparum* cases detected and enrolled per facility (last entry column F)

1 This number may vary according to overall sample size of the survey and the number of clusters selected.

Annex 7. Survey case report form

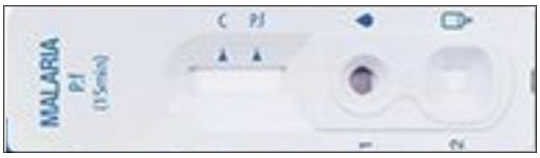
Note: This is an example survey case report form. It may have to be amended to correspond with the RDTs used in the survey.

Note: Each survey form should be pre-labelled chronologically and there should be sufficient labels to place on RDTs, DBSs and plastic bags. Ideally, the *form should be produced in duplicate*.

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 (N/A).

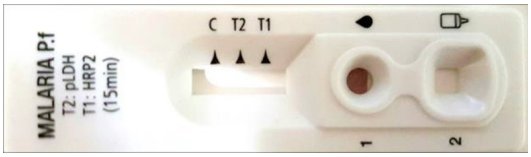
<table>
<tr><th colspan="3">To be completed prior to participant interview</th></tr>
<tr><td>1.</td><td>Barcode/patient ID</td><td>Place label</td></tr>
<tr><td>2.</td><td>Health centre</td><td>Pre-entered for each health centre on printed form or combined with survey ID</td></tr>
<tr><td>3.</td><td>Name of health worker/lab assistant</td><td></td></tr>
<tr><td>4.</td><td>Date of visit</td><td>Day _____ Month _____ Year _____</td></tr>
<tr><td>5.</td><td>Pre-entered for each health centre on printed form:

RDT 1 (must include HRP2 – national programme RDT)

a. Name:
b. Product code:
c. Lot number:
d. Expiry date:
e. Target antigens:
1. T1:</td><td> 

<table><tr><td></td><td>Box 1</td></tr><tr><td>Control</td><td>Pf HRP2</td></tr><tr><td>+ / –</td><td>+ / –</td></tr></table>Circle correct result in each box above.

Circle result of RDT: 1. Negative
2. P. falciparum</td></tr>
<tr><td>6.</td><td>RDT 2 (survey RDT)

a. Name:
b. Product code:
c. Lot number:
d. Expiry date:
e. Target antigens:
1. T1:
2. T2:
3. T3:</td><td> 

<table><tr><td></td><td>Box 2</td><td></td></tr><tr><td>Control</td><td>T2 Pf-LDH</td><td>T1 HRP2</td></tr><tr><td>+ / –</td><td>+ / –</td><td>+ / –</td></tr></table>Circle correct result in each box above.

Circle result of RDT: 1. Negative
2. P. falciparum</td></tr>
<tr><td>7.</td><td>a. Is RDT 1 positive for P. falciparum?
b. Is RDT 2 positive for P. falciparum?</td><td>Y / N

Y / N
If YES to EITHER question, provide treatment.</td></tr>
</table>

8.	DBS taken?	a. Y / N b. Consent for long term storage Y/N
9.	Microscopy	

Microscopy	Positive/ Negative/ N/A	Species	Parasite count (parasites per microlitre)	Initials of microscopist
Field health facility				
National laboratory cross-check (read 1)				
National laboratory cross-check (read 2)				

	To be obtained from each malaria suspect	
10.	Age in years	_____
11.	Sex	a. M b. F
12.	Village where malaria suspect resides	_____ *From pre-populated list if possible*
13.	In the past two weeks, have you had a test for malaria?	a. No → Go to question 15 b. Yes
14.	What was the result of the test?	a. Positive b. Negative
15.	In the past two weeks, have you taken any medicine for malaria?	a. No → Go to question 17 b. Yes
16.	Antimalarial medicine taken	From pre-populated list d. ACT (whichever ACT is first-line drug in the country) e. Other ACTs (could be other names for first-line drug) f. Sulfadoxine/pyrimethamine g. Quinine h. Paracetamol (antipyretics available in country) i. Other _____
17.	Have you travelled to another locality of country in the past 30 days?	a. No → End b. Yes → Go to question 18
18.	Where did you travel?	a. Country _____ b. Region _____ c. District _____ d. City/Village _____ e. N/A

FOR SUPERVISOR USE ONLY		
S1	a. Is box 1 negative? b. Is box 2 positive?	Y / N Y / N If YES to part a and part b, the result is discordant.
REFERENCE LABORATORY USE ONLY		
17a.	Molecular analysis (Indicate the method chosen and make sure all the assay controls and analysis procedures are correctly followed)	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 exon 1 – 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
17b.	Molecular method used	a. Conventional PCR b. Multiplex PCR c. Digital PCR
18a.	Serology (indicate the method chosen and relevant controls used)	a. pfhrp2+/pan-LDH+ b. pfhrp2-/pan-LDH- c. pfhrp2+/pan-LDH- d. pfhrp2-/pan-LDH+
18b.	Method of serological analysis	a. Bead-based immunoassay b. ELISA

Annex 8. Tabulation plan for prevalence of *pfhrp2/3* deletions

Characteristic	Suspected false-negative HRP2 RDT prevalence[b] (n=XX) (95% CI)	Confirmed *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		
Domain (e.g. province, state)		
Domain 1		
Domain 2		
Domain 3		
Domain 4		
Domain 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		

[a] Tabulations are based on *pfhrp2/3* deletion screening only in *P. falciparum* cases with discordant results. If all *P. falciparum* 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 = # of discordant results (HRP2-negative & Pf-LDH- or microscopy-positive) / all *P. falciparum* cases confirmed by Pf-LDH RDT or microscopy as established under the protocol for this study.

[c] *pfhrp2/3* deletion prevalence = # of *P. falciparum* cases with *pfhrp2/3* deletion causing false-negative HRP2 RDT results / total # of *P. falciparum* cases (Pf-LDH RDT-positive or microscopy-positive)

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