# Extending the WHO scheme for external quality assessment of nucleic acid amplification testing for monitoring antimalarial drug resistance

Meeting report, 14 July 2023

World Health Organization

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ISBN 978-92-4-010689-5 (electronic version)

ISBN 978-92-4-010690-1 (print version)



**Suggested citation.** Extending the WHO scheme for external quality assessment of nucleic acid amplification testing for monitoring antimalarial drug resistance: meeting report, 14 July 2023. Geneva: World Health Organization; 2025. Licence: CC BY-NC-SA 3.0 IGO.

**Cataloguing-in-Publication (CIP) data.** CIP data are available at https://iris.who.int/.



This publication contains the meeting report of the subregional networks of antimalaria drug efficacy and resistance in Eastern Africa and Horn of Africa countries and does not necessarily represent the decisions or policies of WHO.

# Contents

# Abbreviations

| | |
|---|---|
| ACT | artemisinin-containing therapy |
| DBS | dried blood sample |
| EQA | external quality assessment |
| IS | international standard |
| LB | lyophilized blood |
| MM | molecular markers |
| NAAT | nucleic acid amplification testing |
| *PfK13* | *Plasmodium falciparum Kelch 13* |
| q3CR | quantitative polymerase chain reaction |
| SNP | single nucleotide polymorphism |
| TES | therapeutic efficacy study |
| UKNEQAS (P) | United Kingdom National External Quality Assessment Service (Parasitology) |
| WHO | World Health Organization |

# Executive summary

The World Health Organization (WHO) recommends use of artemisinin-based combination therapies (ACTs) for treating uncomplicated malaria, with therapeutic efficacy studies (TES) to monitor treatment efficacy. In view of the threat of antimalarial resistance, including confirmed partial resistance to artemisinins in four countries in Africa, use of molecular tools in tracking resistance patterns has become critical.

WHO established the External Quality Assessment (EQA) scheme for nucleic acid amplification testing (NAAT) in 2015 to ensure reliable results from clinical and research laboratories. The scheme is coordinated by WHO and operated by the United Kingdom National EQA Service for Parasitology (UK NEQAS (P)), which provides quality-controlled specimen panels and scoring reports to laboratories to assess and improve the accuracy of testing.

On 14 July 2023, WHO held a virtual meeting with experts to discuss extension of the NAAT EQA scheme to include antimalarial resistance markers. The objectives included identifying priority resistance markers, discussing logistics and specimen types and addressing operational concerns, such as cost and capacity-building. The conclusions of the meeting were as follows.

- **Extension of the NAAT EQA scheme**: Consensus was reached on extending the NAAT EQA scheme to include drug resistance markers and, eventually, molecular correction methods.
- **Integration with EQA of markers of antimalarial resistance**: The antimalarial resistance marker EQA scheme could be included in the WHO and UK NEQAS (P) collaboration as a combined WHO malaria NAAT and molecular markers (MM) of drug resistance EQA scheme, to optimize resources and reduce costs.
- **Specimen panel sourcing**: External collaborators will provide specimen panels and work with UK NEQAS in their preparation and distribution.
- **Preferred sample type: dried blood spots**: Dried blood spots (DBS) were identified as the preferred sample type because of the ease of preparation, storage and shipping.
- **Markers for initial inclusion**: *Plasmodium falciparum Kelch 13* (*PfK13*) markers were considered to be critical for immediate inclusion in panels. Partner drug resistance markers are considered a lower priority but could be included in the future according to their relevance. The importance of molecular correction methods was emphasized, and they should be included as soon as possible in laboratory evaluation.
- **Adaptability of the scheme**: The scheme should be adaptable with respect to the resistance markers to be included, in view of the changing epidemiology of malaria. Specimen panels should include various mixed specimens with different mutations or genotypes in different ratios in order to assess detection limits, sensitivity and accuracy.
- **Learning from other EQA schemes**: Insights from other EQA schemes, such as those for proficiency in cancer diagnosis, may offer guidance for designing the new scheme.

## Next steps

The meeting strongly supported extension of the EQA scheme to include antimalarial resistance markers, which was considered an essential step in strengthening global reporting on resistance. It was proposed that a survey of meeting participants be conducted to gauge their interest and capacity for pilot-testing the extended scheme by current EQA participants.

# 1. Background

## 1.1 Role of molecular tools in monitoring antimalarial resistance

WHO recommends ACTs for the treatment of uncomplicated malaria, with TES to monitor the efficacy of antimalarial treatment. WHO recommends that, when the TES failure rate exceeds 10%, failing ACTs be replaced with alternative, more effective ACTs *(1)*.

Currently, NAAT is used in two aspects of monitoring for antimalarial drug resistance. First, molecular markers associated with drug resistance are characterized to monitor evolving trends in drug resistance patterns. For example, partial resistance to artemisinins is associated with several single nucleotide polymorphisms (SNPs) in the *P. falciparum Kelch 13* (*PfK13*) BTB/POZ and propeller domain. SNPs associated with other antimalarial drugs, such as chloroquine (*pfcrt*) and sulfadoxine (*pfdhps*)–pyrimethamine (*pfdhfr*), have been well established. Variation in the copy number in *pfmdr1* has been implicated in resistance to mefloquine, and SNPs in this gene have been associated with modulation of the response to some antimalarials. Secondly, molecular tools are used to discriminate *P. falciparum* recrudescence from reinfection in TES for molecular correction of drug efficacy, as recommended by WHO *(2)*.

As molecular tools are increasingly important in monitoring the spread of drug resistance and assessing the efficacy of antimalarial drugs, it is essential to ensure the accuracy of NAATs. As partial resistance to artemisinins has been confirmed in four countries in Africa *(3)*, it is even more important to improve the quality of molecular assays. Characterization of drug-resistant markers involves various molecular platforms, and quality standards are required to verify the accuracy of results.

## 1.2 The WHO malaria external quality assessment of nucleic acid amplification testing scheme

In 2014, following the recommendation of a technical consultation on malaria diagnostics in low-transmission settings to use NAAT for research and surveys, the Malaria Policy Advisory Committee endorsed a recommendation to establish an international EQA scheme for NAAT assays to ensure reliable, comparable results. In June 2015, WHO convened a consultation in London, United Kingdom of Great Britain and Northern Ireland, to develop a plan for establishing a WHO EQA scheme for malaria NAAT *(4)*. As recommended at this meeting, a WHO malaria NAAT EQA scheme was established.

The WHO malaria NAAT EQA scheme offers an independent means for clinical, reference and research laboratories to verify periodically the quality of their NAAT malaria diagnostic methods and to monitor their performance over time. Participants are provided with well-characterized, quality-controlled panels of a blinded mix of *Plasmodium*-positive and -negative samples. Participants are given an EQA report after their results have been analysed. Participation in the scheme is voluntary, and the results are confidential.

The scheme is coordinated by the WHO Global Malaria Programme and distributed by the United Kingdom National External Quality Assessment Service for Parasitology (UK NEQAS (P)). Priority is given to not-for-profit laboratories based in developing countries. WHO is responsible for the overall coordination and promotion of the scheme, while UK NEQAS (P) holds the central repository of EQA materials and manages the scheme's operations, which includes the preparation, characterization, storage and shipping of EQA panels; issuance of EQA reports; and handling of any logistical queries. The EQA panels are tested independently by referee laboratories before distribution to laboratories participating in the scheme.

The scheme offers an opportunity for participating laboratories to obtain an independent assessment of their NAAT-based diagnostic methods to determine whether they meet the minimum quality required. According to performance outcomes, opportunities are also provided for corrective action. Participating laboratories that under-perform can identify and address the source(s) of error in order to improve the quality and reliability of their methods.

### 1.3 Virtual meeting

WHO proposed that the scheme could be extended to include monitoring of antimalarial drug resistance markers, including SNPs, variations in copy numbers and markers used for molecular correction (hereafter referred to as antimalarial resistance markers). An online meeting was held on 14 July 2023 with a panel of experts to discuss how the existing EQA scheme could be extended to include antimalarial resistance markers and the most relevant markers to be included in the scheme (See Annex 1 for the agenda and Annex 2 for the list of participants). An overview of the existing WHO malaria NAAT EQA scheme was presented and discussion points for the new antimalarial resistance scheme were put to the group.

The objectives of the consultation were to:

- agree on the antimalarial resistance markers to be prioritized for inclusion in the scheme;
- identify the EQA materials and panels necessary to extend the NAAT EQA scheme; and
- reach consensus on the costing, implementing partners, capacity-building and timeline of the scheme.

Before the meeting, declarations of interests were collected from all external contributors and assessed for conflicts of interest. The declared interests and how they were managed by the WHO Secretariat are summarized in Annex 3.

## 2. Presentation 1: role and functions of providers

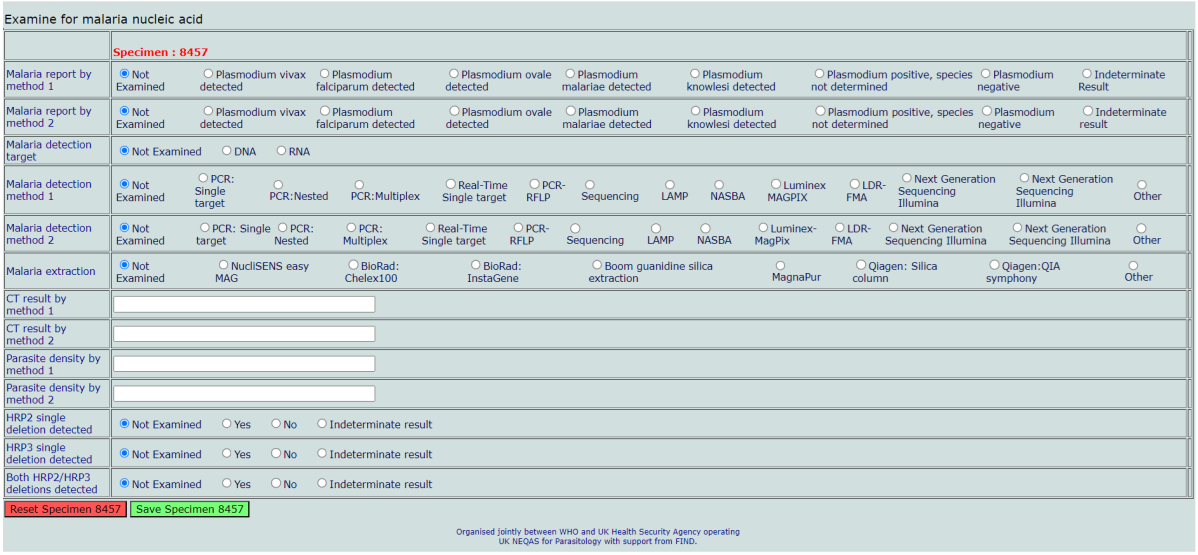
### 2.1 Panel composition and scoring

The WHO malaria EQA scheme is designed to represent real specimens as closely as possible. Various sources are used, including leftover *Plasmodium*-positive samples consisting of EDTA-anti-coagulated peripheral blood and contrived specimens consisting of in vitro cultured *P. falciparum* or *P. knowlesi*. The samples are diluted to the desired parasite density in *Plasmodium*-negative whole blood supplied by United Kingdom National Health Service Blood and Transport, with negative samples. All positive and negative samples are confirmed with NAAT by a panel of seven WHO referee laboratories after sample production to confirm sample content and concentration.

Each panel consists of five lyophilized blood (LB) and five DBS samples. The density of parasites in *Plasmodium*-containing samples varies from 0.018 to 1.1 x $10^6$ parasites per µL of blood. Parasite-positive samples may be obtained from any of the species known routinely to infect humans, i.e. *P. falciparum, P. vivax, P. malariae, P. ovale* (both sub-species) and *P. knowlesi*, depending on availability. The LB and DBS samples have different compositions; thus, different species and/or parasite densities may be included in a given panel. Samples with deletions of *pfhrp2* and/or *pfhrp3* are also included.

After the panels have been distributed, laboratories are given 8 weeks to submit their results to the online portal (Fig. 1).

**Fig. 1. Submission portal for laboratories to submit EQA results**


Examine for malaria nucleic acid

Specimen : 8457

| | |
|---|---|
| Malaria report by method 1 | Not Examined; Plasmodium vivax detected; Plasmodium falciparum detected; Plasmodium ovale detected; Plasmodium malariae detected; Plasmodium knowlesi detected; Plasmodium positive, species not determined; Plasmodium negative; Indeterminate Result |
| Malaria report by method 2 | Not Examined; Plasmodium vivax detected; Plasmodium falciparum detected; Plasmodium ovale detected; Plasmodium malariae detected; Plasmodium knowlesi detected; Plasmodium positive, species not determined; Plasmodium negative; Indeterminate result |
| Malaria detection target | Not Examined; DNA; RNA |
| Malaria detection method 1 | Not Examined; PCR: Single target; PCR:Nested; PCR:Multiplex; Real-Time Single target; PCR-RFLP; Sequencing; LAMP; NASBA; Luminex MAGPIX; LDR-FMA; Next Generation Sequencing Illumina; Next Generation Sequencing Illumina; Other |
| Malaria detection method 2 | Not Examined; PCR: Single target; PCR: Nested; PCR: Multiplex; Real-Time Single target; PCR-RFLP; Sequencing; LAMP; NASBA; Luminex-MagPix; LDR-FMA; Next Generation Sequencing Illumina; Next Generation Sequencing Illumina; Other |
| Malaria extraction | Not Examined; NucliSENS easy MAG; BioRad: Chelex100; BioRad: InstaGene; Boom guanidine silica extraction; MagnaPur; Qiagen: Silica column; Qiagen:QIA symphony; Other |
| CT result by method 1 | |
| CT result by method 2 | |
| Parasite density by method 1 | |
| Parasite density by method 2 | |
| HRP2 single deletion detected | Not Examined; Yes; No; Indeterminate result |
| HRP3 single deletion detected | Not Examined; Yes; No; Indeterminate result |
| Both HRP2/HRP3 deletions detected | Not Examined; Yes; No; Indeterminate result |

Reset Specimen 8457 | Save Specimen 8457

Organised jointly between WHO and UK Health Security Agency operating UK NEQAS for Parasitology with support from FIND.

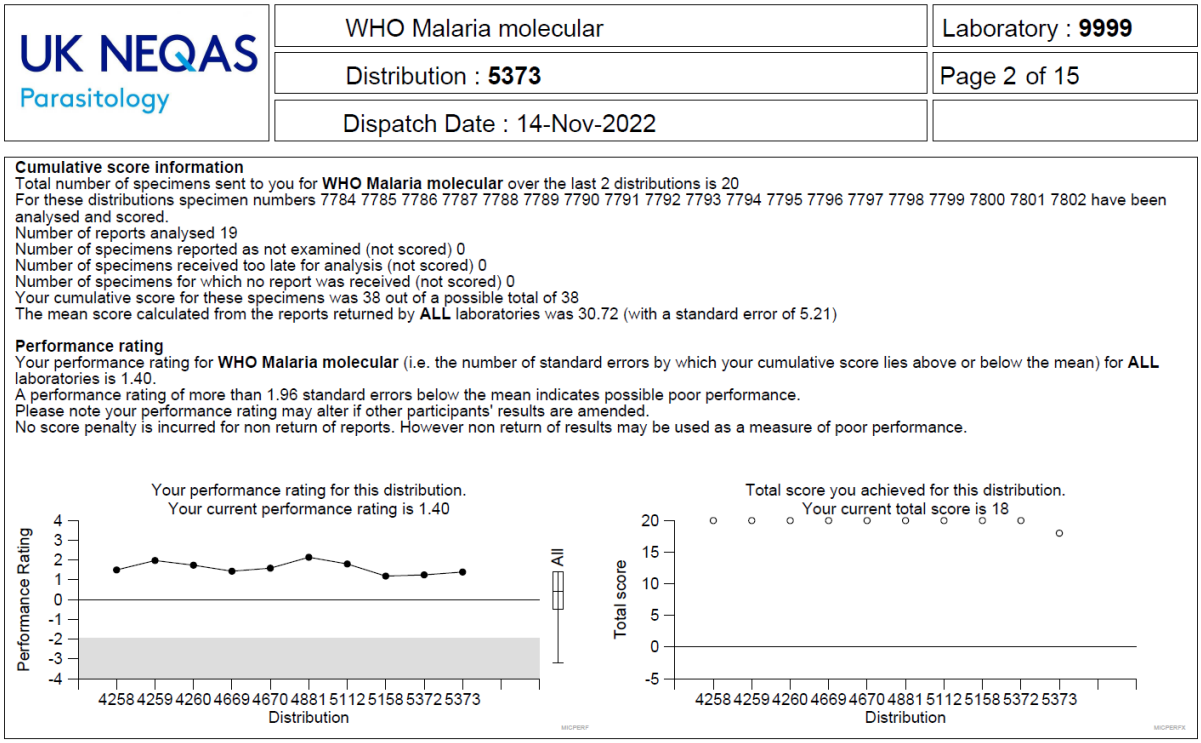
For each sample, a score of 2 to –1 is given, depending on the results submitted and the capacity of each laboratory to analyse that species (an example is shown in Table 1). For example, if a *Plasmodium*-positive sample with a species that the laboratory can identify is identified correctly, the laboratory is awarded a 2; if the response is incorrect, the laboratory is given –1; and, if the sample is correctly identified to genus level, a 1 is given. If the sample contains a species that the laboratory cannot detect and they report a negative sample, a score of 2 is given. The scoring criteria are complex, as they differ for positive and negative samples and according to the capacity of each laboratory to identify each species. The laboratory is given an overall score for each distribution and can compare its results with those of other laboratories in the scheme and over time.

**Table 1. Scoring scheme for detection of *Plasmodium* and determination of the species**

| Code | Description | Report | Score |
|---|---|---|---|
| **Positive specimens** | | | |
| 1 | Genus and species correctly identified (e.g. *P. falciparum* nucleic acid present) | *P. falciparum* nucleic acid present | 2 |
| 2 | Correct genus but wrong species (incorrect *Plasmodium* species) | Incorrect *Plasmodium* species | 0 |
| 3 | Only *Plasmodium* genus identified (e.g. *Plasmodium* spp. detected) | *Plasmodium* spp. detected | 1 |
| 4 | Indeterminate result | Indeterminate result | 0 |
| 5 | No *Plasmodium* nucleic acid detected | *Plasmodium* spp. not detected | –1 |
| 6 | *Plasmodium falciparum* nucleic acid not detected (relevant for labs doing *Pf* identification only) | *P. falciparum* nucleic acid not detected | –1 |
| **Negative specimens** | | | |
| 7 | *Plasmodium* nucleic acid present | Plasmodium nucleic acid present | –1 |
| 8 | Indeterminate result | Indeterminate result | 0 |
| 9 | No *Plasmodium* nucleic acid detected | No *Plasmodium* nucleic acid detected | 2 |

Fig. 2 presents a summary of the performance of one laboratory for one distribution.

**Fig. 2. Example summary results page**



UK NEQAS Parasitology

| WHO Malaria molecular | Laboratory : **9999** |
|---|---|
| Distribution : **5373** | Page 2 of 15 |
| Dispatch Date : 14-Nov-2022 | |

**Cumulative score information**
Total number of specimens sent to you for **WHO Malaria molecular** over the last 2 distributions is 20
For these distributions specimen numbers 7784 7785 7786 7787 7788 7789 7790 7791 7792 7793 7794 7795 7796 7797 7798 7799 7800 7801 7802 have been analysed and scored.
Number of reports analysed 19
Number of specimens reported as not examined (not scored) 0
Number of specimens received too late for analysis (not scored) 0
Number of specimens for which no report was received (not scored) 0
Your cumulative score for these specimens was 38 out of a possible total of 38
The mean score calculated from the reports returned by **ALL** laboratories was 30.72 (with a standard error of 5.21)

**Performance rating**
Your performance rating for **WHO Malaria molecular** (i.e. the number of standard errors by which your cumulative score lies above or below the mean) for **ALL** laboratories is 1.40.
A performance rating of more than 1.96 standard errors below the mean indicates possible poor performance.
Please note your performance rating may alter if other participants' results are amended.
No score penalty is incurred for non return of reports. However non return of results may be used as a measure of poor performance.



## 2.2 Challenges for the EQA provider

Finding adequate volumes of *P. vivax*, *P. ovale* and *P. malariae* is difficult, as 85% of imported malaria cases in the United Kingdom are due to *P. falciparum*. To obtain samples of these rarer species, partnerships and memoranda of understanding have been established with countries in which malaria is endemic.

Provision of *P. falciparum* samples with gene deletions or resistance markers is also difficult, as such parasite isolates must be established in continuous culture, which has implications for access and cost.

## 2.3 Characterization of material

WHO international standard (IS) DNA can be used to quantify *P. falciparum* samples. The standard consists of a freeze-dried whole-blood preparation collected from a patient (see nibsc.org for further details). Before each distribution, the seven referee laboratories are asked to report the parasite density of *P. falciparum*-positive samples in international units. The IS is, however, unsuitable for RNA-based assays (i.e. 18S rRNA). Therefore, as more laboratories adopt RNA-based assays, a plan may be required to adopt an RNA standard or calibrator.

As there is no IS for the other four *Plasmodium* species included in the EQA scheme, guidance may be required from referee laboratories on accurate quantification of these species. In general, the IS is detected by most pan-*Plasmodium*-type NAAT components.

Inclusion of antimalarial resistance markers in the existing EQA scheme will add additional complexity to administration of the scheme, scoring of results, analysing the data and preparing reports. More resources will be required, including an additional supply of cultured parasites with relevant antimalarial markers and additional laboratory staff for the specimen bank for preparing and supplying test specimens twice a year in the new scheme.

# 3. Presentation 2: overview of current EQA scheme and results

## 3.1 Distribution of laboratories

The first round of EQA panels was distributed in January 2017; panels are provided twice a year. To date, there have been 11 distributions to the 80 laboratories in the scheme. Laboratories in all six WHO regions have been enrolled (Table 2), and further laboratories are being enrolled at each distribution as information about the scheme is disseminated, including through presentations and on the WHO webpage.

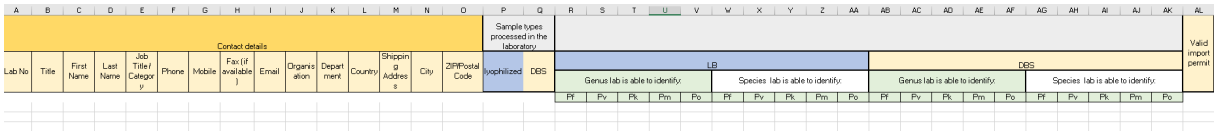
**Table 2. Distribution of laboratories enrolled in the EQA scheme**

| Region | Number of laboratories | Number of countries |
|---|---|---|
| Africa | 24 | 13 |
| Asia | 19 | 12 |
| Australia | 3 | 1 |
| Europe | 9 | 6 |
| North America | 11 | 2 |
| South and Central America | 13 | 8 |

## 3.2 Laboratory profile

Before enrolling in the scheme, laboratories are sent a laboratory profile (Fig. 3) to complete, including whether the sample type is LB or DBS and laboratory analytical methods. This informs the coordinators of the scheme about the Plasmodium species that can be detected to species and genus level for each of the five Plasmodium species included.

**Fig. 3. Laboratory profile to be completed before enrolment**

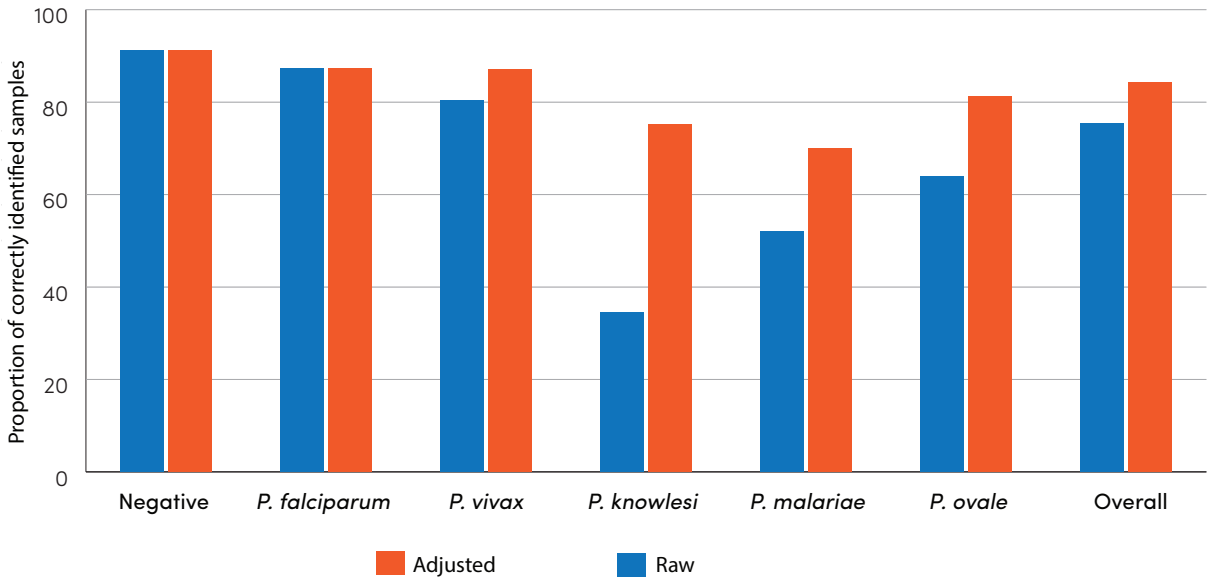



After each round, laboratories are sent a form by UK NEQAS, and the compiled results are sent to WHO to be appended to one dataset. Overall performance is then assessed according to the percentage of samples correctly identified.

### 3.3 Results up to distribution 9

Fig. 4 shows the results of raw and "adjusted" results from data up to distribution 9 according to the percentage of samples that were correctly identified. The raw results include whether a sample was correctly identified, regardless of laboratory capacity for each species. The adjusted results indicate performance after adjustment for the capacity of each laboratory to detect each species. Thus, if a *Plasmodium*-positive sample is identified as having no nucleic acid and the laboratory has reported that it cannot detect that species, the results for the sample will be scored as correct in the adjusted results.

**Fig. 4. Raw and adjusted results for correct identification of species up to distribution 9**




During the scheme, performance was significantly better with LB samples than with DBS (Table 3). This was true for all samples and also in analysis of paired samples, in which two samples in the round were of identical species and concentration but one was in DBS and one in LB format. This allowed direct comparison of performance by sample type.

**Table 3. Performance by sample type**

| | Dried blood spots | Lyophilized blood | *P* | No. of samples |
|---|---|---|---|---|
| All samples | 79.8% | 88.0% | < 0.01 | 2573 |
| Paired samples | 77.8% | 91.8% | < 0.01 | 797 |

Performance was significantly better in samples with a higher parasite density, with a threshold of 2 and 200 parasites/µL (Table 4). This was true for *P. falciparum* and *P. vivax* samples and overall but not for the other three species. A density of 2 parasites/µL was the target threshold set by WHO at the consultation in 2013 *(5)* and is the target limit of detection that should be achieved for NAATs, especially in settings in which elimination is the goal.

**Table 4. Performance by sample species and concentration**

| Plasmodium species | Samples correctly identified (%) | | *P* | Samples correctly identified (%) | | *P* |
|---|---|---|---|---|---|---|
| | ≤ 2 parasites/µL | > 2 parasites/µL | | ≤ 200 parasites/µL | > 200 parasites/µL | |
| *P. falciparum* | 51.5 | 93.4 | < 0.01 | 77.8 | 96.9 | < 0.01 |
| *P. vivax* | 80.2 | 89.2 | < 0.01 | 85.9 | 92.8 | 0.03 |
| *P. knowlesi* | 80.0 | 74.5 | 0.40 | 73.0 | 79.8 | 0.19 |
| *P. malariae*[a] | NA | 69.9 | NA | 70.9 | 63.9 | 0.31 |
| *P. ovale*[a] | NA | 79.4 | NA | 74.3 | 89.5 | < 0.01 |
| **Overall** | **65.6** | **83.6** | **< 0.01** | **77.8** | **90.7** | **< 0.01** |

[a] All samples of *P. malariae* and *P. ovale* contained > 2 parasites/µL.

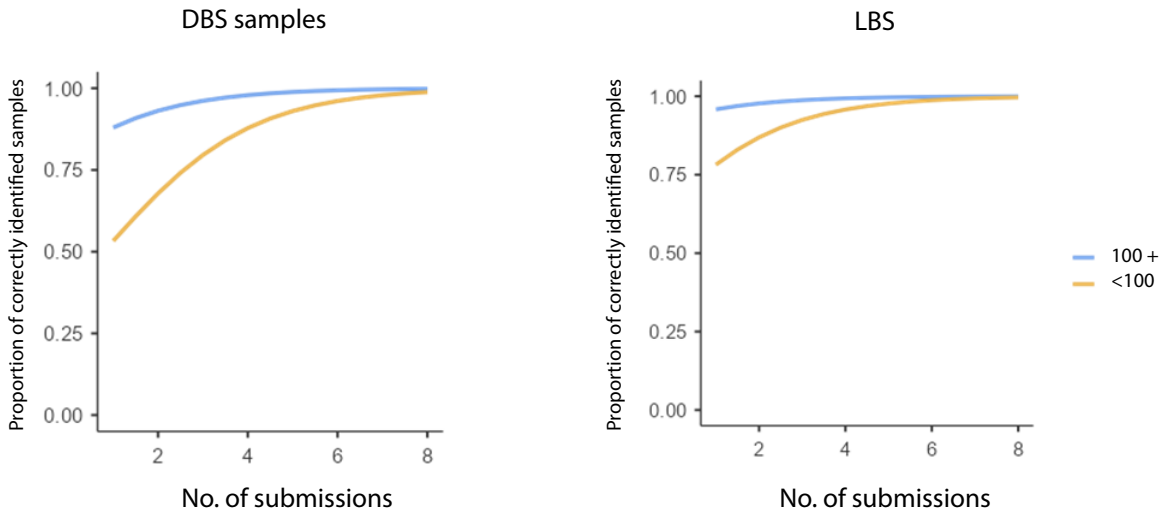
One of the main outputs that can be used to evaluate the success of the EQA scheme is a change in performance over time. This evaluation was, however, complex, because of:

- differences in the composition of sample species in each panel;
- differences in the concentrations of samples;
- different performance outcomes with DBS and LB;
- constant changes by each participating laboratory in joining or submitting in all rounds; and
- laboratory capacity to detect different species.

A generalized estimating equation was used to account for these complexities. A submission number was created for each laboratory for each round according to the number of distributions for which the laboratory had submitted results. For example, if a laboratory had submitted results for three distributions by distribution seven, their submission number in round seven would be three, regardless of when they joined the scheme.

Performance improved as the number of submissions for *P. falciparum* samples increased, for both sample types (Fig. 5). The improvement was greater for lower-density samples, as performance for these samples was lower at the start of enrolment into the scheme.

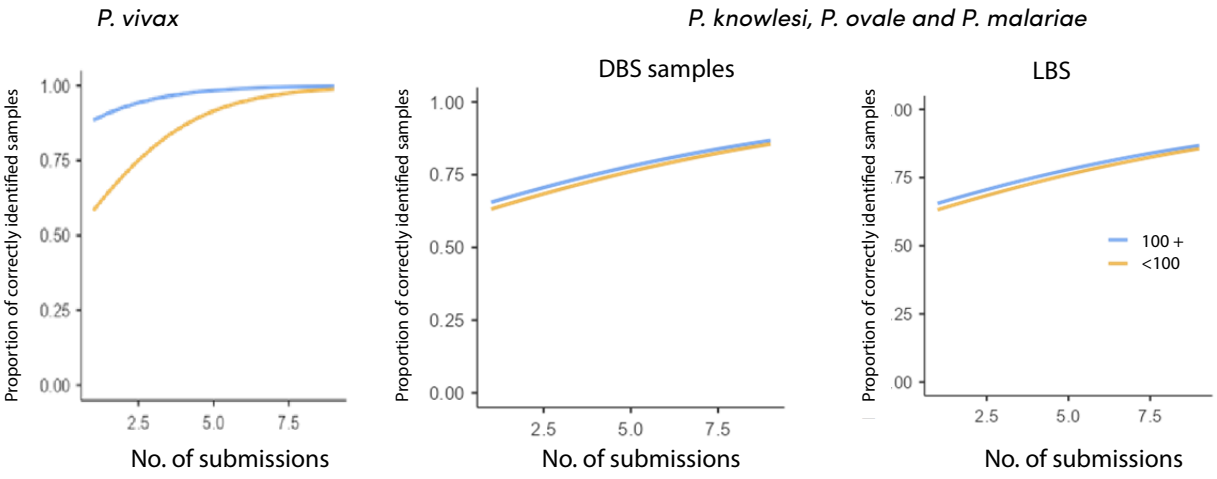
**Fig. 5. Performance for *P. falciparum* samples by number of submissions and sample type**




*Samples broken down by parasite density (parasites/µL)

A similar trend of increasing performance by number of submissions was seen with the other Plasmodium spp. (Fig. 6).

**Fig. 6. Performance by number of submissions for other *Plasmodium* species**




Samples shown by parasite density (parasites/µL)

## 3.4 Challenges for participating laboratories

Laboratories have encountered a number of challenges in the scheme, often due to issues that are out of their control. These include:

- obtaining import permits, which often causes delays, as samples may be held up or even destroyed;
- availability of reagents or of laboratory staff;

- departure of the United Kingdom from the European Union, which led to disruption for some laboratories;
- coronavirus disease 2019 (COVID-19), which led to shutdown of transport routes, diversion of laboratory staff to other projects and lack of access to laboratories for staff; and
- fees introduced in 2020, which led to difficulties for a few laboratories, although not for the majority.

### 3.5 Overall benefits of the EQA scheme

The EQA scheme is growing as new laboratories enrol. The trend in performance by the number of submissions shows a clear improvement over time. Laboratories that were weaker at the start of the scheme have shown the most marked improvement in performance.

## 4. Presentation 3: priorities and models for inclusion of drug resistance markers in the current EQA scheme

### 4.1 Need for an antimalarial resistance marker

There is currently no EQA scheme for malaria drug resistance markers and molecular correction genotyping methods. Several genotyping methods have been used to detect antimalarial resistance markers, including next-generation sequencing. As partial resistance to artemisinins is evolving in Africa, it is essential to ensure that molecular data are collected and reported accurately. An EQA scheme for antimalarial resistance markers will make it possible to standardize the outputs of different genotyping methods and to compare the result from different studies and laboratories. In addition, the scheme will provide constructive feedback for improving the ability of participating laboratories to collect molecular data. An EQA scheme will be valuable for the International Standards Organization and for other purposes of accreditation.

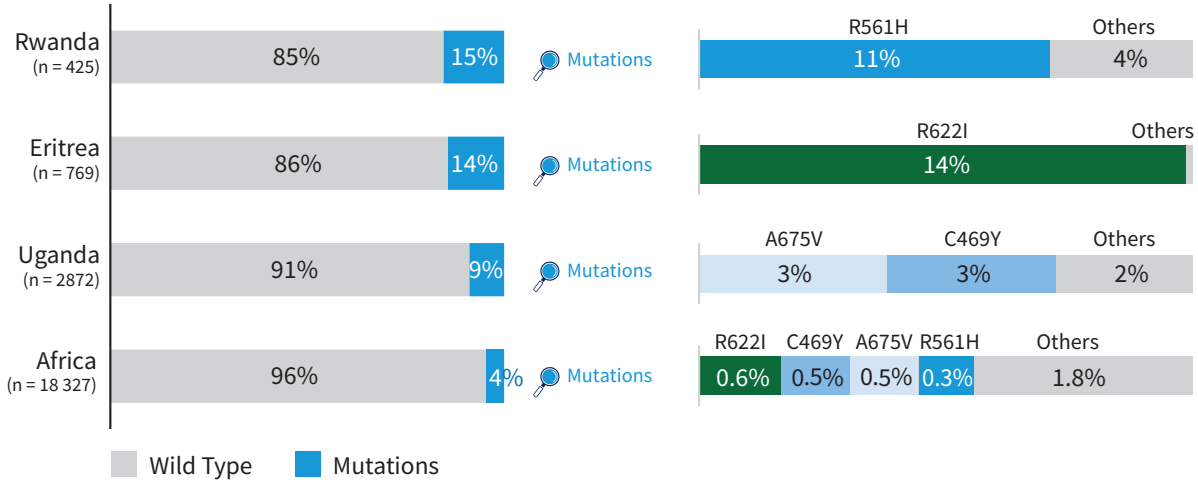
Partial resistance to artemisinins has been associated with various mutations in the *PfK13* propeller region of *P. falciparum* parasites. To confirm partial resistance to artemisinins at a site, good-quality evidence is required on the presence of a validated marker (≥ 5%) and of delayed clearance. Delayed clearance of parasites alone does not cause ACT treatment failure, while partial resistance to both artemisinins and the partner drugs has been the cause of high rates of ACT treatment failure.

### 4.2 Evolving antimalarial resistance in Africa

In November 2022, experts convened by the WHO Global Malaria Programme reviewed data on antimalarial resistance in Africa *(3)*. They found that the prevalence of molecular markers of partial resistance to artemisinins was high (> 5%) in three African countries: Eritrea, Rwanda and Uganda (Fig. 7). Subsequently, a high frequency of *PfK13* molecular markers of resistance was confirmed in the United Republic of Tanzania in patients with delayed clearance. Thus, partial resistance to artemisinins has been confirmed in four countries, with different *PfK13* markers predominating in each country (Fig. 7, without data for the United Republic of Tanzania). The data reveal emerging patterns of evolutionary diversity in *PfK13* genetic polymorphism in this African region. They do not

represent the current prevalence of *PfK13* resistance markers, which was not in the scope of this meeting. Nevertheless, ongoing studies in the region suggest that the prevalence of artemisinin resistance markers is evolving and should be carefully monitored. These observations highlight the importance of collecting quality-assured molecular data for taking public health action, including changing policy.

**Fig. 7. Prevalence of antimalarial resistance markers in eastern Africa**




Source: WHO *(3)*

## 4.3 Molecular markers of resistance

Table 5 lists the known molecular markers of resistance to various antimalarial. The table shows that there are well-characterized molecular markers for some of the partner drugs used in current ACTs, including mefloquine, sulfadoxine–pyrimethamine and piperaquine. For some partner drugs, however, there are no validated molecular markers of resistance, although research is under way. An EQA scheme for antimalarial resistance markers must cover all relevant markers of resistance and a molecular correction method.

**Table 5. Molecular markers of antimalarial resistance and the drugs against which they confer resistance**

| Antimalarial drug | Molecular marker |
|---|---|
| Artemisinin | *pfK13* SNPs |
| **Partner drugs** | |
| Mefloquine | *pfmdr1* copy number increase |
| Piperaquine | *Plasmepsin 2/3* copy number |
| Sulfadoxine | *Pfdhps* SNPs |
| Pyrimethamine | *pfdhfr* SNPs |
| Lumefantrine | No validated marker |
| Amodiaquine | No validated marker |
| Pyronaridine | No validated marker |
| **Other drugs** | |
| Chloroquine | *pfcrt* SNPs |
| Atovaquone | Cytochrome B SNPs |

## 4.4 Discussion of the presentations

One of the main points of discussion was the priorities for molecular markers to be included in the scheme. Those proposed during the meeting were:

- phase 1: *PfK13* SNPs;
- phase 2: ACT partner drug markers for SNPs and variation in copy number;
- phase 3: markers (currently *msp1, msp2* and microsatellites) used for molecular correction.

The other decisions discussed during the meeting were:

- whether the antimalarial resistance scheme could be included in the EQA scheme or whether a separate scheme would be necessary;
- the specimen types to be included: e.g. DBS and LB;
- the number of DBS and LB specimens to be included in each panel;
- whether mixed genotypes should be included;
- the parasite density in the specimens; and
- reporting format: only mutant SNPs, variation in copy number, allelic types and microsatellite marker length or some other format?

## 4.5 Parasite resource material

Dr David Fidock, University of Columbia (United States of America), described a specimen bank of *P. falciparum* parasites representing a wide geographical area over Africa, the Americas and Asia, that were genetically modified to carry various *PfK13* mutants. Table 6 lists the parasites available in the repository.

**Table 6. Parasites available in the University of Columbia repository**

| Parasite | Geographical origin | K13 mutants available |
|---|---|---|
| Dd2 | South-East Asia | WT, E252Q, P413A, G436V, F446I, C469Y, M476I, Y493H, R539T, I543T, P553L, P574L, C580Y, R622I, A675V, |
| Cam 3.I | Western Cambodia | WT, R539T |
| Cam 3.II | Western Cambodia | WT, A212T, E252Q, F446I, R539T, P553L, P574L, C580Y |
| Cam2 | Western Cambodia | WT, C580Y |
| Cam5 | Western Cambodia | WT, I543T |
| CamWT | Western Cambodia | A212T, C580Y |
| V1/S | Viet Nam | WT, R539T, C580Y |
| FCB | South-East Asia | WT, C580Y |
| Thai6 | Thailand | E252Q |
| Thai7 | Thailand | E252Q |
| RF7 | Cambodia | WT, A212T, R539T, C580Y |
| NF54 | Africa | WT, C580Y |
| F32 | United Republic of Tanzania | WT, M476I, R561H, M579I, C580Y |
| UG659 | Uganda | WT, M579I, C580Y, R622I |
| UG815 | Uganda | WT, M579I, C580Y, R622I |
| MAS-136 | Uganda | WT, C469Y, A675V |
| 3D7 | Africa | WT, M579I, R561H, C580Y |
| PAT-015 | Uganda | WT, C469Y, A675V |

### 4.6 Discussion of the presentation

Some unpublished studies in African countries such as Uganda were mentioned that show much higher levels of *PfK13* mutations (30–54% in some areas) than reported in the presentation, in which data from 2022 were used to highlight the evolutionary diversity of *PfK13*. One of the studies was published after the meeting *(6)*.

Participants also noted differences and similarities between the pattern of partial resistance to artemisinins in the African countries and those observed in South-East Asia. A greater range of mutations that confer resistance is found in Africa (e.g. R561H, R622I, A675V, C469Y, C469F), while C580Y is the predominant mutation in South-East Asia (with other mutations also present). Partial resistance to artemisinins associated with *PfK13* mutations appears to be less frequent on the African continent as a whole but is increasing rapidly in East Africa and the Horn of Africa. Partial resistance is evolving and is in its early phase in Africa, while resistance, including to partner drugs, is well established in South-East Asia. In East Africa and the Horn of Africa, the *561H* mutation currently appears to confer the most resistance.

The participants emphasized the importance of an EQA scheme for collecting reliable, quality-assured molecular data that are relevant for both Africa and other regions with endemic malaria.

## 5. Discussion

A general group discussion was held to consider the questions posed during the presentations.

### 5.1 Provider and scheme logistics

Participants agreed that WHO should include antimalarial resistance markers in the existing EQA scheme. They noted that, while inclusion of the new antimalarial resistance marker scheme into the existing EQA scheme would theoretically save time and costs, there are several barriers. First, most samples in the EQA panels are not *P. falciparum*. Secondly, not all the current participating laboratories test for antimalarial resistance. Thirdly, even if antimalarial-resistant parasites could be added to the EQA panels, inclusion of all relevant resistance markers (*PfK13*, partner drug resistance markers and molecular correction markers) in the scheme would require a larger specimen panel and result in a complex reporting system. With other logistical needs, it was considered that inclusion of molecular diagnosis in the current EQA scheme would be difficult.

A necessary step, therefore, is acquisition of additional *P. falciparum* samples to create entirely new panels of *P. falciparum* with and without antimalarial resistance markers. The additional EQA antimalarial scheme could be conducted with a separate panel of samples sent to laboratories that test for antimalarial resistance markers. If most of the laboratories that conduct EQA for antimalarial resistance markers also participate in the existing EQA scheme, a possibility would be an "EQA plus EQA MM of drug resistance" approach, whereby the new EQA antimalarial markers are included in the existing scheme and laboratories that participate in the antimalarial resistance scheme are sent the standard EQA panel plus an additional set of EQA antimalarial samples. This would save on logistics and shipping costs. The laboratories would analyse samples for the existing EQA scheme and also an additional panel of *P. falciparum* samples with and without antimalarial resistance markers. The choice of option will depend on the practicality of implementing the new antimalarial resistance markers scheme without compromising the current EQA scheme for molecular diagnostics.

## 5.2 Sample type

While the current scheme includes panels with both the DBS and the LB format, most participants agreed that DBS samples are the most useful for analysing antimalarial resistance markers, as most of the samples they receive and test are in this format. Use of DBS would ensure the best assessment of their performance in testing for antimalarial resistance markers.

## 5.3 Molecular markers

Substantial discussion was conducted on the molecular markers to be included in the scheme. Agreement was reached on the sets of markers that should be the highest priority for inclusion from the outset according to epidemiological evidence and the shared interests of the laboratories. Other markers should be considered for potential future use and may become more important as the scheme continues. Thus, as the markers included will probably evolve over time, it is not possible to decide beforehand which panels will be used in the future. While it was difficult to decide on the panels of markers to be included, markers for inclusion were prioritized as follows.

### 5.3.1 Artemisinin resistance marker PfK13

Participants agreed that the most important markers to be included in the scheme are those of the *PfK13* propeller domain, which confers partial resistance to artemisinin drugs. They agreed that the case of *PfK13* mutations is continuously evolving and that the scheme must therefore be adaptable to amending the list of mutations in the panel over time. This is especially true for African countries, and both commonly observed markers and rare markers should be included.

Although the case of *PfK13* is in flux and varies by region, it was considered that some of the mutations that predominate in different regions could be good proxies for detection of other emerging SNPs, particularly for laboratories in which sequencing-based assays are used. The recommended targets for the initial stage of the EQA MM of drug resistance programme were:

- *R561H* – Rwanda, Uganda and United Republic of Tanzania
- *R622I* – Eritrea and Ethiopia
- *C469Y/F* – Uganda
- *A675V* – Uganda
- *580Y* – South-East Asia, Papua New Guinea

These common mutations were considered of highest importance for inclusion from the onset, although they are by no means the only ones currently present in *P. falciparum* parasites. As the scheme advances, rarer mutations should be included, especially for laboratories in countries in which they are present.

While inclusion of a range of mutations is important to mirror the wide range present in circulating *P. falciparum* parasites, participants questioned whether all mutations should be included. For example, if *469Y* is tested, should every other mutation be assessed? Some members of the group considered that not all mutation should be included but only predominant mutations associated with resistance and a few rare mutations. The composition of the specimen panel could be modified for each round of EQA, so that a broad range of mutations is covered over time. Testing for a narrower range of mutations would allow the scheme to start earlier, as a wide range of mutations need not be included from the beginning.

It was also suggested that a DNA plasmid containing several versions of *PfK13* mutations be used rather than relying on naturally occurring *PfK13* mutants from clinical samples or cultured parasites. While a DNA plasmid might not represent natural specimens, it could be used as a positive control, the plasmid being diluted in blood before preparation of DBS for the scheme. The choice of specimen also depends on the method used to detect mutations, such as whole or partial genome sequencing or *PfK13*-specific Sanger sequencing as opposed to non-sequencing methods such as quantitative polymerase chain reaction (qPCR). In laboratories in which either sequencing method is used, a *PfK13* mutation in the specimen panel could be identified if those laboratories use methods for sequencing the entire *PfK13* gene and not just a segment. For laboratories that detect specific SNPs by other methods, such as qPCR, inclusion of any and all *PfK13* mutations in the panel might result in EQA failures that reflect methodological differences. One way of resolving the problem of selection of *PfK13* mutants would be to survey the methods that participating laboratories use to identify *PfK13* mutations in a questionnaire and use the information to develop a specimen panel that can be tested by the laboratory methods identified.

It was agreed that, while it is relevant to provide "pure" samples (i.e. samples containing only wild-type parasites or one particular mutant genotype), some samples of mixtures of wild-type and mutant samples, with more than one mutant in a sample, could also be provided. This would enable laboratories to assess their capacity to detect mutants present in samples at a low frequency, such as 30%, and inclusion of samples with a range of frequencies would allow laboratories to determine their limits of detection. While such low-frequency mutants would be more difficult to detect, they represent those that might be present in real samples collected in epidemiological studies or TES. It is therefore important to evaluate performance with samples of this type. Assessment of much lower frequencies could be considered in the future but was deemed of low priority for the initial EQA MM of drug resistance programme.

At the outset of the scheme, a small number of defined SNPs in various mixtures could be included to challenge the group and to assess their ability to detect SNPs. This would be challenging for laboratories but not impossible. It would be achievable for expert laboratories and would help other laboratories to improve their performance. Over time, the complexity and limits of detection could become more stringent. The scheme could include specimen types that test the sensitivity, limit of detection and accuracy of laboratory methods for detecting mutations.

### 5.3.2 Molecular correction

Molecular correction is an important tool for interpreting TES outcomes, especially in areas with high transmission, where new infections can occur during follow-up (28 or 42 days, depending on the ACT combination). Therefore, it is important to differentiate recrudescence from a new infection when an infection is observed during the TES follow-up period. Quality-assured molecular analysis is essential for assessing TES outcomes, as it is used as a basis for decisions about changing first-line treatment.

In 2021, the WHO Global Malaria Programme updated its recommendations for a genotyping method to distinguish reinfection from recrudescence in Africa *(2)*. The recommendation states that *msp1* and *msp2* should continue to be used, but *glurp* should be replaced by a microsatellite (*Poly-alpha*, *Pfpk2* or *TA1*). Many laboratories are optimizing the newly recommended method, while some may still be using other methods, including previously recommended WHO methods. Interpretation of the results of TES is difficult when different methods are used. An EQA scheme for molecular correction methods could improve collection of quality-assured results. A number of participants considered that molecular correction is extremely relevant and should be included in the scheme as soon as possible.

It was recognized that molecular correction is a different method from SNP detection and is a powerful tool for assessing treatment failure in a TES. Therefore, laboratories should be able to evaluate their performance. Paired samples could be tested and the laboratories asked whether the two samples were the same or different (indicating recrudescence or reinfection) and, potentially, how they differed. The group debated whether results could be "all or nothing", such as whether two samples were identical or not, or whether more detail would be necessary about how they differ. While minimal results might be required initially, in the future, laboratories could be asked to submit additional information such as the test and markers they used, definition of a match, full genotyping data and assay parameters.

A further suggestion was to include samples with a low level of a recrudescent *P. falciparum* strain with another strain, such as at a certain percentage that could reasonably be detected by currently used methods in a sample containing the same strain as in the paired sample, while the remainder of the sample would be of a different strain. This could be used to evaluate identification of the presence of mixtures with a recrudescent sample and a new infection. With different ratios of mixtures that include recrudescent samples at the lowest threshold of detection, the limit of detection for a mixed infection could be evaluated for different laboratories.

#### 5.3.3 Partner drug resistance markers

Resistance of partner drugs can result in failure of ACT treatment. Although inclusion of markers of partner drug resistance was discussed, there are currently no validated markers for the partner drugs in the most widely used ACT, lumefantrine and amodiaquine. Inclusion of these markers was therefore considered not to be of highest importance at the beginning of the scheme but could be considered for later inclusion if laboratories that conduct such analyses wish them to be included. The markers include those for sulfadoxine–pyrimethamine drug resistance – *pfdhps* and *pfhfr* SNPS – and chloroquine resistance markers such as *pfcrt* SNPs, as some mutations in this gene have been implicated in resistance to piperaquine, a partner drug in ACT.

Markers of mefloquine and piperaquine resistance (*pfmdr1* and *plasmepsin 2/3* copy number) could be included in the scheme later if laboratories express an interest. Piperaquine resistance was considered currently less relevant in Africa but may become more important.

Thus, while a focus on *PfK13* was considered a good starting point, other markers may become relevant in the future. As the range of mutations in malaria-endemic countries varies widely among regions, laboratories in different regions will probably focus on different markers. The range of markers included in the scheme will therefore have to be broad to accommodate such differences.

Inclusion of wild type samples was highlighted.

### 5.4 Methods

The group considered that the most common methods for detecting antimalarial resistance markers are Sanger sequencing, whole-genome sequencing and targeted genome sequencing. Some laboratories, however, use probe or amplification assays, such as qPCR and gel electrophoresis. Laboratories will be instructed to use their routine method, in the same way that they are requested to use their usual method in the current malaria EQA scheme. This may affect the level of detail that laboratories can report, which should be accounted for in the submission.

## 5.5 Material sourcing

Material could be obtained in various ways. Parasites could be cultured by partner laboratories that prepare final blood samples, or the cultured parasites could be frozen and shipped to UK NEQAS (P) to be mixed with whole blood and samples created in London. The consensus of the group was for the latter option, which would ensure that the sample panel is prepared exactly as intended and would avoid any potential mix with other strains. The parent material (pre-mixing) could be sent to reference laboratories to confirm the pedigree of the material before mixing it with whole blood and other strains.

The group considered that the density of samples should not be too low at the start of the new scheme, as it would be very difficult for laboratories to detect resistance markers. Real samples from TES and surveillance studies could, however, include some with very low parasite densities, such as from asymptomatic patients. Inclusion of low-density samples would be important to assess a laboratory's limit of detection, which varies by laboratory. Therefore, the preferred option was to start with a higher density panel and provide lower densities in the future. That would also allow laboratories to assess their limit of detection. Agreement was not reached on the threshold, but it was suggested that it not be below 200 parasites/µL, which may be the lower threshold of cutoff for inclusion in most TES.

Participants were invited to share samples for the specimen panel. It was agreed that use should be made of the large repository of mutant strains compiled by Dr David Fidock, who has also developed recombinant technology for making new mutant genotypes and introducing them into the genetic backgrounds of different parasites. It was agreed that his resources could be used as a specimen bank that is open to inclusion of appropriate specimens from other investigators.

As noted above, use of plasmids was discussed, whereby plasmids could be created that contain a number of resistance-conferring mutations, which could be spiked into whole blood. This approach would be useful for creating unique samples with particular mixes of markers, which would be difficult to create from existing strains. It was acknowledged that samples in this format would be different from samples from epidemiological studies that laboratories test routinely, which are mainly in the form of DBS. As the aim of the scheme is to assess the performance of laboratories' usual methods, use of plasmids would affect the results. Some participants considered that addition of plasmids to blood and use of the mixture to prepare DBS would result in samples that were substantially similar to samples prepared from parasite-containing blood. Others considered that use of plasmids would be a challenge for DNA isolation methods in some laboratories. The performance of contrived plasmid-based samples could be considered and tested in the EQA programme. Nonetheless, it was agreed that plasmid DNAs are a valuable source of positive controls for molecular assays. It was noted that plasmids have been used in other schemes, such as during the outbreaks of Ebola virus disease, as shipping of virus-containing samples is prohibited. While clinical samples are preferable, inclusion of plasmid-spiked samples in each panel of samples could result in a larger mix of markers.

## 5.6 Reporting

Differences in the methods and capacities of laboratories will complicate the design of a submission portal that allows straightforward input of identified markers while allowing input of extended information. An interface that can capture relevant information while avoiding complex data analysis from free-text answers will be required. Participants discussed whether the input should comprise simply the absence or presence of

resistance markers or whether the results should include identification and quantification of SNPs. The input will depend on the method used, as laboratories that can sequence the whole *PfK13* gene, for example, will be able to detect multiple SNPs, while laboratories that use qPCR will be limited to certain SNPs.

The group accepted that a "less is more" approach would be sufficient for the EQA scheme, especially at the outset, with simpler samples and simpler reporting during the first few rounds of distribution. The requirements could become more complex as the scheme evolves.

As the complexity of the interface is similar to that of proficiency testing for cancer diagnosis, it was suggested that cancer EQA schemes be studied. It was further suggested that a trial version of the submission portal be designed, that could be reviewed by the panel and then amended.

### 5.7 Preparation for extension of the scheme and cost

Participants agreed that laboratories should be asked to participate in a survey in order to assess interest in a new antimalarial resistance marker scheme. The survey would include questions on the capacity of a laboratory to conduct such analyses, the molecular markers they can detect, the method used, limit of detection and willingness to pay, similar to the survey conducted in 2014 before the malaria EQA scheme was established. This information would assist the organizers in planning extension of the scheme and the design of a submission portal.

Participants agreed that a "pilot" round would be useful, including the laboratories of the participants in the meeting. The laboratories could provide feedback on what did and did not work for them, and the coordinators of the scheme could consider improvements in aspect such as source material preparation, shipment and the results submission portal. Such a pilot round could allow resolution of problems before the scheme is extended to the full list of participating laboratories.

Costing was not discussed at the meeting. WHO will estimate the costs of the scheme and identify potential funding sources.

## 6. Overall conclusions and next steps

The conclusions of the meeting are listed below.

- **Extension of the NAAT EQA scheme**. Consensus was reached on extending the NAAT EQA scheme to include drug resistance markers and, eventually, molecular correction methods.
- **Integration with EQA of markers of antimalarial (AM) resistance**. The antimalarial resistance marker EQA scheme could be included in the WHO and UK NEQAS (P) collaboration as a combined WHO malaria NAAT and MM of drug resistance EQA scheme, to optimize resources and reduce costs.
- **Specimen panel sourcing**. External collaborators will provide specimen panels and work with UK NEQAS in their preparation and distribution.

- **Preferred sample type: dried blood spots**. Dried blood spots (DBSs) were identified as the preferred sample type because of the ease of preparation, storage and shipping.

- **Markers for initial inclusion**. *PfK13* markers were considered to be critical for immediate inclusion in panels. Partner drug resistance markers are considered a lower priority but could be included in the future according to their relevance. The importance of molecular correction methods was emphasized and should be included as soon as possible in laboratory evaluation.

- **Adaptability of the scheme**. The scheme should be adaptable with respect to the resistance markers to be included, in view of the changing epidemiology of malaria. Specimen panels should include various mixed specimens with different mutations or genotypes in different ratios in order to assess detection limits, sensitivity and accuracy.

- **Learning from other EQA schemes**. Insights from other EQA schemes, such as those for proficiency in cancer diagnosis, may offer guidance for designing the new scheme.

## Next steps

The meeting strongly supported extension of the EQA scheme to include antimalarial resistance markers, which was considered an essential step in strengthening global reporting of resistance data. It was proposed that a survey of meeting participants be conducted to gauge their interest and capacity for pilot-testing the extended scheme by current EQA participants.

# References[1]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1 All references accessed on 29 January 2025.

# Annex 1. Agenda

| Friday 14 July 2023 | | |
|---|---|---|
| 15:00 –15:10 | Welcome and introductions | Sean Murphy |
| 15:10–15:20 | Review of meeting objectives and agenda | Charlotte Rasmussen |
| 15:20–15:50 | UK NEQAS – EQA provider's role and functions | Peter Chiodini |
| 15:50–16:20 | WHO Malaria NAAT EQA – Overview and challenges (materials and sourcing) | Rebecca Thomson |
| 16:20–17:00 | Priorities and models for inclusion of drug resistance markers | Venkatachalam Udhayakumar |
| 17:00–18:00 | Discussion | All |
| 18:00 | Closure of the meeting | |

# Annex 2. List of participants

## Experts

Jeffrey A. Bailey
Department of Pathology and Laboratory Medicine, Warren Alpert Medical School, Brown University, Providence, United States of America

Khalid Beshir
London School of Hygiene and Tropical Medicine, Faculty of Infectious and Tropical diseases, London, United Kingdom of Great Britain and Northern Ireland

Qin Cheng
Drug Resistance and Diagnostics, Australian Defence Force Malaria and Infectious, Disease Institute, Brisbane, Australia

Peter Chiodini
University College London Hospitals, NHS Foundation Trust, London, United Kingdom of Great Britain and Northern Ireland

David A. Fidock
Department of Microbiology and Immunology, Columbia University, New York, United States of America

Sean C. Murphy (chair)
Laboratory Medicine, University of Washington Medical Center, Seattle, United States of America

Christian Nsanzabana
Swiss Tropical and Public Health Institute, Basel, Switzerland

Lynette Isabella Ochola-Oyier
Biosciences Department, Kemri-Wellcome Trust Research Programme, Kilifi, Kenya

Philip Rosenthal
Department of Medicine, University of California, San Francisco, United States of America

Sarah Volkman
Harvard School of Public Health, Boston, United States of America

## Rapporteurs

Rebecca Thompson
Independent consultant, London, United Kingdom of Great Britain and Northern Ireland

Venkatachalam Udhayakumar
Senior consultant in public health and biotechnology, Decatur, United States of America

Charlotte Rasmussen
Diagnostics, Medicines and Resistance Unit, Global Malaria Programme, WHO, Geneva, Switzerland

Marian Warsame
WHO consultant, Gothenburg, Sweden

## Observers

Molly M. Freeman
Malaria Laboratory Research and Development Team, Centers for Disease Control and Prevention, Atlanta, United States of America

Mateusz Plucinski
Malaria Branch, Centers for Disease Control and Prevention, President's Malaria Initiative, Atlanta, United States of America

Estee Torok
Global Health – Malaria, Bill & Melinda Gates Foundation, London, United Kingdom of Great Britain and Northern Ireland

## WHO Secretariat

Andrea Bosman, Diagnostics, Medicines and Resistance Unit, Global Malaria Programme

Anderson Chinorumba, WHO consultant, Harare, Zimbabwe

# Annex 3. Declarations of interest

All experts have submitted their declarations of interests, which were assessed by Ms Charlotte Rasmussen, Technical Officer, Diagnostics, Medicines and Resistance Unit, WHO Global Malaria Programme. Of the eleven independent experts, ten independent experts could fully participate in the discussions and deliberations of the consultation.

Six reported no interests and five reported interests. For four of the five experts who declared interests, these interests were considered not relevant to the subject of the technical consultation and its outcome. One expert, Dr Peter Chiodini, declared interests that were considered significant and relevant to the subject of the meeting. For this reason, Dr Chiodini participated in the meeting but could not contribute to the final meeting deliberations.

For further information please contact:

**Global Malaria Programme**
**World Health Organization**
20 avenue Appia
1211 Geneva 27
Switzerland
Email: GMPinfo@who.int