WHO Malaria NAAT EQA scheme: Using EQA specimens as controls

Numerous participants of our WHO Malaria molecular EQA scheme have approached us in the past regarding control materials. The queries include:

1. Can the EQA specimens be used for validation of assays/techniques in their labs?
2. Can the EQA specimens be used as positive or negative controls in their assays?
3. Can the EQA specimens be used as standardisation material for quantification?
4. Can the EQA specimens be used for measuring limit of detection of their assays?
5. Can the WHO scheme provide standardised material that can serve as controls for participating labs especially for purposes such as 1-4 stated above?

This document has been prepared to answer these queries.

1. Can the EQA specimen be used for validation of assays/techniques?

The EQA materials sent to participants in the WHO Malaria Molecular EQA scheme are not standards and therefore cannot be used for validation of assays/techniques. A recognised International Standard is required for that purpose.

The 1st WHO International Standard for *P. falciparum* DNA NAT-based assays is available via NIBSC (https://www.nibsc.org/documents/ifu/04-176.pdf) and is intended to:

- ensure consistent assay implementation between laboratories
- allow inter-laboratory comparisons
- provide materials for assay validation
- and in particular, to generate secondary standards to monitor routine assay use (Padley et al, 2008)

Participants are strongly advised to acquire and use the WHO International Standard for this purpose. The attached Instructions for use, NIBSC code: 04/176, provide details on reconstitution, preparation and storage of aliquots.

2. Can the EQA specimens be used as positive or negative controls in their assays?

Participants might choose to use the leftover EQA material as a positive or negative run controls in their assays. However, the participant must ensure appropriate storage and aseptic handling of the EQA specimen; especially once the freeze-dried specimen has been re-suspended in molecular grade water.

We highly recommend making smaller aliquots of the EQA specimen sent and storing them as soon as possible at -20 or -80°C for future use.

Please note that neither WHO nor UK NEQAS can be held accountable for contamination of any kind once the EQA specimen vials have been opened.

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WHO/UCN/GMP/2023.02

3. Can the EQA specimens be used as standardisation material for quantification?

Qualitative vs. quantitative nature of the specimens: The EQA specimens provided to participants go through rigorous quality checks. This includes pre-distribution PCR checks by 6 independent Referee laboratories. The Referee labs are international and world-leaders in their field. Those Referee labs that are able to perform quantitative analyses, submit those results to UK NEQAS Parasitology. However, there is variation among the Referee labs primarily due to one or more of the reasons below:

- Lack of an International Standard for non-falciparum species
- Difference in units reported:
  - Copy number
  - Total DNA content
  - Parasite count [parasites/mL or parasites/uL]
- Difference in assays used (DNA-based versus RNA-based)

As a result of the above, whilst there is always a consensus regarding the qualitative presence or absence of malaria parasites (all 5 species) in the specimens, there is not uniform consensus in the way that the absolute quantity of parasites in the specimens is reported.

Therefore, we do not advise using the EQA specimens provided as quantitative controls. We will not be able to inform the participants of an absolute parasitaemia value for each positive specimen due to the multiple reasons for potential variation described above. However, if needed, the participant can perform quantification of the EQA specimens using their own protocols. The samples can then be serially diluted to create a series of samples of known parasitaemia. Please note that WHO or UK NEQAS cannot be held accountable for this parasitaemia estimation.

Commercially available standards or calibrators:

DNA Standard: As mentioned above, the 1st WHO International Standard for P. falciparum DNA for use in NAT-based assays is available via NIBSC (https://www.nibsc.org/documents/ifu/04-176.pdf). Secondary and Tertiary Standards can be prepared from International Standards as described in https://apps.who.int/medicinedocs/en/m/abstract/Js23325en/

RNA calibrator: Participants using RNA-based assays are advised to contact Dr Sean Murphy (http://depts.washington.edu/labweb/Faculty/MurphySean.htm) regarding a *P. falciparum* RNA calibration standard for RNA-based molecular assays.

4. Can the EQA specimens be used for measuring limit of detection of their assays?

The WHO International Standard is the only validated Standard for malaria NAAT assays. Once reconstituted as per the attached Instructions for Use, it may be serially diluted in parasite-negative whole blood and used to determine a limit of detection for *P. falciparum* DNA. An illustrative example is given in Appendix A.

P. falciparum EQA specimens may be calibrated by participants against the International Standard and also used to provide additional data on limit of detection.

At present, there are no International DNA standards for non-falciparum malaria parasites, so the EQA samples for those species cannot yet be calibrated in that way.

5. Can the WHO scheme provide standardised material that can serve as controls for participating labs especially for purposes such as 1-4 stated above?

The samples distributed in this EQA Scheme are provided solely for that purpose. They are not produced as Standards or as run controls. As described above, the additional ways in which participants may choose to make use of material left over, after testing and reporting EQA results, is their own responsibility.

6. How to cite these materials in any reference? : we understand and appreciate that the EQA specimens provided might be used by participants for various studies and that they would like to identify the source of such specimens (especially in publications). Please note that both WHO and UK NEQAS support the use of these specimens for **research or quality assurance purposes, only**. Within these remits, if the participants would like to publish and acknowledge the source of the specimens, the material can be attributed to WHO c/o UK NEQAS Parasitology, hosted by Public Health England, UK.

Reference:

Padley (2008) https://malariajournal.biomedcentral.com/articles/10.1186/1475-2875-7-139

Appendix A: Measuring a limit of detection for *P. falciparum* DNA

Example: The WHO International Standard (Int Std) contains $1x10^9$ IU per mL ($1x10^6$ IU per µL)

It was produced from a blood sample containing approximately 9.8% parasitaemia which approximates to 490,000 parasites per µL. However, whilst participants may find this a useful comparison, only results in IU derived from calibration against the Int Std can be considered valid.

Dilution series:

NB Dilute in parasite-free whole blood (e.g. out of date whole blood from a blood bank if available and permitted). Make serial dilutions and mix thoroughly each time before making the next one using a roller device as commonly used in haematology laboratories.

Reconstituted Int Std neat	= $1x10^9$ IU per mL	Approx 490,000 parasites per µL*
1 in 10 dilution	= $1x10^8$ IU per mL	Approx 49,000 parasites per µL*
1 in 100 dilution	= $1x10^7$ IU per mL	Approx 4900 parasites per µL*
1 in 1000 dilution	= $1x10^6$ IU per mL	Approx 490 parasites per µL*
1 in 10,000 dilution	= $1x10^5$ IU per mL	Approx 49 parasites per µL*
1 in 100,000 dilution	= $1x10^4$ IU per mL	Approx 4.9 parasites per µL*
1 in 1,000,000 dilution	= $1x10^3$ IU per mL	Approx 0.49 parasites per µL*
1 in 10,000,000 dilution	= $1x10^2$ IU per mL	Approx 0.049 parasites per µL*

*Expressed per microlitre as this is the unit most commonly used in practice. This is for comparison only, to help put a limit of detection in IU into context.

When an end point has been reached, make intermediate dilutions. For example, if the assay is positive at $1x10^4$ IU per mL but negative at 1×10^3 IU per mL, repeat with dilutions at $1x10^4$ IU per mL 0.75×10^4 IU per mL, 0.5×10^4 IU per mL, 0.25×10^4 IU per mL and 1×10^3 IU per mL.

In addition, please consult https://apps.who.int/medicinedocs/en/m/abstract/Js23325en/