



# False-negative RDT results and *P. falciparum* histidine-rich protein 2/3 gene deletions

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INFORMATION NOTE

## TARGET READERSHIP

National malaria control programme managers and their implementing partners, procurement agencies, national regulatory authorities for in-vitro diagnostics and manufacturers of malaria rapid diagnostic tests (RDTs).

## PURPOSE

To provide updated information on the implications of reports of histidine-rich protein 2/3 (*pfhrp2/pfhrp3*) gene deletions in *Plasmodium falciparum* parasites for case management and to advise on procedures for investigating suspected false-negative RDT results.

## BACKGROUND

Most of the currently available commercial RDT kits work by detecting a specific protein expressed only by *P. falciparum*, called HRP2, in the blood of people infected with falciparum malaria. The antibodies on the test strip recognize the HRP2 antigen but may cross-react with protein expressed by another member of the HRP gene family, *pfhrp3*, because of the strong similarity of the amino acid sequence. The general preference for HRP2-based RDTs in procurement is due largely to the finding in some studies that they are more sensitive and heat-stable than RDTs that detect other malaria antigens, such as plasmodium lactate dehydrogenase (pLDH) – pan (all species) or *P. falciparum*-specific – or aldolase.

In certain situations, HRP2-detecting tests are less sensitive, particularly for parasites that express little or no target antigen, resulting in a false-negative result. In 2010, Gamboa et al.<sup>1</sup> reported the first confirmed identification of *P. falciparum* parasites with *pfhrp2/pfhrp3* gene deletions, which expressed neither HRP2 or HRP3, in the Amazon River basin in Peru. Subsequent retrospective analyses<sup>2</sup> at different sites in the Loreto region of the Peruvian Amazon showed a statistically significant increase in the number (and percentage) of parasites with gene deletions between specimens collected in 1998–2001 (20.7%) and in 2003–2005 (40.6%). The prevalence of parasites with *pfhrp2/pfhrp3* deletions varies, however, from locality to locality. Publications followed from other countries, such as India, Mali and Senegal, but with much lower prevalence estimates, and some studies were based on a flawed design and/or had incomplete analyses.<sup>3</sup> There have been no reports of parasites failing to express pLDH or aldolase, the other antigens targeted by malaria RDTs, as these targets are essential enzymes for parasite metabolism and survival.

In light of reports of HRP2 deletions in parasites in several African countries, including the Democratic Republic of the Congo,<sup>4</sup> Eritrea,<sup>5</sup> Ghana,<sup>6</sup> Kenya,<sup>7</sup> Rwanda<sup>8</sup> and India,<sup>9</sup> WHO is providing guidance and periodic updates to RDT manufacturers, procurers, implementers and users on confirming (or excluding) new geographical foci of parasites with deleted *pfhrp2/pfhrp3* and on investigating other causes of suspected false-negative RDT results.

This update specifically includes revisions to reflect the results of round 8 of WHO malaria RDT product testing (<https://www.who.int/malaria/publications/atoz/9789241514965/en/>); ad hoc WHO testing of a selection of RDTs against *pfhrp2/3* deleted parasite panels; the WHO survey protocol template for determining the prevalence of *pfhrp2/3* deletions causing false negative RDT results (<https://www.who.int/malaria/publications/atoz/hrp2-deletion-protocol/en/>), and the Malaria Threat Maps (<http://apps.who.int/malaria/maps/threats/>).

## POTENTIAL CAUSES AND INVESTIGATIONS INTO SUSPECTED FALSE-NEGATIVE RDT RESULTS

In most settings, genetic mutations like deletion of *pfhrp2/pfhrp3* in parasites are not likely to be the main cause of false-negative results in RDTs, and more studies are required to determine the true prevalence of these mutations. False-negative RDT results are more likely to be due to the procurement and use of poor-quality RDTs or use of the wrong comparator for the diagnostic test, such as poor-quality microscopy for cross-checking negative RDT results.<sup>10</sup> Poor transport and storage conditions for RDTs, with sustained exposure to high temperature, can affect their diagnostic performance. More rarely, operator errors during performance and/or interpretation of RDT results can result in false-negative results. Table 1 lists the product, operator, supply chain, host and parasite factors that can lead to false-negative RDT results and suggested means to investigate such cases. Many of the potential causes of false-negative results can be prevented or minimized by procuring good-quality RDTs, by improving the quality control of procured RDTs (lot verification) and by good training of users.

TABLE 1.

## Causes of false-negative RDT results and investigative actions

CLASSIFICATION	CAUSE OF FALSE-NEGATIVE RDT RESULT	SUGGESTED ACTIONS
<b>Operator factors</b>	Operator error in preparing the RDT, performing the test or interpreting the result	Verify whether RDTs are used by untrained staff; assess RDT competence on site.
<b>Use of an imperfect "gold standard" as a comparator</b>	Thick or thin films from a patient with a negative RDT result are incorrectly interpreted as "positive" by microscopy.	Verify microscopy procedures and interpretation by a qualified microscopist.
<b>Product design or quality</b>	Poor sensitivity of an RDT due to poor specificity, affinity or insufficient quantity of antibodies. Poor packaging can result in exposure to humidity, which will rapidly degrade RDTs.	Inspect the instructions for errors; inspect the integrity of the packaging, including the colour indicator desiccant for evidence of moisture. Cross-check suspected false-negative RDT results against microscopy performed by two qualified microscopists or, if microscopy is not available, against a high-quality non-HRP2-detecting RDT; retrieve RDTs from affected areas, and send for lot testing to WHO-recognized laboratories.*
<b>Transport or storage conditions</b>	Poor visibility of test bands due to strong background colour on the test Incorrect instructions for use	Assess RDT performance and training on site; if the strong background colour persists, notify the manufacturer. Review the instructions for use for accuracy.
<b>Parasite factors</b>	Antibody degradation due to poor resistance to heat or incorrect transport or storage, e.g. exposure to high temperatures, freeze-thawing Parasites lack or express low levels of the target antigen, i.e. HRP2	Inspect temperature monitoring of RDT transport and storage chain to determine whether temperatures exceed maximum storage temperature, typically 30 °C or 40 °C or < 2 °C. If temperatures are not within those in the manufacturers' instructions, send the RDTs to the WHO lot testing laboratory. * Train health workers to respect storage conditions, and improve storage places (e.g. add fans). Patient samples are negative on an HRP2 test line of at least two quality-assured malaria RDTs <b>and</b> positive on the pan- or pf-PLDH test line when a combination RDT is used <b>and</b> the sample is confirmed to be positive microscopically for <i>P. falciparum</i> by two qualified microscopists. If these conditions are met, place fresh blood samples or dried blood spots (50–60 µL) on e.g. Whatman® 3MM filter paper or other collection cards, in frozen storage (-20 °C) until shipment for PCR and <i>pfhrp2/pfhrp3</i> gene analysis.
<b>Host parasite density</b>	Variation in the amino acid sequence of the epitope targeted by the monoclonal antibody Very low parasite density or target antigen concentration Very high parasite load (severe malaria) causing prozone effect (hyperparasitaemia and antigen overload)	Repeat test with an RDT of a different brand or different manufacturer that targets the same antigen or an RDT that targets a different antigen, e.g. pan-PLDH or Pf-PLDH. Manufacturers may use monoclonal antibodies that target different epitopes of the same antigen. Perform high-quality microscopy and record the parasite count; if high-quality microscopy is not available, repeat the RDT if symptoms persist. Repeat testing with a 10 x and if needed a subsequent 50 x dilution of the sample, with dilutions in 0.9% NaCl **

**Notes:**\* Information about lot testing can be found here: <http://www.who.int/malaria/areas/diagnosis/rapid-diagnostic-tests/evaluation-lot-testing/en/>\*\* Gillet et al. Prozone in malaria rapid diagnostics tests: how many cases are missed? *Malar J* 2011;10:166. <https://doi.org/10.1186/1475-2875-10-166>

Thousands of febrile children with negative RDT results have been followed up in several studies,<sup>11,12</sup> which showed no malaria-related deaths or hospitalizations. In many endemic areas, malaria prevalence rates have fallen to low levels, and the majority of accurately performed RDTs give negative results. Treatment of individuals with negative RDT results promotes drug resistance, wastes resources and can delay diagnosis of non-malaria causes of fever. In some circumstances, however, false-negative RDT results should be suspected, and an investigation should be carried out to determine the quality of the RDTs, the competence of the operator and/or the presence of *hrp2/hrp3* deletions.

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#### **When should false-negative RDT results be suspected for individual patients?**

- A symptomatic patient with an initially negative RDT who presents with persistent signs or symptoms of malaria and repeated negative RDT results but a positive blood film interpreted by a qualified microscopist or a positive result with a different quality-assured RDT that targets a different falciparum-specific malaria antigen (e.g. pf-pLDH) or is of the same brand but from a different lot.
- A patient with signs or symptoms of malaria with a negative HRP2-based RDT result, who recently visited an area that is known to have a high prevalence of *pfhrp2/hrp3*-deleted parasites, such as Eritrea and Peru.

#### **When should false-negative RDT results be suspected for a population living in a certain geographical area?**

- Discordance between RDT and microscopy results, with  $\geq 10$ –15% higher positivity rates by microscopy and routine quality control by cross-checking or when both tests are performed on the same individuals (e.g. during surveys).
  - The national malaria control programme and/or the RDT manufacturer receives multiple formal complaints or anecdotal evidence of RDTs returning inaccurate results.
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## **WHEN AND HOW SHOULD FALSE-NEGATIVE HRP2-DETECTING RDT RESULTS DUE TO SUSPECTED PFHRP2 DELETION BE INVESTIGATED?<sup>13</sup>**

A *pfhrp2* deletion should be strongly suspected if a patient sample gives negative results on an HRP2 test line of at least two quality-assured malaria RDTs<sup>14</sup> **and** positive on the pan- or pf-pLDH test line when a combination test is used, **and** the sample is confirmed microscopically to be positive for *P. falciparum* by two qualified microscopists.

If a *pfhrp2* gene deletion is suspected and the conditions described above are met:

- Immediately inform the National Malaria Control Programme and WHO;
- Archive the labelled RDTs and slides in a dry, clean area;

- Collect at least two separate blood drops (50 µL x 2) onto filter paper (e.g. Whatman® 3MM) or appropriate collection cards optimized for DNA analysis;<sup>15</sup> air-dry filter paper or cards overnight in a clean environment, sealed in air-tight plastic bags with desiccant.<sup>16</sup>
- Confirm the presence of *P. falciparum* infection by PCR analysis according to established protocols and with appropriate standards and quality control measures.
- If PCR is positive, confirm *pfhrp2/hrp3* gene deletion by PCR and antigen analysis at laboratories experienced in this kind of assay. WHO/GMP can facilitate linkages with such laboratories and provide further guidance. Contact: Malaria\_rdt@who.int, with the subject line: “Laboratory support for investigations into suspected *pfhrp2/3* gene deletions”.

## SURVEYS AND SURVEILLANCE OF PFHRP2/HRP3 DELETIONS

Attributing false-negative results to *pfhrp2/pfhrp3* deletion has significant implications for public health. Alternative RDTs will have to be procured, and case management decisions will have to be revised, with re-training in algorithms and RDTs. Therefore, all investigations must be carried out systematically and accurately.

Following confirmation of *pfhrp2* deletions in initial case investigations and/or other sources e.g. published reports, the affected country and neighbouring countries should conduct a baseline survey to determine the prevalence of *pfhrp2/3* deletions. WHO has developed survey protocol templates (<https://apps.who.int/iris/bitstream/handle/10665/260140/WHO-CDS-GMP-2018.03-eng.pdf>) to determine the prevalence of *pfhrp2/3* deletions causing negative HRP2 RDTs amongst symptomatic patients and based on the estimated prevalence whether a change in diagnostic strategy or ongoing surveillance/repeat survey is indicated. This protocol includes a sampling tool, case report forms and consent/assent forms.

## ALTERNATIVES TO HRP2-BASED RDTs

If *pfhrp2* deletions causing negative HRP2 RDTs are found to be prevalent among symptomatic individuals (lower 95% confidence interval is > 5%), as, e.g. in Eritrea and several countries in South America (Brazil, Colombia, Peru), country programmes will have to switch to RDTs that do not rely exclusively on HRP2 for detecting *P. falciparum*. A threshold of 5% was selected because it somewhere around this point that the proportion of cases missed by HRP2 RDTs due to non-*hrp2* expression may be greater than the proportion of cases that would be missed by less-sensitive pLDH-based RDTs. A recommendation to switch is further informed by mathematical models that show whether parasites lacking *pfhrp2* genes will spread<sup>17</sup> under HRP2-only RDT pressure; a switch may also be decided because of the complexity of procuring and training in use of multiple RDTs. Any change should be applied nationwide, although roll-out might be prioritized on the basis of the prevalence of *pfhrp2* deletions.

Until recently, the laboratory evaluation component of the WHO prequalification process, also known as WHO product testing, assessed RDTs only against

*P. falciparum* culture and clinical samples that express HRP2. This was particularly problematic for assessing the performance of products in which HRP2 and pf-pLDH are on the same test line but also it assumed that pf-LDH and pan-LDH detecting RDTs would perform similarly against HRP2 expressing and non-expressing parasites. To address this problem and test this assumption, WHO and collaborators established a panel of wild-type and cultured single and double *pfhrp2/3* deleted parasites for round 8 of the WHO malaria RDT product testing programme,<sup>18</sup> the results are summarized in Table 2.

Specifically, Table 2 illustrates the performance of RDTs for diagnosis of *P. falciparum* malaria by detection of non-HRP2 antigens, namely Plasmodium lactate dehydrogenase (pLDH), pan (pan-pLDH; all species) and *P. falciparum*-specific (pf-pLDH). It shows if the products met recommended case management performance criteria for detection of HRP2 expressing and non-HRP2 expressing *P. falciparum*. Overall, only the pan-LDH-only RDTs met case management performance criteria on both HRP2 expressing and non-expressing *P. falciparum* panels and therefore, appear to be the best RDT option for areas with high prevalence of parasites lacking HRP2. Performance of Pf-LDH-detecting RDTs against wild type *P. falciparum* did not necessarily predict performance against *pfhrp2*-deleted parasites. Furthermore, no Pf-LDH detecting RDT met performance criteria required on both wildtype and *pfhrp2/3* deleted parasite panels. However, several performed well at detecting the higher density *pfhrp2/3* deleted samples ( $\approx 2000$  parasites/ $\mu\text{L}$ ) and can be used in parallel with HRP2 RDTs to screen for suspected *pfhrp2/3* deletions in surveys,<sup>19</sup> as most patients presenting with symptomatic falciparum malaria present with parasite densities at or above these thresholds. A solution is still urgently needed for areas with a high prevalence of *pfhrp2/3* deletions causing negative RDTs, and where falciparum and non-falciparum infections need to be distinguished ie. pan-LDH RDTs are not alone adequate for case management. Ultimately, further research including larger studies from a range of geographical settings are needed to further delineate RDT performance against single and double deletion of *pfhrp2* and *pfhrp3*.<sup>20</sup>

Further details to complement Table 2, e.g. heat stability, false-positive results for non-*P. falciparum* infections and test band intensity should be consulted in product testing reports.

Given the weakness in the current RDT armamentarium, where microscopy is available, services should be strengthened to ensure that parasitological confirmation of malaria continues until gaps are filled and transitions to new RDTs are completed as well as to support investigations of new foci of suspected *pfhrp2/3*-deleted parasites.

TABLE 2

**WHO Malaria RDT Product Testing: Rounds 5-8: Performance of RDTs not based exclusively on HRP2 for the detection of low density HRP2-expressing and non-expressing *P. falciparum* malaria**

Product	Manufacturer	<i>P. falciparum</i> HRP2 expressing, <i>P. vivax</i> and malaria negative panels						<i>P. falciparum</i> HRP2 non-expressing panel					
		PDS <sup>a</sup>		FP	IR	Meets WHO procurement criteria <sup>b</sup>	PDS	E <sup>i</sup>	Meets WHO procurement criteria for detection of <i>pfhrp2</i> / <i>3</i> deleted <i>P.falciparum</i> <sup>m</sup>	Applicable for use with a HRP2 RDT, as screening tool for surveys of <i>pfhrp2</i> deletions <sup>n</sup>	Round		
		A <sup>b</sup>	B <sup>c</sup>	C <sup>d</sup>	D <sup>e</sup>	Pf @ 2000p/μL							
<b>Pf only</b>													
CareStart™ Malaria Pf (HRP2/pLDH) Ag Combo 3-line RDT <sup>i</sup>	RMSM-02571	82 (81/40) <sup>f</sup>	NA	0.5	0.0	No	12.5 (0/12.5) <sup>f</sup>	100	No	Yes	8		
CareStart™ Malaria Pf (HRP2/pLDH) Ag RDT	RMPM-02591	88.0	NA	0.0	0.0	No	17.5	100	No	No	8		
CareStart™ Malaria Pf (HRP2/pLDH) Ag RDT	RMPM-02571	96.0	NA	0.0	0.0	Yes <sup>g</sup>	60.0	100	No	No	8		
careUS™ Malaria Combo Pf (HRP2/pLDH) Ag	RMP-M02582	88.0	NA	0.0	0.0	No	22.5	100	No	No	8		
SD BIOLINE Malaria Ag Pf (HRP2/pLDH) <sup>i</sup>	05FK90	90 (88/77) <sup>f</sup>	NA	0.0	0.1	Yes <sup>g</sup>	32.5 (0/32.5) <sup>f</sup>	100	No	Yes	8		
SD BIOLINE Malaria Ag Pf. (HRP2/pLDH) 2 Lines	05FK130-40-0	90.0	NA	0.0	0.1	No	UK	UK	UK	UK	7		
EzDx Malaria Pf Rapid malaria Antigen detection test (pLDH)	RK MAL 024-25 Pvt. Ltd.	10.0	NA	5.8	0.0	No	12.5	100	No	Yes	8		
<b>Pf and Pan</b>													
CareStart™ Malaria Pf/PAN (pLDH) Ag RDT	RMLM-02571	83.0	97.1	1.0	0.1	No	0.0	82.5	No	No	8		
CareStart™ Malaria Screen RDT	RMAM-05071	93.0	94.3	0.0	0.1	No	UK	UK	UK	UK	7		
Malaria pf (pLDH) / PAN-pLDH Test Device	MFV-124	41.0	8.6	81.3 (235)	0.1	No	UK	UK	UK	UK	5		
MERISCREEN Malaria pLDH Ag	MVLRPD-02	27.0	100.0	1.0	0.0	No	10.0	100	No	Yes	8		
<b>Pf and Pv/Pvom</b>													
BIOCREDIT Malaria Ag Pf/Pv (pLDH/pLDH)	C6ORHA25	75.0	100.0	0.0	0.6	Yes	UK	UK	UK	UK	7		
<b>Pf, Pf and Pv</b>													
SD BIOLINE Malaria Ag Pf/Pf/Pv <sup>f</sup>	05FK120	89 (89/62) <sup>f</sup>	97.1	0.0	0.0	Yes <sup>g</sup>	20 (0/20) <sup>f</sup>	100	No	Yes	8		
<b>Pan only</b>													
Advantage Pan Malaria Card	IR013025	77.0	100.0	0.4	0.0	Yes	UK	UK	UK	UK	5		
CareStart™ Malaria PAN (pLDH) Ag RDT	RMNM-02571	84.0	88.6	0.0	0.0	Yes <sup>g</sup>	100 <sup>h</sup>	100 <sup>i</sup>	Yes	No	5 <sup>h,i</sup>		
CareStart™ Malaria PAN (pLDH) Ag RDT	RMNM-02591	98.0	97.1	9.1	0.0	Yes	90.0		Yes	No	8		
careUS™ Malaria PAN (pLDH) Ag	RMN-M02582	98.0	85.7	5.3	0.0	Yes	85.0		Yes	No	8		

**Abbreviations:** UK: unknown; Pf: *Plasmodium falciparum*; Pv: *Plasmodium vivax*; pan: *Plasmodium species*; Pvom: *Plasmodium vivax, ovale and malariae*

**Performance criteria (highlighted in green if met):**

- A: *P. falciparum* panel detection score (PDS)  $\geq 75\%$  at 200 parasites/ $\mu\text{L}$
  - B: *P. vivax* panel detection score (PDS)  $\geq 75\%$  at 200 parasites/ $\mu\text{L}$
  - C: false-positive (FP) rate against clean negatives  $< 10\%$
  - D: invalid rate (IR)  $< 5\%$
  - E: *pfhrp2* negative *P. falciparum* panel detection score (PDS)  $> 75\%$  at 200 parasites/ $\mu\text{L}$  (in areas where *pfhrp2* deletions are prevalent)
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- a A sample is considered detected only if all RDTs from both lots read by the first technician, at minimum specified reading time, are positive
  - b Round 1, n=79; Round 2, n=100; Round 3, n=99; Round 4, n=98; Round 5, n=100; Round 6, n=100; Round 7, n=100; Round 8, n=100
  - c Round 1, n=20; Round 2, n=40; Round 3, n=35; Round 4, n=34; Round 5, n=35; Round 6, n=35; Round 7, n=35; Round 8, n=35
  - d Round 1, n=168; Round 2, n=200; Round 3, n=200; Round 4, n=232; Round 5, n=236; Round 6, n=208; Round 7, n=220; Round 8, n=208
  - e Round 1, n=954; Round 2, n=1240; Round 3, n=1204; Round 4, n=1192; Round 5, n=1214; Round 6, n=1210; Round 7, n=1210; Round 8, n=1210
  - f PDS presented in the table is based on a positive Pf test line (either HRP2 or Pf-LDH). The results in brackets are the PDS based alone on HRP2 and Pf-LDH test lines, respectively.
  - g Indicates a WHO prequalified product (as 15 February 2019), see updates at: [https://www.who.int/diagnostics\\_laboratory/evaluations/pq-list/malaria/public\\_report/en/](https://www.who.int/diagnostics_laboratory/evaluations/pq-list/malaria/public_report/en/)
  - h <https://www.who.int/malaria/news/2019/rdt-procurement-criteria/en/>
  - i Round 8, n=40 (18 double deletion: *pfhrp2*-/*pfhrp3* -; 22 single deletion; *pfhrp2*-/*pfhrp3*+) )
  - j Results (PDS) of adhoc assessment of pLDH containing round 8 RDTs against high density HRP2 negative panel : n=40 (18 double deletion: *pfhrp2*-/*pfhrp3* -; 22 single deletion; *pfhrp2*-/*pfhrp3*+) )
  - k Results (PDS) of adhoc assessment of this product against the round 8 low density HRP2 negative panel n=40 (18 low density double deletion: *pfhrp2*-/*pfhrp3* -; 22 single deletion; *pfhrp2*-/*pfhrp3*+) )
  - l Results (PDS) of adhoc assessment of this product against a high density HRP2 negative panel n=40 (18 low density double deletion: *pfhrp2*-/*pfhrp3* -; 22 single deletion; *pfhrp2*-/*pfhrp3*+) )
  - m These results should be considered when procuring RDT for use in areas where *pfhrp2* + or - *pfhrp3* deletions are prevalent.
  - n RDTs including pf-LDH individual test lines that have a PDS  $>90\%$  against *pfhrp2* deleted parasite samples of 2000 parasites/ $\mu\text{L}$  may be used to screen for *pfhrp2* deletions as per WHO survey protocol template (33)



## INTERIM WHO RECOMMENDATIONS

1. Suspected false-negative RDT results should be investigated.
2. *Pfhrp2/3* gene deletions should be suspected and the national malaria control programme and WHO informed when:
  - a. a sample from an individual tests negative on the HRP2 line of at least two quality-assured malaria RDTs **and** positive on the pan- or pf-pLDH test line, when a combination RDT is used **and** the sample is confirmed by microscopy to be positive for *P. falciparum* by two qualified microscopists;
  - b. in a programme, the rates of discordance between the results of RDTs and microscopy are systematically  $\geq 10\text{--}15\%$ , with higher positivity rates in microscopy, where quality is controlled routinely by cross-checking or both are performed on the same individuals (e.g. during surveys) and/or when the national malaria control programme receives multiple formal complaints or anecdotal evidence of RDTs that give false-negative results for *P. falciparum*.
3. When *pfhrp2/hrp3* gene deletions have been reported, the baseline prevalence should be determined in the affected country and neighbouring countries. This may require specific surveys or adaptation of planned surveys, such as therapeutic efficacy studies. WHO has published survey protocol templates to assist countries in designing their own survey for determining the prevalence of *pfhrp2/3* deletions causing negative HRP2-RDT results amongst symptomatic patients.
4. Confirmatory evidence of *pfhrp* deletions should include PCR for *pfhrp2* and *pfhrp3*, as HRP3 proteins can show cross-reactivity in HRP2-based RDTs.<sup>13, 18, 21</sup> Although useful analysis of flanking genes for *pfhrp2* (and *pfhrp3*) and serological confirmation of the absent HRP2 antigen (by immunological assay, e.g. ELISA, bead-based immunoassay or a second brand of RDT) are considered optional.
5. A nationwide change to an RDT that includes non-HRP2 target antigens for *P. falciparum* is recommended when the lower 95% confidence interval of the prevalence of symptomatic patients carrying *pfhrp2*-deleted parasites (causing false-negative HRP2 RDT results) is  $\geq 5\%$ .<sup>22</sup> If *pfhrp2* deletions are confirmed but the prevalence is  $< 5\%$ , it is recommended that a change be planned over a longer period, as it is anticipated that *pfhrp2/3*-deleted parasites will persist and spread. A repeated survey after 1–2 years will inform a prioritized roll-out of RDTs that include non-HRP2-based antigens.
6. In all other cases, if *pfhrp2* deletions are confirmed in samples from any source, the suggested action is to establish the prevalence of false-negative HRP-based RDT results secondary to *pfhrp2* deletion through representative surveys.
7. Well-preserved archived specimens may be analysed to identify the existence and geographical location of *pfhrp2/pfhrp3*-deleted parasite populations and thereby guide future investigations to establish prevalence.

8. In the absence of confirmed reports of *pfhrp2*/*pfhrp3* gene deletions, it is not recommended that new initiatives be taken to find these gene deletions, unless they are prompted by findings described under 2 above.

## WHO/GMP RESPONSE

Given the complexity of investigating suspected false-negative RDT results and the risk that parasites that do not express HRP2/HRP3 emerge but are not detected, WHO has or is conducting the following activities:

- published a global response plan which outlines the critical areas of work needed to mount an effective response and begins with mapping the scope and scope of *pfhrp2/3* deletions causing negative HRP2-RDTs (<https://www.who.int/malaria/publications/atoz/response-plan-pfhrp2-gene-deletions/en/>);
- preparing survey protocols (and tools) for conducting baseline assessments to determine whether the prevalence of *pfhrp2* deletions that cause negative HRP2 RDT results among symptomatic patients with confirmed *P. falciparum* malaria, has reached a threshold for a change in diagnostic strategy ([https://www.who.int/malaria/publications/diagnostic\\_testing/en/](https://www.who.int/malaria/publications/diagnostic_testing/en/));
- establishing and expanding a panel of *pfhrp2/3*-deleted parasites (cultured and wild-type) for evaluating the performance of non-HRP2 Pf-detecting RDTs;
- established a network of laboratories to review and build consensus on laboratory methods for characterizing *pfhrp2/3* gene deletions and linking reference laboratories with field investigators to ensure reliable, accurate reporting of *pfhrp2/3* gene deletions;
- working with relevant groups to adapt planned surveys to include collection of blood samples for molecular testing for malaria, including analysis of *pfhrp2/3*, based on WHO-recommended protocols. Areas affected by these mutations, including neighbouring countries, will be a priority;
- working with research groups that hold collections of recently archived samples to screen for the presence of *pfhrp2/pfhrp3*-deleted parasites;
- monitoring the peer review and grey literature for reports of *pfhrp2/3* deletions and incorporating findings regularly in the WHO Malaria Threat Maps (<http://apps.who.int/malaria/maps/threats/>);
- rigorously reviewing manuscripts submitted for publications and published reports of *pfhrp2/pfhrp3* deletions to determine the accuracy of claims; and
- encouraging test developers and RDT manufacturers to improve the performance of pLDH-based tests and identify new target antigens.

## Endnotes

1. Gamboa D, Ho MF, Bendezu J, Torres K, Chiodoni PL, Barnwell J et al. A large proportion of *P. falciparum* isolates in the Amazon region of Peru lack *pfhrp2* and *pfhrp3*: implications for malaria rapid diagnostic tests. *PLoS One*. 2010;5:e8091.
2. Akinyi S, Hayden T, Gamboa D, Torres K, Bendezu J, Abdallah JF et al. Multiple genetic origins of histidine-rich protein 2 gene deletion in *Plasmodium falciparum* parasites from Peru. *Sci Rep*. 2013;3:2797.
3. Cheng Q, Gatton M, Barnwell J, Chiodini P, McCarthy J, Bell D et al. *Plasmodium falciparum* parasites lacking histidine-rich protein 2 and 3: a review and recommendations for accurate reporting. *Malar J*. 2014;13:283.
4. Parr JB, Verity R, Doctor SM, Janko M, Carey-Ewend K, Turmanet BJ et al. *pfhrp2*-deleted *Plasmodium falciparum* parasites in the Democratic Republic of the Congo: a national cross-sectional survey. *J Infect Dis*. 2017;216:36–44.
5. Berhane A, Mihreteab S, Mohammed S, Embaye G, Hagos F, Zehaie A et al. PfHRP2 Detecting malaria RDTs: alarming false negative results in Eritrea 2016. American Society of Tropical Medicine and Hygiene 65th Annual Conference. *ASTMH 2016;95(Suppl.5):Poster 879*.
6. Amoah LE, Abankwa J, Opong A. *Plasmodium falciparum* histidine rich protein-2 diversity and the implications for PfHRP 2-based malaria rapid diagnostic tests in Ghana. *Malar J*. 2016;15:101.
7. Beshir KB, Sepúlveda N, Bharmal J, Robinson A, Mwanguzi J, Obukosia Busula A et al. *Plasmodium falciparum* parasites with 1 histidine-rich protein 2 (*pfhrp2*) and *pfhrp3* gene deletions in two endemic regions of Kenya (submitted for publication).
8. Kozycki CT, Umulisa N, Rulisa S, Mwikarago EI, Musabyimana JP, Habima JP et al. False-negative malaria rapid diagnostic tests in Rwanda: impact of *Plasmodium falciparum* isolates lacking *hrp2* and declining malaria transmission. *Malar J*. 2017;16:123.
9. Bharti PK, Chandel HS, Ahmad A, Krishna S, Udhayakumar V, Singh N. Prevalence of *pfhrp2* and/or *pfhrp3* gene deletion in *Plasmodium falciparum* population in eight highly endemic states in India. *PLoS One*. 2016;11:e0157949.
10. Kahama-Maró J, D'Acremont V, Mtasiwa D, Genton B, Lengeler C. Low quality of routine microscopy for malaria at different levels of the health system in Dar es Salaam. *Malar J* 2011;10:332.
11. Senn N, Rarau P, Manong D, Salib M, Siba P, Robinson, LJ et al. Rapid diagnostic test-based management of malaria: an effectiveness study in Papua New Guinean infants with *Plasmodium falciparum* and *Plasmodium vivax* malaria. *Clinical infectious diseases* 2012; 54, H. 5. S. 644–651.
12. Baiden F, Webster J, Tivura M, Delimini R, Berko Y, et al. Accuracy of rapid tests for malaria and treatment outcomes for malaria and non-malaria cases among under-five children in rural Ghana. *PLoS ONE* 2012;7(4): e34073.
13. Cheng Q, Gatton M, Barnwell J, Chiodini P, McCarthy J, Bell D et al. *Plasmodium falciparum* parasites lacking histidine-rich protein 2 and 3: a review and recommendations for accurate reporting. *Malar J*. 2014;13:283.
14. Quality-assured RDTs are selected on the basis of WHO-recommended procurement criteria, lot-tested by a WHO -recognized laboratory (<https://www.who.int/malaria/areas/diagnosis/rapid-diagnostic-tests/lot-testing/en/>) and transported and stored in accordance with the manufacturer's recommendations.
15. Stored blood slides and used RDTs could be used as sources of DNA, but they are not ideal.
16. The desiccant in the RDT cassette packaging can be used.
17. Gatton ML, Dunn J, Chaudhry A, Ciketic S, Cunningham J, Cheng Q. Use of PfHRP2-only RDTs rapidly selects for PfHRP2-negative parasites, with serious implications for malaria case management and control. *J Infect Dis*. 2017. doi: 10.1093/infdis/jix094.
18. Malaria rapid diagnostic test performance: results of WHO product testing of malaria RDTs: round 8 (2016–2018). Geneva: World Health Organization; 2018.
19. Panel detection score is > 90 at 2000 parasites/μL and their false-positive and invalid rates are < 2%.

20. Comparison of performance of malaria rapid diagnostic tests for detection of wild type and HRP2-negative *Plasmodium falciparum*. Michelle L. Gatton, Alisha Chaudhry, Jeff Glenn, Scott Wilson, Yong Ah, Amy Kong, Rosalynn L. Ord, Roxanne R. Rees-Channer, Peter Chiodini, Sandra Incardona, Qin Cheng, Michael Aidoo, Jane Cunningham. Submitted for publication, April 2019
21. Parr, J. B., Anderson, O., Juliano, J. J., & Meshnick, S. R. (2018). Streamlined, PCR-based testing for pfhrp2- and pfhrp3-negative *Plasmodium falciparum*. *Malaria journal*, 17(1), 137. doi:10.1186/s12936-018-2287-4
22. To allow for sampling variation.
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