Global **Malaria** Programme

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

MAY 2016 (REV. SEPTEMBER 2017 AND JULY 2019) 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

TTo 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.

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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.[1] 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[2] 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.[3] 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,[4] Eritrea,[5] Ghana,[6] Kenya,[7] Rwanda[8] and India,[9] 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.[10] 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
Operator factors	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.
Use of an imperfect "gold standard" as a comparator	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.
Product design or quality	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.*
	Poor visibility of test bands due to strong background colour on the test	Assess RDT performance and training on site; if the strong background colour persists, notify the manufacturer.
	Incorrect instructions for use	Review the instructions for use for accuracy.
Transport or storage conditions	Antibody degradation due to poor resistance to heat or incorrect transport or storage, e.g. exposure to high temperatures, freeze-thawing	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).
Parasite factors	Parasites lack or express low levels of the target antigen, i.e. HRP2	Patient samples are negative on an HRP2 test 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 to be positive microscopically for *P. falciparum* 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 *pfhrp2/pfhrp3* gene analysis.
	Variation in the amino acid sequence of the epitope targeted by the monoclonal antibody	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.
Host parasite density	Very low parasite density or target antigen concentration	Perform high-quality microscopy and record the parasite count; if high-quality microscopy is not available, repeat the RDT if symptoms persist.
	Very high parasite load (severe malaria) causing prozone effect (hyperparasitaemia and antigen overload)	Repeat testing with a 10 × and if needed a subsequent 50 × 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,[11,12] 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.

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 ≥ 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.

WHEN AND HOW SHOULD FALSE-NEGATIVE HRP2-DETECTING RDT RESULTS DUE TO SUSPECTED PFHRP2 DELETION BE INVESTIGATED?[13]

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[14] **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;[15] air-dry filter paper or cards overnight in a clean environment, sealed in air-tight plastic bags with desiccant.[16]

- 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 symptomatics 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[17] 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,[18] 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 (≈ 2000 parasites/µL) and can be used in parallel with HRP2 RDTs to screen for suspected *pfhrp2/3* deletions in surveys,[19] 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*.[20]

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/pfhrp3*-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	*P. falciparum* HRP2 expressing, *P. vivax* and malaria negative panels					*P. falciparum* HRP2 non-expressing panel				
			PDS[a]		FP	IR	Meets WHO procurement criteria[h]	PDS		Meets WHO procurement criteria for detection of *pfhrp2/3* deleted *P.falciparum*[m]	Applicable for use with a HRP2 RDT, as screening tool for surveys of *pfhrp2* deletions[n]	Round
			A[b]	B[c]	C[d]	D[e]		E[i]	Pf @ 2000p/µL[j]			
Pf only												
CareStart™ Malaria Pf (HRP2/pLDH) Ag Combo 3-line RDT [f]	RMSM-02571	Access Bio Inc.	82 (81/40)[f]	NA	0.5	0.0	No	12.5 (0/12.5)[f]	100	No	Yes	8
CareStart™ Malaria Pf (HRP2/pLDH) Ag RDT	RMPM-02591	Access Bio Ethiopia	88.0	NA	0.0	0.0	No	17.5	100	No	No	8
CareStart™ Malaria Pf (HRP2/pLDH) Ag RDT	RMPM-02571	Access Bio Inc.	96.0	NA	0.0	0.0	Yes[g]	60.0	100	No	No	8
careUS™ Malaria Combo Pf (HRP2/pLDH) Ag	RMP-M02582	WELLS BIO, INC	88.0	NA	0.0	0.0	No	22.5	100	No	No	8
SD BIOLINE Malaria Ag P.f (HRP2/pLDH) [f]	05FK90	Standard Diagnostics Inc. (Alere)	90 (88/71)[f]	NA	0.0	0.1	Yes[g]	32.5 (0/32.5)[f]	100	No	Yes	8
SD BIOLINE Malaria Ag P.f. (HRP2/pLDH) 2 Lines	05FK130-40-0	Standard Diagnostics, Inc.	90.0	NA	0.0 (231)	0.1	No	UK	UK	UK	UK	7
EzDx Malaria Pf Rapid malaria Antigen detection test (pLDH)	RK MAL 024-25	Advy Chemical Pvt. Ltd.	10.0	NA	5.8	0.0	No	12.5	100	No	Yes	8
Pf and Pan												
CareStart™ Malaria Pf/PAN (pLDH) Ag RDT	RMLM-02571	Access Bio Inc.	83.0	97.1	1.0	0.1	No	0.0	82.5	No	No	8
CareStart™ Malaria Screen RDT	RMAM-05071	Access Bio, Inc.	93.0	94.3	0.0 (231)	0.1	No	UK	UK	UK	UK	7
Malaria pf (pLDH) / PAN-pLDH Test Device	MFV-124	AZOG, Inc.	41.0	8.6	81.3 (235)	0.1	No	UK	UK	UK	UK	5
MERISCREEN Malaria pLDH Ag	MVLRPD-02	Meril Diagnostics Pvt. Ltd.	27.0	100.0	1.0	0.0	No	10.0	100	No	Yes	8
Pf and Pv/Pvom												
BIOCREDIT Malaria Ag Pf/Pv (pLDH/pLDH)	C60RHA25	RapiGEN Inc.	75.0	100.0	0.0 (230)	0.6	Yes	UK	UK	UK	UK	7
Pf, Pf and Pv												
SD BIOLINE Malaria Ag P.f/P.f/P.v [f]	05FK120	Standard Diagnostics Inc. (Alere)	89 (89/62)[f]	97.1	0.0	0.0	Yes[g]	20 (0/20)[f]	100	No	Yes	8
Pan only												
Advantage Pan Malaria Card	IR013025	J. Mitra & Co. Pvt. Ltd.	77.0	100.0	0.4	0.0	Yes	UK	UK	UK	UK	5
CareStart™ Malaria PAN (pLDH) Ag RDT	RMNM-02571	Access Bio, Inc.	84.0	88.6	0.0	0.0	Yes[g]	100[k]	100[l]	Yes	No	5[k,l]
CareStart™ Malaria PAN (pLDH) Ag RDT	RMNM-02591	Access Bio Ethiopia	98.0	97.1	9.1	0.0	Yes	90.0		Yes	No	8
careUS™ Malaria PAN (pLDH) Ag	RMN-M02582	WELLS BIO, INC	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) a ≥ 75% at 200 parasites/µL
B: *P. vivax* panel detection score (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* panel detection score (PDS) > 75% at 200 parasites/uL (in areas where pfhrp2 deletions are prevalent)

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/
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 pfLDH 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/µL may be used to screen for pfhrp2 deletions as per WHO survey protocol template (33)

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