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Geneva, 24 to 28 August 2020

Collaborative study to evaluate the proposed First World Health Organization International Standard for *Plasmodium vivax* antigens

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NOTE:
This document has been prepared for the purpose of inviting comments and suggestions on the proposals contained therein, which will then be considered by the Expert Committee on Biological Standardization (ECBS). Comments MUST be received by **10 August 2020** and should be addressed to the World Health Organization, 1211 Geneva 27, Switzerland, attention: Technologies, Standards and Norms (TSN). Comments may also be submitted electronically to the Responsible Officer: **Dr Ivana Knezevic** at email: knezevici@who.int.

Summary

An international collaborative study was conducted to assess the suitability of a candidate *Plasmodium vivax* preparation, assigned product code 19/116, for use as a World Health Organization (WHO) International Standard (IS) in the detection of *P. vivax* antigen lactate dehydrogenase (PvLDH). The potencies of the candidate IS and a range of *P. vivax* clinical isolates were evaluated using commercially available Enzyme-linked immunosorbent assays (ELISA) and a wide variety of malaria rapid diagnostic tests (RDT).

The samples for evaluation were distributed to 16 laboratories in 11 different countries. Seven participating laboratories carried out ELISAs and fifteen participants performed RDTs. The samples were assayed on four separate days and the data were collated and analysed by the National Institute for Biological Standards and Control (NIBSC). The results demonstrated that PvLDH was detected consistently by the majority of participants and that reporting potency and limit-of-detection relative to this preparation resulted in a large reduction in variance between laboratories. The candidate standard is stable under recommended storage conditions of -70 ℃ or below, and therefore suitable for long term use. Real-time and accelerated stability studies of the candidate IS are ongoing.

Based on the results of the collaborative study, it is proposed that the lyophilised *P. vivax* candidate IS preparation, code number 19/116 should be established at the 1st IS for *P. vivax* antigen (LDH), with 1000 International Units (IU) per ampoule. The unitage proposed is arbitrary but, on the basis of the data presented in this study the limit of detection of the tests analysed would range from 0.24 – 62.52 IU / mL.

The intended use for the candidate IS is for the standardisation, and evaluation of performance and sensitivity of *P. vivax* antigen detection tests as well as for the calibration of secondary reference materials.

Introduction

Plasmodium vivax is the second most abundant of the human malaria parasites with an estimated 7.5 million cases (95% CI: 5.9 – 9.3 million) across a broad range of WHO regions; African, Americas, Eastern Mediterranean, South East Asia and Western Pacific[1]. *Plasmodium vivax* accounts for >75% of all malaria in the Americas and 50% in South East Asia. Accurate diagnosis of vivax malaria is of critical importance to inform the appropriate treatment[1].

Thus far, the development of WHO International Standards for malaria has focused on *Plasmodium falciparum* and standards for; nucleic acid amplification technologies (NAAT)[2], antigens[3], as well as a reference reagent for serology [4,5]. These have all been completed and products established. Current work is now addressing the development of reagents specifically for *Plasmodium vivax*. At the 2017 meeting of the Expert Committee on Biological Standardization (ECBS) projects to develop; an antigen standard to support rapid diagnostic testing and a serology standard to support vaccine development and immunoepidemiology, were endorsed[6]. This study addresses the development of an International Standard for *P. vivax* antigens that will support the development and quality control of Rapid Diagnostic Tests (RDTs).

The WHO recommends that all suspected cases of malaria are confirmed by parasitological diagnosis with microscopy or RDTs prior to the initiation of antimalaria chemotherapy[7]. Malaria RDTs detect *Plasmodium*-specific antigens in patient blood samples and provide a convenient alternative to light microscopy. Malaria RDTs can have a range of different antigen targets that can be; specific for *P. falciparum* (Pf), specific for *P. vivax* (Pv), Pan-specific (for all human malaria), and also a line for Pvom (*P. vivax*, *ovale* and *malariae*). The main *P. vivax* antigen targeted by RDTs is lactate dehydrogenase (Pv-pLDH) and is the focus of this study. Large-scale use of RDTs in the field has revealed the performance of RDTs for detecting *P. vivax* to be highly variable in endemic settings[8]. This variability is due to product-to-product and lot-to-lot differences, lack of stability at high temperatures, and variable product formats.

The WHO-FIND product testing programme started in 2007 with the objective of assessing the performance of commercially available malaria RDTs with standard *P. falciparum*, *P. vivax*, and negative samples from diverse geographical endemic settings[9]. The programme has been pivotal in ensuring quality control of RDTs and informing countries on their procurement decisions. However, the collection, preparation, characterization, and transport of clinical samples for product testing was proven to be difficult and expensive to maintain in the long-term; therefore, the role of product evaluation was transferred to WHO Prequalification of IVDs Programme in 2018. The testing, for pre-qualification, remains centralized at the USCDC but there remains a desire to enable decentralized testing for downstream lot monitoring and for this it is essential that high quality reference materials are available. Critical to this cascade is the presence of an International Standard.

This report describes the preparation of a candidate International Standard and a collaborative study to assess its suitability to serve as the 1st WHO International Standard for *P. vivax* antigen (PvLDH). The study uses several different ELISA kits and currently available RDTs to study *P. vivax* clinical samples. It assesses the performance of the candidate material in comparison to clinical isolates from the WHO malaria specimen bank as well as to recombinant protein. It is expected that both locally sourced clinical samples as well as recombinant proteins will have a role to play as secondary standards in future quality control of RDTs and it is intended that

this source of parasite derived PvLDH in the candidate will provide the primary reference point for these materials as they are developed. The antigen detection tests used in this study consist of two different parts; ELISA and RDTs. Study participants took part in either or both parts of the study. The aim of the collaborative study was to determine whether the candidate IS is:

- Detectable in a wide range of *P. vivax* antigen detection assays
- Facilitates harmonisation of measurements across different assays/laboratories
- Equivalent (behaves similarly in *P. vivax* antigen detection tests) to Pv-pLDH found in *P. vivax* clinical isolates

Materials and Methods

Biosamples

A. Candidate International Standard – 19/116

Source material for the IS comprises red blood cell (RBC) lysates from *P. vivax*-infected donors. RBC lysates were obtained from 20 adults with a confirmed *P. vivax* malaria diagnosis between February and June 2018 as part of a specimen collection study implemented in health centers/posts in Iquitos, Peru by Foundation for Innovative New Diagnostics (FIND) in collaboration with Universidad Peruana Cayetano Heredia (UPCH). Signed informed consent forms were collected prior to sample collection. The study was approved by the Institutional Committee for Research Ethics of UPCH. Prior to blood donation, each donor was screened for and found negative for human immunodeficiency virus (HIV-1/2), hepatitis C virus (HCV) and hepatitis B virus (HBV). For the viral screening, the following RDTs according to the manufacturers' instructions: Determine HIV-1/2 (Alere; 7D2342), SD BIOLINE HCV (Standard Diagnostics, Inc.; 02FK10), and VIKIA HBS AG CE (Biomerieux; 31124). Based on the on-site microscopic analysis of blood samples, the samples had a mean average parasitaemia of 6575 parasites/µL and constituted a total volume of ~ 425 mL (Table 2).

The samples were transferred to NIBSC, U.K. for further characterization and preparation of candidate material. Results of the on-site viral screening were subsequently confirmed by serological testing in-house at NIBSC. HBV and HCV tests were performed using viral marker detection on a Roche cobas 6800 by method detailed in manufacturers operation manual. HIV-1/2 and HBsAg tests were done with the use of DiaSorin Liason XL for testing plasma pools for the presence of HBsAg and HIV Ab/Ag by method detailed in manufacturers operation manual.
The concentration of Pv-pLDH in each red blood cellRBC fraction was estimated by quantitative ELISA (Qualisa, Qualpro Diagnostics). Serial dilutions of each sample were assayed and potency calculated relative to a reference curve generated with recombinant PvLDH (Span Diagnostics). One sample (ID: MA00703P0147) was found to be negative for PvLDH and excluded from all further work. The estimated PvLDH antigen concentration in the remaining 19 RBC fractions of the Peruvian clinical isolates are listed in Table 2.

B. P. vivax clinical isolates

Three *P. vivax* clinical isolates, at 2,000 parasites/µL (Table 3), were provided by the WHO malaria specimen bank at the Centers for Disease Control and Prevention (CDC) for use in the collaborative study. These were selected to increase geographic coverage in the collaborative study and came from Ethiopia, The Philippines and Cambodia.

C. Recombinant Plasmodium vivax lactate dehydrogenase (PvpLDH)

Recombinant PvLDH with batch number PV-1905001 and catalogue number A130112-03 at a concentration of 1 mg/mL and molecular weight of 36kDa was obtained from Span Diagnostics SARL. This product was produced in *E. coli* and purified by immobilised metal affinity chromatography.

An additional recombinant protein was obtained from Microcoat (Catalogue number: 890025). This product was formulated and dried down in a defined matrix. The protein is fused to a Glutathione-S-transferase (GST)-tag and produced recombinantly in a heterogenous

expression system and purified via affinity chromatography. The GST-Pv-LDH had a molecular weight of 60.5 kDa.

Product development

A. Trial pool: ELISA and RDT testing

0.5 mL of each of the 19 RBC lysates were pooled in a 15 mL falcon tube to form a representative trial sample. The objective was to establish a balance between batch size and reactivity on the kits and assess suitable parameters for the final product.

The pool was also titrated in duplicate on four *P. vivax* detecting RDT products in listed below to determine a limit of detection (LoD);

1. SD BIOLINE Malaria Ag P.f/P.f/P.v supplied by Standard Diagnostics (Abbott Diagnostics Korea Inc.), Cat number 05FK120.
2. SD BIOLINE Malaria P.f/P.v supplied by Standard Diagnostics (Abbott Diagnostics Korea Inc.), Cat number 05FK80
3. EzDx^{TM} Malaria Pan/Pf Rapid Test Detection kit supplied by Advy Chemical Private limited, Cat number RKMAL001
4. Advantage Pan Malaria card supplied by J.Mitra and Co. Pvt. Ltd. Cat number IR013050.

B. Trial pool: Lyophilisation

A trial lyophilisation was performed on the pooled material diluted 1:7 in 10 mM Tris pH 7.4, 5% trehalose, 1 mM EDTA. The dilution selected reflects the desire to have a complete dose response curve whilst enabling a significant batch size. 50 µµ of buffer diluted pooled 19 RBC lysates were sent to the Centre for Biological Reference Materials (CBRM) of the NIBSC. Two different fill options were evaluated: 0.25 mL and 0.5 mL. Filling was performed in 2.14/2.15 CBRM using the Hamilton dispenser. Filling was on ice with stirring throughout the fill. Three checkweights were measured for all fill options. 13mm diameter igloo closures were inserted halfway down each ampoule.

The trays were loaded onto the top shelf of a Virtis Genesis dryer (serial number 216249) which had been precooled to -50°C. The freeze-drying cycle ran over 4 days (5692 minutes, 94.9 hours). The ampoules were backfilled with N_2 gas and stoppered before removing. The ampoules were sealed manually using an Adelphi sealer.

C. Definitive fill of candidate material (19/116)

Conditions for the definitive fill were established from the trial data. A fill volume of 0.5 mL was selected for preferential stability and improved cake but with the intention to resuspend in 0.25 mL in order to achieve sufficient PvLDH concentration for dose-response curves and RDT detection. The final average parasitemia in a reconstituted vial would be equivalent to approximately 3300 parasites per µL whole blood, or approximately 2200 ng / mL based on an average of the values listed in **Table 2**. This is higher than the top theoretical concentrations used in the product testing programme (which is 2000 p/µL) and would be expected to be sufficient for all good RDTs.

A larger pool of the same 19 samples was created in a class II microbiological safety cabinet with aseptic technique to maintain sterility. All RBC fractions were moved from -80ºC to -20ºC freezer a day before pooling. On the morning of the fill the RBC fractions were thawed on the bench at room temperature. The pool was diluted 1:4 into 11.43 mM Tris pH 7.4, 6.43 % trehalose, 1.14 mM EDTA buffer. The pool was dispensed in 0.5 mL aliquots into 2.5 mL autoclaved DIN glass ampoules using a Bausch & Strobel automated AFV5090 ampoules filling line. The bulk was maintained at ambient temperature and continually stirred to maintain homogeneity. Freeze drying was performed using a 4-day cycle with freeze-drier template cycle number FD0154 V01. The definitive fill commenced on 16th May 2019 at the Centre for Biological Reference Materials (CBRM) of the NIBSC.

D. Accelerated degradation studies

An accelerated degradation study has been set up in order to predict the long-term stability of the candidate *P. vivax* preparation (19/116). Ampoules of 19/116 were placed at -150 ºC, -70 ºC, -20 ºC, +4 ºC, +20 ºC, +37 ºC, +45 ºC and +56 ºC and will be retrieved at specific time points to check PvLDH stability. To date accelerated degradation data have been obtained for the 36 and 40 weeks (9 and 10 months respectively). Samples were reconstituted in whole blood and PvLDH levels measured. The ELISA protocol used was the same QUALISA SOP for pLDH ELISA for malaria antigen used in the collaborative study (Appendix 3 Instructions for use

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WHO International Standard
1st WHO International Standard for Plasmodium vivax antigen (LDH)
NIBSC code: 19/116
Instructions for use
(Version 1.00, Dated)

1. INTENDED USE

This preparation contains red blood cell (RBC) lysates from P. vivax-infected donors from Peru. The intended use for is for the standardisation, and evaluation of performance and sensitivity of P. vivax antigen detection tests that detect P. vivax lactate dehydrogenase (PvLHDH) as well as for the calibration of secondary reference materials.

2. CAUTION

This preparation is not for administration to humans or animals in the human food chain.

The preparation contains material of human origin, and either the final product or the source materials, from which it is derived, have been tested and found negative for HBsAg, anti-HIV and HCV RNA. As with all materials of biological origin, this preparation should be regarded as potentially hazardous to health. It should be used and discarded according to your own laboratory's safety procedures. Such safety procedures should include the wearing of protective gloves and avoiding the generation of aerosols. Care should be exercised in opening ampoules or vials, to avoid cuts.

3. UNITAGE

This material has an assigned unitage of 1000 International Units of PvLDH per ampoule.

4. CONTENTS

Country of origin of biological material: Peru.
Each ampoule is a lyophilate of a preparation that contained 0.5 ml of lysed red blood cells from P. vivax infected donors diluted 1:4 into 11.43 mM Tris pH 7.4, 6.43 % trehalose, 1.14 mM EDTA buffer.

5. STORAGE

This preparation should be stored at -20°C or below on receipt. If the material is to be stored for more than 3 months prior to use storage at -70°C is recommended.
Please note: because of the inherent stability of lyophilized material, NIBSC may ship these materials at ambient temperature.

6. DIRECTIONS FOR OPENING

DIN ampoules have an 'easy-open' coloured stress point, where the narrow ampoule stem joins the wider ampoule body. Various types of ampoule breaker are available commercially. To open the ampoule, tap the ampoule gently to collect material at the bottom (labelled) end and follow manufactures instructions provided with the ampoule breaker.

7. USE OF MATERIAL

No attempt should be made to weigh out any portion of the freeze-dried material prior to reconstitution
This material is supplied lyophilised and before use should be reconstituted in 0.25 mL of whole blood. Reconstituted material should be used on the day of reconstitution.

8. STABILITY

Reference materials are held at NIBSC within assured, temperature-controlled storage facilities. Reference Materials should be stored on receipt as indicated on the label.

NIBSC follows the policy of WHO with respect to its reference materials.

9. REFERENCES

Charles Olomu, Lynne M. Harris, Peter Rigsby, Eleanor Atkinson, Seda Yerlikaya, Xavier Ding, Paul W. Bowyer,* and the Collaborative Study Group (2020). Collaborative study to evaluate the proposed First World Health Organization International Standard for Plasmodium vivax antigens. WHO/BS/2020.xxxx

10. ACKNOWLEDGEMENTS

This material was developed in collaboration with the Foundation for Innovative New Diagnostics (FIND). We gratefully acknowledge the significant contributions of the collaborative study group in the development of this IS. We would like to thank Professor Dionicia Gamboa and Dr. Katherine Torres from Malaria Laboratory in UPCH (Lima, Peru) and the study staff in Iquitos, Peru for undertaking the specimen collection study, Further acknowledgment is extended to the patients who kindly donated samples for this project. We also thank the WHO malaria specimen bank at the US CDC for providing clinical isolates for the collaborative study.

11. FURTHER INFORMATION

Further information can be obtained as follows;
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JCTLM Higher order reference materials:
http://www.bipm.org/en/committees/jc/jctlm/
Derivation of International Units:
http://www.nibsc.org/standardisation/international_standards.aspx
Ordering standards from NIBSC:
http://www.nibsc.org/products/ordering.aspx
NIBSC Terms & Conditions:
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13. CITATION

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14. MATERIAL SAFETY SHEET

Classification in accordance with Directive 2000/54/EC, Regulation (EC) No 1272/2008: Not applicable or not classified

Physical and Chemical properties			
Physical appearance: Freeze-dried		Corrosive:	No
Stable:	Yes	Oxidising:	No
Hygroscopic:	No	Irritant:	No
Flammable:	No	Handling:See caution, Section 2	
Other (specify):	Contains material of human origin		

Toxicological properties	
Effects of inhalation:	Not established, avoid inhalation
Effects of ingestion:	Not established, avoid ingestion
Effects of skin absorption:	Not established, avoid contact with skin

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Suggested First Aid	
Inhalation:	Seek medical advice
Ingestion:	Seek medical advice
Contact with eyes:	Wash with copious amounts of water. Seek medical advice
Contact with skin:	Wash thoroughly with water.

Action on Spillage and Method of Disposal
Spillage of ampoule contents should be taken up with absorbent material wetted with an appropriate disinfectant. Rinse area with an appropriate disinfectant followed by water. Absorbent materials used to treat spillage should be treated as biological waste.

15. LIABILITY AND LOSS

In the event that this document is translated into another language, the English language version shall prevail in the event of any inconsistencies between the documents.

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16. INFORMATION FOR CUSTOMS USE ONLY

Country of origin for customs purposes*: United Kingdom * Defined as the country where the goods have been produced and/or sufficiently processed to be classed as originating from the country of supply, for example a change of state such as freeze-drying.
Net weight: 0.523 g
Toxicity Statement: Non-toxic
Veterinary certificate or other statement if applicable. **Attached:** No

17. CERTIFICATE OF ANALYSIS

NIBSC does not provide a Certificate of Analysis for WHO Biological Reference Materials because they are internationally recognised primary reference materials fully described in the instructions for use. The reference materials are established according to the WHO Recommendations for the preparation, characterization and establishment of international and other biological reference standards http://www.who.int/bloodproducts/publications/TRS932Annex2_Inter_biolefstandardsrev2004.pdf (revised 2004). They are officially endorsed by the WHO Expert Committee on Biological Standardization (ECBS) based on the report of the international collaborative study which established their suitability for the intended use.

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Appendix 4). The stability of the candidate IS was predicted using the Arrhenius model for accelerated degradation studies with potencies expressed relative to the -70 °C baseline sample [10,11].

E. In use stability after reconstitution

An in-use reconstitution study was set up to determine the short-term stability of PvLDH in 19/116. Each ampoule of 19/116 was reconstituted in 250 µL whole blood and stored for 2 days at temperatures of -70°C, -20°C, +4°C and ambient temperature. After storage each sample was characterized alongside a freshly reconstituted sample by ELISA (

Appendix 3
Instructions for use

Medicines & Healthcare products Regulatory Agency

NIBSC Confidence in Biological Medicines

WHO International Standard
1st WHO International Standard for Plasmodium vivax antigen (LDH)
NIBSC code: 19/116
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(Version 1.00, Dated)

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2. CAUTION

This preparation is not for administration to humans or animals in the human food chain.

The preparation contains material of human origin, and either the final product or the source materials, from which it is derived, have been tested and found negative for HBsAg, anti-HIV and HCV RNA. As with all materials of biological origin, this preparation should be regarded as potentially hazardous to health. It should be used and discarded according to your own laboratory's safety procedures. Such safety procedures should include the wearing of protective gloves and avoiding the generation of aerosols. Care should be exercised in opening ampoules or vials, to avoid cuts.

3. UNITAGE

This material has an assigned unitage of 1000 International Units of PvLDH per ampoule.

4. CONTENTS

Country of origin of biological material: Peru.
Each ampoule is a lyophilate of a preparation that contained 0.5 ml of lysed red blood cells from P. vivax infected donors diluted 1:4 into 11.43 mM Tris pH 7.4, 6.43 % trehalose, 1.14 mM EDTA buffer.

5. STORAGE

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Please note: because of the inherent stability of lyophilized material, NIBSC may ship these materials at ambient temperature.

6. DIRECTIONS FOR OPENING

DIN ampoules have an 'easy-open' coloured stress point, where the narrow ampoule stem joins the wider ampoule body. Various types of ampoule breaker are available commercially. To open the ampoule, tap the ampoule gently to collect material at the bottom (labelled) end and follow manufactures instructions provided with the ampoule breaker.

7. USE OF MATERIAL

No attempt should be made to weigh out any portion of the freeze-dried material prior to reconstitution
This material is supplied lyophilised and before use should be reconstituted in 0.25 mL of whole blood. Reconstituted material should be used on the day of reconstitution.

8. STABILITY

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Derivation of International Units:
http://www.nibsc.org/standardisation/international_standards.aspx
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http://www.nibsc.org/products/ordering.aspx
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14. MATERIAL SAFETY SHEET

Classification in accordance with Directive 2000/54/EC, Regulation (EC) No 1272/2008: Not applicable or not classified

Physical and Chemical properties			
Physical appearance: Freeze-dried		Corrosive:	No
Stable:	Yes	Oxidising:	No
Hygroscopic:	No	Irritant:	No
Flammable:	No	Handling:See caution, Section 2	
Other (specify):	Contains material of human origin		

Toxicological properties	
Effects of inhalation:	Not established, avoid inhalation
Effects of ingestion:	Not established, avoid ingestion
Effects of skin absorption:	Not established, avoid contact with skin

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Suggested First Aid	
Inhalation:	Seek medical advice
Ingestion:	Seek medical advice
Contact with eyes:	Wash with copious amounts of water. Seek medical advice
Contact with skin:	Wash thoroughly with water.
Action on Spillage and Method of Disposal	
Spillage of ampoule contents should be taken up with absorbent material wetted with an appropriate disinfectant. Rinse area with an appropriate disinfectant followed by water. Absorbent materials used to treat spillage should be treated as biological waste.	

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16. INFORMATION FOR CUSTOMS USE ONLY

Country of origin for customs purposes*: United Kingdom * Defined as the country where the goods have been produced and/or sufficiently processed to be classed as originating from the country of supply, for example a change of state such as freeze-drying.
Net weight: 0.523 g
Toxicity Statement: Non-toxic
Veterinary certificate or other statement if applicable. **Attached:** No

17. CERTIFICATE OF ANALYSIS

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Appendix 4).

Collaborative study

A. Participants

Sixteen participants from 11 different countries took part in the study and included ELISA and RDT manufacturers, research institutes and universities as well as end-users involved in the field of malaria diagnostics. Participants filled questionnaires to state their willingness to take part and their ability to carry out ELISA and/or malaria RDT as well as any other routine malaria antigen detection tests performed in their laboratories. A list of participants, organisation type and country can be seen in Appendix 2.

B. Study Design and Protocol

ELISA and RDTs were evaluated in the collaborative study.
The commercially available ELISA kit 'Qualisa' (Tulip group limited) was used for the multi-laboratory analysis based on previous evaluation of suitability. In addition, participants were encouraged to use alternative ELISAs with which they were familiar. CELISA (Cell labs), Malaria AG ELISA (ApDia) and Malaria AG ELISA (DRG diagnostics) were evaluated by individual laboratories.
Eighteen commercially available RDTs from a range of manufacturers (

Table 5) were used to detect PvLDH proteins in biosamples. Six of these tests were evaluated by multiple laboratories each and were used for inter-laboratory analysis. The remaining tests, generally manufacturers testing their own products, were analysed by one laboratory and were used for fitness-for-purpose analysis.
All participants were sent sets of biosamples containing the three *P. vivax* clinical isolates, recombinant Pv pLDH, and coded duplicate candidate IS ampoules. Participating laboratories were asked to carry out four independent runs of each assay using fresh biosamples on four different days. The candidate IS and clinical isolates were serially diluted in parasite-free whole blood, sourced by each participant, for all assays.

C. RDTs

All study participants were supplied with 4 different RDT products for testing and each RDT product was randomly distributed to two or more participants - except RDT manufacturers, who only tested their own products. The protocol for RDT testing is given in Appendix 7. Study participants tested in duplicate, 3 *P. vivax* clinical isolates, the recombinant PvLDH panel and the coded duplicate candidate IS samples on the allocated RDTs.

- All laboratories tested the same 3 *P. vivax* isolates: ET01V09-2000, PH09V02-2000 and KH07V10-2000. Eight 2-fold serial dilutions, from an initial 1:10 starting dilution of the 2,000 parasites/µL clinical isolate stock solutions, were tested in the RDT products. Top concentration ca. 200 parasites/µL.
- The recombinant PvLDH panel samples were diluted to 91 µg / mL in distilled water and made up to a starting dilution of 1:50 with whole blood and seven 2-fold dilutions were made prior to loading onto the RDT. Top concentration 1.82 µg / mL.
- The IS ampoules were reconstituted with 250 µL of whole blood. For the first experimental run participants were asked to test seven 2-fold serial dilutions from an initial reconstituted neat concentration. Top concentration ca. 3300 parasites/µL.

Samples were loaded onto RDTs as specified by the manufacturer's instructions for each RDT. Each biosample dilution tested on an RDT was scored 0, 1, 2, 3 or 4 based on band colour intensity chart with 0 being negative and 4 being highest band intensity. Each RDT was scored independently by two readers and the scores recorded on the RDT results form. For the first experimental run, participants were asked to serially dilute clinical isolate and candidate IS samples until 3 consecutive scores of zero were recorded for each RDT and for each band. For experimental runs 2, 3, and 4, participants were asked to only test 3 dilutions above and below the limit of detection (LOD) established in the first experimental run.

D. ELISA

The final versions of the collaborative study protocols are given in; Appendix 3 Instructions for use

Medicines & Healthcare products Regulatory Agency

NIBSC Confidence in Biological Medicines

WHO International Standard
1st WHO International Standard for Plasmodium vivax antigen (LDH)
NIBSC code: 19/116
Instructions for use
(Version 1.00, Dated)

1. INTENDED USE

This preparation contains red blood cell (RBC) lysates from P. vivax-infected donors from Peru. The intended use for is for the standardisation, and evaluation of performance and sensitivity of P. vivax antigen detection tests that detect P. vivax lactate dehydrogenase (PvLHDH) as well as for the calibration of secondary reference materials.

2. CAUTION

This preparation is not for administration to humans or animals in the human food chain.

The preparation contains material of human origin, and either the final product or the source materials, from which it is derived, have been tested and found negative for HBsAg, anti-HIV and HCV RNA. As with all materials of biological origin, this preparation should be regarded as potentially hazardous to health. It should be used and discarded according to your own laboratory's safety procedures. Such safety procedures should include the wearing of protective gloves and avoiding the generation of aerosols. Care should be exercised in opening ampoules or vials, to avoid cuts.

3. UNITAGE

This material has an assigned unitage of 1000 International Units of PvLDH per ampoule.

4. CONTENTS

Country of origin of biological material: Peru.
Each ampoule is a lyophilate of a preparation that contained 0.5 ml of lysed red blood cells from P. vivax infected donors diluted 1:4 into 11.43 mM Tris pH 7.4, 6.43 % trehalose, 1.14 mM EDTA buffer.

5. STORAGE

This preparation should be stored at -20°C or below on receipt. If the material is to be stored for more than 3 months prior to use storage at -70°C is recommended.
Please note: because of the inherent stability of lyophilized material, NIBSC may ship these materials at ambient temperature.

6. DIRECTIONS FOR OPENING

DIN ampoules have an 'easy-open' coloured stress point, where the narrow ampoule stem joins the wider ampoule body. Various types of ampoule breaker are available commercially. To open the ampoule, tap the ampoule gently to collect material at the bottom (labelled) end and follow manufactures instructions provided with the ampoule breaker.

7. USE OF MATERIAL

No attempt should be made to weigh out any portion of the freeze-dried material prior to reconstitution
This material is supplied lyophilised and before use should be reconstituted in 0.25 mL of whole blood. Reconstituted material should be used on the day of reconstitution.

8. STABILITY

Reference materials are held at NIBSC within assured, temperature-controlled storage facilities. Reference Materials should be stored on receipt as indicated on the label.

NIBSC follows the policy of WHO with respect to its reference materials.

9. REFERENCES

Charles Olomu, Lynne M. Harris, Peter Rigsby, Eleanor Atkinson, Seda Yerlikaya, Xavier Ding, Paul W. Bowyer,* and the Collaborative Study Group (2020). Collaborative study to evaluate the proposed First World Health Organization International Standard for Plasmodium vivax antigens. WHO/BS/2020.xxxx

10. ACKNOWLEDGEMENTS

This material was developed in collaboration with the Foundation for Innovative New Diagnostics (FIND). We gratefully acknowledge the significant contributions of the collaborative study group in the development of this IS. We would like to thank Professor Dionicia Gamboa and Dr. Katherine Torres from Malaria Laboratory in UPCH (Lima, Peru) and the study staff in Iquitos, Peru for undertaking the specimen collection study, Further acknowledgment is extended to the patients who kindly donated samples for this project. We also thank the WHO malaria specimen bank at the US CDC for providing clinical isolates for the collaborative study.

11. FURTHER INFORMATION

Further information can be obtained as follows;
This material: enquiries@nibsc.org
WHO Biological Standards:
http://www.who.int/biologicals/en/
JCTLM Higher order reference materials:
http://www.bipm.org/en/committees/jc/jctlm/
Derivation of International Units:
http://www.nibsc.org/standardisation/international_standards.aspx
Ordering standards from NIBSC:
http://www.nibsc.org/products/ordering.aspx
NIBSC Terms & Conditions:
http://www.nibsc.org/terms_and_conditions.aspx

12. CUSTOMER FEEDBACK

Customers are encouraged to provide feedback on the suitability or use of the material provided or other aspects of our service. Please send any comments to enquiries@nibsc.org

13. CITATION

In all publications, including data sheets, in which this material is referenced, it is important that the preparation's title, its status, the NIBSC code number, and the name and address of NIBSC are cited and cited correctly.

14. MATERIAL SAFETY SHEET

Classification in accordance with Directive 2000/54/EC, Regulation (EC) No 1272/2008: Not applicable or not classified

Physical and Chemical properties			
Physical appearance: Freeze-dried		Corrosive:	No
Stable:	Yes	Oxidising:	No
Hygroscopic:	No	Irritant:	No
Flammable:	No	Handling:	See caution, Section 2
Other (specify):	Contains material of human origin		

Toxicological properties	
Effects of inhalation:	Not established, avoid inhalation
Effects of ingestion:	Not established, avoid ingestion
Effects of skin absorption:	Not established, avoid contact with skin

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WHO International Laboratory for Biological Standards,
UK Official Medicines Control Laboratory

World Health Organization

Medicines & Healthcare products Regulatory Agency

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Suggested First Aid	
Inhalation:	Seek medical advice
Ingestion:	Seek medical advice
Contact with eyes:	Wash with copious amounts of water. Seek medical advice
Contact with skin:	Wash thoroughly with water.

Action on Spillage and Method of Disposal
Spillage of ampoule contents should be taken up with absorbent material wetted with an appropriate disinfectant. Rinse area with an appropriate disinfectant followed by water. Absorbent materials used to treat spillage should be treated as biological waste.

15. LIABILITY AND LOSS

In the event that this document is translated into another language, the English language version shall prevail in the event of any inconsistencies between the documents.

Unless expressly stated otherwise by NIBSC, NIBSC's Standard Terms and Conditions for the Supply of Materials (available at http://www.nibsc.org/About_Us/Terms_and_Conditions.aspx or upon request by the Recipient) ("Conditions") apply to the exclusion of all other terms and are hereby incorporated into this document by reference. The Recipient's attention is drawn in particular to the provisions of clause 11 of the Conditions.

16. INFORMATION FOR CUSTOMS USE ONLY

Country of origin for customs purposes*: United Kingdom * Defined as the country where the goods have been produced and/or sufficiently processed to be classed as originating from the country of supply, for example a change of state such as freeze-drying.
Net weight: 0.523 g
Toxicity Statement: Non-toxic
Veterinary certificate or other statement if applicable. **Attached:** No

17. CERTIFICATE OF ANALYSIS

NIBSC does not provide a Certificate of Analysis for WHO Biological Reference Materials because they are internationally recognised primary reference materials fully described in the instructions for use. The reference materials are established according to the WHO Recommendations for the preparation, characterization and establishment of international and other biological reference standards http://www.who.int/bloodproducts/publications/TRS932Annex2_Inter_biolefstandardsrev2004.pdf (revised 2004). They are officially endorsed by the WHO Expert Committee on Biological Standardization (ECBS) based on the report of the international collaborative study which established their suitability for the intended use.

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UK Official Medicines Control Laboratory

World Health Organization

Appendix 4 (Qualisa), Appendix 5 (CELISA), Appendix 6 (apDia and DRG diagnostics).

- All laboratories tested the same 3 *P. vivax* isolates: ET01V09-2000, PH09V02-2000 and KH07V10-2000. Eight 2-fold serial dilutions, from an initial 1:5 starting dilution of the 2,000 parasites/µL clinical isolate stock solutions, were tested in the ELISAs. Top concentration ca. 400 parasites/µL
- The recombinant PvLDH sample was diluted to 91 µg / mL in distilled water and made up to a starting dilution of 1:200 with whole blood and seven 2-fold dilutions were made prior to loading onto the ELISA plate. Top concentration ca. 455 ng / mL.
- The IS samples were reconstituted in 250 µL and participants were asked to test seven 2-fold serial dilutions from an initial 1:3 dilution of reconstituted candidate IS ampoules. Top concentration ca. 1100 parasites/µL.

Participants were requested to record raw optical densities (OD) in datasheet provided

E. Statistical Analysis

RDT

Sample median endpoint titres were calculated for each laboratory and RDT. These were also expressed relative to sample IS-A in order to assess the impact of harmonisation between laboratories and RDT types when titres are normalised to a common reference sample.

Intra-run variability was assessed using the duplicate end-point titres obtained for each sample on each RDT, within an assay run. Where duplicate results differed by more than 4-fold, no result for that sample, on that RDT, in that run, was used. Such exclusions were made in only ~0.5% and ~0.3% of cases for RDT Pv and Pan bands, respectively.
To assess the inter-run variability for each sample on each RDT, the ratio of the maximum and minimum endpoint titres across assay runs was calculated. Where the ratio for a sample exceeded 16, all results for that sample for that RDT were excluded. Such exclusions were made in ~5% of cases for both RDT Pv and Pan bands. “n/a” in Table 6 and Table 7 indicate where no result is available due to these exclusions.

A variance components analysis was performed using $\log_{10}$-transformed titres to determine the levels of variability in the results. Variability was expressed as Geometric Coefficient of Variation i.e. GCV = $(10^s - 1) \times 100\%$ where s denotes the standard deviation of the $\log_{10}$-transformed titres.

ELISA

Potency estimates were calculated relative to 19/116 using CombiStats[12] with a sigmoid curves model and no transformation of the assay response. Instances where the ratio of fitted slopes was outside the range 0.80-1.25 were considered non-parallel and no estimates are reported. These samples were omitted from further analysis.

Results from all valid assays were combined as unweighted geometric means (GM) for each laboratory and these laboratory means were used to calculate overall unweighted geometric means. Variability between laboratories has been expressed using geometric coefficients of variation (GCV = $\{10^s-1\}\times100\%$ where s is the standard deviation of the $\log_{10}$ transformed estimates).

Results

A. Characterisation of individual biosamples and definitive fill

PvLDH concentration determination
Data on the individual biosamples characterised by ELISA and quantified by comparison to a recombinant protein (Table 2). The concentration of samples ranged from 51-19781 ng / mL by this quantification. The most commonly used quantification is parasites / µL blood. By this measure the samples ranged from 306 – 17612 parasites / µL. A plot of these two different, but commonly used, approaches to concentration is shown. The Pearson correlation coefficient is 0.56 supporting the importance of a common and standardised approach to concentration determination.

Trial studies on clinical isolate pools
A pool, comprising 0.5 mL of each of the 19 RBC lysates was created. The objective was to establish a balance between batch size and reactivity on the tests. The mean parasitemia of the neat sample, based on an average of the individual samples, is 6575 parasites / ul (4360 ng / mL).

The pool was titrated, in duplicate, on 4 *P. vivax*-detecting RDT products in order to determine end-point titres (Table 4). The titrations observed (to 1:64 and 1:256) correspond to detection limits in the region of 100 and 25 parasites per ul of blood for these RDTs. These trial data allowed an optimum balance of sufficient antigenemia and batch size to be selected.

Definitive fill
Based on accelerated degradation studies of a trial lyophilisation it was determined a filling volume of 500 µL gave increased stability and an improved cake compared to using 250 µL. For this reason, it was decided that a larger volume of a more dilute sample should be lyophilised (500 µL) but to achieve enough antigenemia the product will be resuspended in 250 µL.

A total of 2,511 ampoules were lyophilized in a single batch and were assigned the NIBSC product code 19/116. The ampoules had an average fill weight of 0.523 g with CV = 0.47%. The mean residual moisture was 0.22 % (CV = 20.74%) and the mean oxygen headspace was 0.13% (CV 57.16%). The filling statistics are summarized in Table 1. Post-fill microbiological assessment for bacterial, mould and yeast colonies were negligible. The samples are stored under assured temperature-controlled conditions (-70°C) within the NIBSC /CBRM. They will remain at -70°C pending longer-term stability studies.

Stability Studies
The potencies of candidate 19/116 stored at the various temperatures for the 9 and 10 months were analysed by ELISA and expressed relative to 19/116 stored at -70 °C (Table 10A). These data were fitted to the Arrhenius model to predict PvLDH loss over time (Table 10B). There was evidence for a reduction in PvLDH antigen potency after 9 and 10 months when stored at +37°C and +45°C but currently the product appears stable at lower temperatures. Accelerated and real-time stability studies will be continued to ensure product integrity. For the accelerated degradation study, samples will be retrieved for analysis after 12 months and 24 months and used to inform the choice of later timepoints in this testing programme. Present data is consistent with good long-term stability when stored at -70 °C, -20°C and +4°C.

Stability after reconstitution

For each experiment it is expected that a new freshly reconstituted ampoule should be used. However, we chose to determine the stability of the standard after reconstitution. The potencies of the stored reconstituted samples were expressed relative to the freshly reconstituted samples shown (Table 11)**.** There was no loss of reactivity of the reconstituted samples in ELISA when stored for 48 hours at -70, -20, +4 and ambient temperature. The data indicated that 19/116 is stable after reconstitution for 48 hours with no evidence of any significant loss in potency as indicated by relative potency estimates in Table 11.

B. Collaborative study results

RDT study

The RDT study was designed to evaluate the fitness-for-purpose of the candidate International Standard on a range of devices that detect *P. vivax*. As described in the methods median values derived from the within lab replicates are reported. The results are grouped by line type; PvLDH-specific (9-devices), Pan-LDH (8 devices), Pf-specific (16-devices), and Pvom (1) (

Table 5). Two devices detect two Pf-specific antigens; PfHRP-2 and PfLDH. A product detecting aldolase instead of lactate dehydrogenase was also included in the study. For all except the Pf-specific lines an analysis of endpoint titre relative to IS-A could be performed in addition to determination of end-point titre alone. A summary of all RDTs and the number of participants that analysed them by can be seen in

Table 5.

Data returned for analysis
Fifteen participants were involved in the RDT evaluation part of the study. A total of 18 RDTs were evaluated. These are summarized in
Table 5. Seven of these RDTs (1-7) were evaluated by more than 1 participant laboratory. RDT 8-18 were evaluated by 1 laboratory each, usually the manufacturer. Some data was excluded from further analysis as described in the methods. These will not be discussed further.

Pv-specific lines
Nine devices with lines specific from PvLDH were analysed. The *P. falciparum* International Standard (16/376) did not give signal with any of the devices as expected. The recombinant protein control (PvLDH) was detected on all these devices as expected. Median LOD could be determined for all of the biosamples although some exclusions were applied. Qualitatively all samples performed as expected against this line (Table 6).

Variability in Pv band reactivity (RDT types 1, 2, 4 and 6) was reduced for samples A, B and C when titres were expressed relative to sample IS-A (Figure 1A), with total GCV values reduced from ~850% to ~200%, although these remain higher than the inherent variability associated with the testing as shown by the total GCV for sample IS-B (72%) which is a coded duplicate of IS-A (Figure 1B and Table 8). The improved harmonisation when using relative titres is due to the resulting lack of significant variability between different RDT types which was evident when using the absolute titres alone (Table 8). Variability was higher for the

recombinant PvLDH (sample D), although a small reduction in GCV was observed when titres were normalised (1278% to 844%) with improved agreement between different RDT types.

Rapid diagnostic test performance is commonly measured using parasites at 200 parasites / µL of blood. This unitage, although variable in terms of antigen content has been a mainstay of product development. Appendix 1B demonstrates that the candidate IS (starting at approximately 3300 parasites / µL) gives a similar range and rank order to the clinical isolates (which started at 200 parasites / µL). The range of detection limits observed 0.2 – 42.7 parasites / µL are similar to those observed for the clinical isolates (0.5-56.3 parasites / µL). The values are in the expected range and demonstrate that the IS is performing similarly to the clinical isolates on this detection line and across this range of RDTs.

Pan-lines - LDH

Eight of the devices analysed have Pan-LDH lines and one device detects Pan-specific aldolase. After exclusions, all devices were able to detect all of the biosamples (IS-A, IS-B and clinical samples A, B, C). The *P. falciparum* International Standard (16/376) gave a signal on all except device 16. This device is listed as Pan-LDH only. The failure of 16/376 to be detected by this line is unexplained. The recombinant protein, pLDH, was detected by all except device (#14). It is not known why no signal could be detected with the recombinant protein on this device while all the clinical isolates were detected perhaps indicating the absence of the appropriate epitope or sufficient avidity for the recombinant protein with this antibody used in this device.

Variability in Pan-LDH band results (for devices 3 and 7) gave similar outcomes for samples A, B, and C (Table 8 and Figure 2) with GCV values reduced from ~322% to ~67%, a reduction from 209% to 117% for 16/376, and a GCV value for IS-B relative to IS-A of 71%. Decreased variance when titres were expressed relative to IS-A was observed for all samples, including the recombinant protein sample (GCV reduced from 1139% to 330%).

Similar to the PvLDH lines it is possible to express in the range in detection limits in parasites / µL (**Appendix 1**A). For the IS-A and B the range was from 0.4 – 51.6 parasites / µL is similar to those observed for the clinical isolates (0.8 – 75.0 parasites / µL). The values are in the expected range and demonstrate that the IS is performing similarly to the clinical isolates on this detection line and across all RDTs tested.

Pan-lines - Aldolase

One device detects Pan-aldolase instead of Pan-LDH or PvLDH. This is reported on the device as a Pan band and is included in Table 7 (device 5). The whole parasite preparations both *P. vivax* (IS-A, IS-B and biosamples A,B,C) and *P. falciparum* (16/376) were recognized by this line. This is expected. Unexpectedly the recombinant PvLDH (Sample D) also reacted with this line. The reason for this is not known. Further analysis of the variance was not conducted.

Pvom-line

The *P. falciparum* International Standard 16/376 is not detected by this line but all of the other biosamples are as expected. Further analysis of the variance was not conducted

Pf-lines – HRP2 and PfLDH

Typically, Pf lines detect histidine rich protein 2 (HRP-2) although lactate dehydrogenase is also a potential target for these lines. This is not the focus of this study but provide a useful negative control for the *P. vivax* standards.
Sixteen devices tested have a Pf-line and one device had an additional PfLDH specific line, and another a combined PfHRP-2/PfLDH line. Some labs reported signal on the Pf-line for the recombinant PvLDH; RDT 1 (one instance across 8 labs), RDT 3 (fours instances across 8 labs), RDT 6 (two instances across 3 labs). This is unexpected. Neither the *P. vivax* clinical samples nor IS-A and IS-B react with this line as expected. The focused testing around the LOD for the expected target antigen detection lines for the later replicates (runs 2-4) was not designed to detect this unexpected cross reactivity. Therefore, in some cases, the test samples were not sufficiently concentrated samples to detect this effect in the later replicates and consequently this was reported as zero. Further evaluation identified that in the complete dose response curve for devices 8 and 10 (replicate 1) this unexpected reactivity was also present as well as two more of the laboratories testing device three.

ELISA study

Complete dose response curves could not be obtained with the additional ELISAs (non-Qualisa) analysed in this study. Insufficient data was obtained to perform any further analysis that would be comparable to the main ELISA study. The subsequent analysis therefore focuses on the multi-laboratory analysis using Qualisa.

Estimates of ED_{50} and relative potency are shown in

* This was a developmental product that has had a design change since the testing and as such is not available

Table 13 and Table 14 respectively. Summaries of laboratory geometric means and inter-laboratory GCV are shown in Figure 3.
In two cases (Lab 3, Run 3 and Lab 4, Run 1) assays were excluded from further calculations as the relative potency estimate for IS-B was highly discrepant from the expected value of 1.0 (in both cases outside the range 0.67-1.50). This resulted in no valid estimates from Lab 4.

The use of potencies relative to IS-A instead of ED_{50} values results in a decreased GCV (Figure 3). Without reporting relative to IS-A the between-lab GCVs were 140.2%, 185.3%, 81.3%, 274.7% for samples IS-B, A, B, C respectively (range 81.3 – 274.7%). When reported relative to IS-A the GCVs are 13.9%, 25.7%, 25.8%, 31.3% for samples IS-B, A, B, C respectively (range 13.9 – 31.3%). This is comparable with data from the development of the analogous International standard for *P. falciparum* that showed a range of 13.4 – 23.6% [3].

Conclusions

Over 300 million RDTs are distributed every year and their use is a critical component in the successful detection and subsequent treatment of malaria. As the quality control framework evolves from centralised to increasingly decentralised testing it is critical to have a robust network of reference materials. Until now the existing reference materials, each usually sufficient for their own local use, have lacked an International Standard to enable cross laboratory and cross reference material harmonisation. This work has reported the development and subsequent collaborative assessment of a material that could serve as an International Standard for *P. vivax* antigens.

It is not currently possible to grow sufficient *Plasmodium vivax* to use as an International Standard in a laboratory. This *in vitro* production was favoured for the *Plasmodium falciparum* antigens International Standard [3]. This leaves clinical isolates as the optimal source of material to form the International Standard. Whilst using a clinical isolate removes concerns about differences between laboratory adapted parasites and their clinically derived comparators it also means that reproducing the same material in the future is essentially impossible. However, there is relatively little genetic diversity in *P. vivax* LDH [13] and it can be expected that future collections of clinical isolates will, at least at the DNA level for this antigen, be similar. Collection of a single *P. vivax* infection in sufficient quantity is challenging because typically this parasite does not achieve the same high parasitemias as *Plasmodium falciparum,* therefore a strategy was developed to create a pool of *P. vivax* clinical samples that was of sufficient antigenemia to create the International standard. The collaborative study reported here compared this candidate IS (comprised of isolates from Peru) with clinical isolates from Ethiopia, The Philippines and Cambodia and assessed; the diversity of rapid diagnostic tests on which the candidate could be detected, the extent to which it could improve harmonisation and similarity with clinical isolates from a wider range of locations.

The candidate material (coded 19/116) was detectable on all of the RDTs analysed. In each case it reacted with the target band PvLDH, PanLDH or Pan aldolase, and was not detected by any of the *P. falciparum* specific bands which served as a negative control. The clinical isolates in the study (A,B,C) showed the same qualitative behaviour on all the devices. Thus, the candidate IS performed the same as the geographically diverse clinical isolates. Furthermore, when expressed in units of parasites per µL there was good agreement between the limits of detection obtained with the IS compared to A,B,C. For the RDTs that were analysed by multiple different laboratories the observed variability in the LOD titre was substantially reduced when reported relative to the candidate IS for all of the biosamples in the study.

The recombinant protein in this study did not show the same performance as the candidate IS with notable and unexpected cross reactivity with the *Plasmodium falciparum* HRP-2 band on some devices and the Pan-aldolase band. This contrasted with the clinically derived material. The recombinant protein could still be assigned a value in IU and standardised to the candidate International Standard and may have a role to play in quality control rather than sensitivity and specificity by virtue of the relative ease of production at larger scale. Importantly the candidate IS was able to reduce variance components for the recombinant protein too and it is expected that this would be true of other recombinant proteins that are available.

The ELISA study focused on results using Qualisa. The other ELISA formats that were used did not yield suitable dose response curves for further analysis, although it should be noted that

the greatest response was seen for the candidate IS in the majority of cases and that the clinical isolates did not perform better. The Qualisa data, although requiring a large number of exclusions, showed similar performance for the clinical isolates and the candidate IS as well as a reduction in inter-laboratory variability (from 81.3 – 274.7% to 13.9 – 31.3% through use of the candidate standard). This variability is similar to that seen for the *P. falciparum* antigen standard[3].

This ELISA format was sufficient to allow accelerated thermal degradation and stability after reconstitution studies. These data indicate that there will be good long-term stability at -70 or -20°C. Decisions on long-term storage will be taken when more data is available particularly in the light of the similarly formulated *P. falciparum* antigen standard currently requiring storage at -70°C.

Taken together, these data suggest that the establishment of candidate 19/116 as the first WHO International Standard for *P. vivax* antigen (LDH) will provide a reference material with long term stability that is detectable on the rapid diagnostic tests providing results similar to clinical isolates and can aid harmonisation of results between different laboratories and RDTs as well as be a standard on which development of secondary standards could be based.

Proposal

Based on the results of the collaborative study, it is proposed that the lyophilised *P. vivax* candidate IS preparation, code number 19/116 should be established at the 1st IS for *P. vivax* antigen (LDH), with 1000 International Units (IU) per ampoule. The unitage proposed is arbitrary but, on the basis of the data presented in this study the limit of detection of the tests analysed would range from 0.24 – 62.52 IU / mL.

The intended use for the candidate IS is for the standardisation, and evaluation of performance and sensitivity of *P. vivax* antigen detection tests as well as for the calibration of secondary reference materials. If alternative diagnostic tests detecting different antigens increase in prevalence this IS may also be useful in their development.

The custodian laboratory is the National Institute for Biological Standards and Control (NIBSC).

Comments from participants – experimental phase

The following comments were received from the participants on the results returned

ELISA (QUALISA).
Laboratory 5 commented pipetting issues were observed after QUALISA run 1 for the ELISA study and pipettes had to be re-calibrated for subsequent runs.

Laboratory 7 commented that the parasite free blood used in the study was A+.

Laboratory 8 commented that TMB reaction was stopped at 15 minutes rather than 10 in runs 1 and 2.

Laboratory 10 made the following comments.

- For dilution run 1 IS-A, when reconstituted with malaria parasite free blood became thick and there was a difficulty in picking the sample with pipette. However, lypholized material was completely dissolved in malaria parasite free blood. The same was observed with IS-A and IS-B in dilution run 2.
- Precipitates were observed in run 1-4 after adding stop solution in wells especially in dilutions 1 and ½ wells of all biosamples. Plates were read at 10 minutes in all runs.

RDT
Laboratory 3 made the following comments.

- For RDT run 1, Many RDTs for dilutions 5-8 were not readable - likely due to the addition of excess assay buffer. Due to time constraints these were not repeated. For CI samples, repeats were not deemed necessary as an approximation of LoD could be estimated from available data and would be refined in runs 2-4. For IS and RP samples repeats were not deemed necessary as 3 further 3 dilutions of each sample needed to be run to confirm the LoD.
- In RDT run 3 CI-B and 16/376 7th dilution not tested on SD Bioline - PROTOCOL DEVIATION.
- In RDT run 4 Negative control not run for SD Bioline RDT and First Response RDT.

Laboratory 4 commented that CareStart (HRP2/pLDH) P.f/Pan RDTs tested positive in P.f test lines against recombinant P.v LDH antigens.

Laboratory 7 made the following comments.

- In RDT run 1 the case of First response-Malaria Ag Pf/Pv card test, additional dilutions were prepared for IS-A and IS-B. The CI-B and Pv-pLDH showed positive results for 1st dilution only for Arkray ParaHit RDT kit.

- In RDT run 2 the Pv-pLDH showed positive results for the dilutions above the LOD (1/2) and negative results for the dilutions of the LOD (1/4) and below the LOD (1/8) for S.D Malaria RDT Kit. Similar results for Pv-pLDH were observed in Arkray ParaHit Total and First Response Malaria RDT Kit. The CI-C shown positive results for all selected 3 dilutions above and below the LOD in First Response Malaria and Carestart Malaria RDT kit. The yellow colour row signify that additional testing was

performed for the confirmation of negative samples for next dilution. The blue colour represent variations in the duplicate reading for the same dilution.

- In RDT run 3 The CI-A showed positive results for the dilutions (1/4) above the LOD and negative results for the dilutions of the LOD (1/8) and below the LOD (1/16) for Arkray ParaHit Total. Similar results for CI-A was observed in CareStart Malaria RDT Kit. The Pv-pLDH showed negative results for selected dilutions in all RDT kits. The yellow color row represent additional testing was performed for the confirmation of negative samples for next dilution.
- In RDT run 4 The Pf - 16/376 showed positive results for all selected 3 dilutions (1/32, 1/64, 1/128) above and below the LOD in S.D Malaria and Carestart Malaria RDT kit. The Pv-pLDH showed negative results for selected dilutions in all RDT kits. The yellow coloured row indicates that additional testing was performed for the confirmation of negative samples for next dilution. The blue color represent variations in the duplicate reading for the same dilution.
- The PFB (Parasite Free Blood) used in the study was A+
- Laboratory 10 commented that RDT kit number 4 was not received.

Laboratory 11 made the following comments.

- For RDT run 1 The Recombinant Pv-pLDH cross-reacted with the HRP2 band on the Carestart Malaria Pf/PAN (HRP2/pLDH) Ag Combo RDT.
- For RDT run 2 and 3 First Response Malaria Ag. Pf/Pv Card test with Cl-A and SD Bioline Malaria Ag Pf/Pv with Cl-B. Carestart Malaria Pf/Pan with Pf IS 16/376 still tested positive beyond LOD in result table while Carestart Malaria Pf/PAN with IS-A and IS-B. were positive beyond LOD for run 5.

Laboratory 13 made the following comments.

- For RDT run 1 IS-A (1 to 1/4 dilution rate) and IS-B (1 to 1/2 dilution rate) samples on parasite free blood were sticky. Sticky blood caused raddish background and late migration speed than other samples.
- Malaria parasite free blood was purchased from Discovery Life Sciences confirmed it was malaria negative by method of microscopy and site in-house RDT. Purchased malaria free blood was also confirmed negative for HBV, HCV, HIV 1&2.

Laboratory 17 reported issues with onward shipping after initial receipt from NIBSC. The cold chain was compromised. The consequence of this in terms of activity of samples is not known.

Comments from participants on manuscript

Several participants provided minor comments that were editorial in nature and we have amended the report accordingly.

Participant 8

Comment: 'In the report, it is mentioned that 2 recombinant pLDHs have been used - one from SPAN (which is His-tagged) and the the other one from Microcoat (which is GST-tagged). I am curious to know, from scientific point of view, if both the recombinant pLDHs showed cross-reactivity with the HRP2 segment in some or all of the RDTs and whether this cross-reactivity observed with both recombinant antigens showed similar positivities in these RDTs.'

Response: For the bulk of the work, and in the collaborative study, we only used SPAN diagnostics His-tagged pLDH and so, sadly, we do not know the answer to your question on cross reactivity with the HRP2 band.

Participant 11

Comment: The PQ program still uses the same specimen bank and slightly tweaked methodology as before and the change reflects more coordination at WHO than the activity itself. Testing is also centralized (at CDC).

Response: Thank you. The introduction will be amended to reflect this

Comment: Please provide pLDH antigen concentration

Response: Similar to the approximate parasitemia values quoted we can use an average of all the biosamples used to give an approximation. We noticed in the early phase that the theoretical mass of PvLDH varies depending on the test used and the recombinant protein it is compared against. But we appreciate that an approximate value based on the observations of the Qualisa test on which each of the individual samples were characterized will be of interest.

Comment: In relation to stability studies 'Is there a time limit for this or will be indefinite till vials are exhausted?

For all International standards we have an on-going programme of real-time stability studies until the batch is exhausted.

Participant 13

Comment: Device 18 was a developmental product that has undergone a design change since this study. It is not an available product. It has a PfLDH line in addition to those listed.

Response: Through discussion, and in agreement with the manufacturer, the text has been changed to reflect the status of this product, but the data has remained in the study. The PfLDH line data has been studied and the results updated.

Acknowledgements

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Table 1: Pv antigen IS trial fill and definitive fill statistics

Parameter	**0.25 mL fill option**	**0.5 mL fill option**	**Definitive fill (19/116)**
Number filled	47	49	2511
Fill weight (g)	0.257	0.499	0.523
Fill CV % (target <1)	0.07	2.65	0.47
Number measured	3	3	107
Dry weight (g)	0.0232	0.0459	0.0435
CV %	0.90	0.92	1.03
Number measured	3	3	6
Residual moisture % w/w	0.04	0.04	0.22
CV %	n/a	52.19	20.74
Number measured	2	3	12
Oxygen %	0.37	2.00	0.13
CV %	58.55	163*	57.16
Number measured	3	3	12
Appearance	Robust cakes that break up on agitation formed across the batch.	Robust cakes formed across the batch. *One outlier included, hence high CV.	

Table 2: *P. vivax* clinical isolates used as source material for developing the proposed International Standard

[A]. The participant code, specimen code, parasitemia and PvLDH antigen concentration of clinical isolates are listed. [B] A plot of parasites / µL versus concentration in ng / ml. Pearson correlation coefficient is 0.56.

[A]

Specimen code	Parasitaemia (parasites/µL)	PvLDH status	Estimated *[PvLDH] ng / mL
MA007030103	1487	Positive	1607
MA007030105	1226	Positive	561
MA007030106	7518	Positive	5666
MA007030107	2478	Positive	234
MA007030108	306	Positive	51
MA007030109	2710	Positive	384
MA007030111	1548	Positive	538
MA007030112	1832	Positive	1841
MA007030113	3234	Positive	251
MA007030114	6310	Positive	2165
MA007030115	12352	Positive	2671
MA007030116	17612	Positive	2615
MA007030117	6511	Positive	3439
MA007030118	5666	Positive	17426
MA007030119	12398	Positive	5782
MA007030120	14050	Positive	1188
MA007030121	7912	Positive	9324
MA007030122	5477	Positive	7369
MA007030123	14294	Positive	19781

[B]

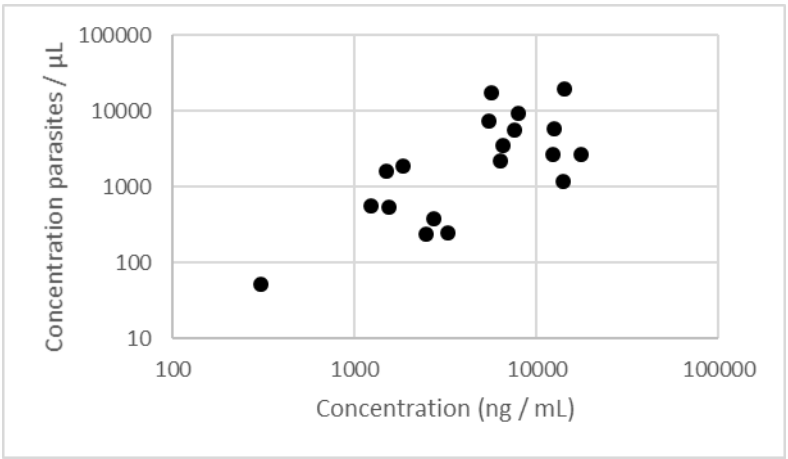


Table 3: *P. vivax* clinical isolates tested in the collaborative study.

ID	Sample code	Sample Origin	Concentration (parasites/µL)
ET01V09-2000	Clinical Isolate A (CIA)	Ethiopia	2000
PH09V02-2000	Clinical Isolate B (CIB)	RITM, Philippines	2000
KH07V10-2000	Clinical Isolate C (CIC)	Cambodia	2000

Table 4: Reactivity of the pre-fill pool in a subset of *P. vivax* detecting RDTs

RDT	Supplier	Catalogue number	Antigen detection lines	Pre-fill pool mean end-point titre PvLDH line
SD BIOLINE Malaria Ag P.f/P.f/P.v	Standard Diagnostics (Abbott Diagnostics Korea Inc.)	05FK120	HRP-2 PfLDH PvLDH	1/256
SD BIOLINE Malaria Ag P.f/P.v	Standard Diagnostics (Abbott Diagnostics Korea Inc.)	05FK80	HRP-2 PvLDH	1/64
EzDx™ Malaria Pan/Pf Rapid Test Detection kit	Advy Chemical Private Limited	RK MAL001	HRP-2 Pan LDH	1/256
Advantage Pan Malaria card	J. Mitra & Co. Pvt. Ltd.	IR013050	Pan LDH	1/256

Table 5: RDT products used in the collaborative study

RDT Code	RDT	Manufacturer	Cat/Lot no	Detection Band	Number of participants testing product
1	First Response® Malaria Ag. P.f./P.v. Card test	Premier Medical Corporation Private Ltd.	P119FRC25	PfHRP2, PvLDH	8
2	SD BIOLINE Malaria Ag P.f/P.v	Standard Diagnostics (Abbott Diagnostics Korea Inc.)	05FK80	PfHRP2, PvLDH	9
3	Access Bio Inc.	CareStart™ Malaria HRP2/pLDH (Pf/PAN) COMBO	RMRM-02571	PfHRP2, PanLDH	8
4	Wondfo	Guangzhou WONDFO Biotech CO., Ltd.	W056-C	PfHRP2, PvLDH	3
5	ParaHIT Total+Rapid test for P.falciparum and Pan malaria	Arkay Health Care Pvt. Ltd.	55IC206-25	PfHRP2, Pan Aldolase	3
6	STANDARD Q Malaria P.f/P.v Ag Test	SD Biosensor	09Mal20D	PfRP2, PvLDH	3
7	Bioperfectus Pf/Pan	Jiangsu Bioperfectus Technologies	SC2020W1T	PfHRP2, PanLDH	2
8	CareStart™ Malaria – Pf/PAN (HRP2/pLDH) Ag Combo	Access Bio Inc	MV19M63	PfHRP2, PanLDH	1
9	CareStart™ Malaria HRP2/pLDH(Pf/VOM) Combo	Access Bio, Inc.	GO171	PfHRP2, Pvom LDH	1
10	CareStart™ Malaria – Pf/Pv (HRP2/pLDH) Ag Combo	Access Bio Inc	MV19M61	PfHRP2, PvLDH	1
11	SD BIOLINE Malaria Ag P.f/Pan	Standard Diagnostics (Abbott Diagnostics Korea Inc.)	05FK60	PfHRP2, PanLDH	1
12	One step Malaria Pv Whole Blood Test	Guangzhou WONDFO Biotech CO., Ltd.	W078-C	PvLDH	1
13	One step Malaria Pf/Pan Whole Blood Test	Guangzhou WONDFO Biotech CO., Ltd.	W054-C	PfHRP2, PanLDH	1
14	STANDARD Q Malaria P.f/Pan Ag Test	SD Biosensor	B25MAL3MLR0	PfHRP2, PanLDH	1
15	Bioperfectus Pf/Pv	Jiangsu Bioperfectus Technologies Pf/Pan	SC20203W-20T	PfHRP2, PvLDH	1
16	CareStart™ Malaria – PAN (pLDH) Ag	Access Bio Inc	MN19M61	PanLDH only	1

17	SD BIOLINE Malaria Ag P.f/P.f/P.v	Standard Diagnostics (Abbott Diagnostics Korea Inc.)	05FK120	PfHRP2, PfLDH, PvLDH	1
18 *	BIOLINE Malaria P.f/P.v Plus	Standard Diagnostics (Abbott Diagnostics Korea Inc.)	05FK160	PfHRP2, PfLDH, PvLDH	1

*RDT 18 was a developmental product that has had a design change. It is no longer available

Table 6: Pv-LDH Median end point titres

RDT	Lab	Pv Median endpoint titres						
		IS-A	**IS-B**	**A**	**B**	**C**	**pLDH**	**16/376**
1	1	12288	49152	1024	256	256	4096	0
1	2	768	512	64	32	64	192	0
1	3	n/a	n/a	8	8	8	n/a	0
1	4	4096	4096	128	48	128	2048	0
1	5	384	384	96	32	96	48	0
1	7	512	768	64	32	64	0	0
1	11	2048	2048	128	96	256	1024	0
1	16	2048	2048	512	64	512	2048	0
2	1	512	512	4	2	3	64	0
2	2	32	32	2	1	2	32	0
2	3	8	8	4	4	4	12	0
2	4	32	128	4	2	2	48	0
2	5	48	32	2	1	4	2	0
2	7	64	32	8	4	8	1	0
2	10	32	64	2	1	2	12	0
2	11	128	128	8	4	8	96	0
2	13	128	128	6	4	16	32	0
4	3	8	8	2	1	2	32	0
4	4	32	32	4	2	2	96	0
4	15	192	256	32	16	32	16	0
6	1	65536	n/a	2048	128	192	1024	0
6	2	512	512	64	32	64	256	0
6	17	1536	2048	128	128	192	0.5	0
10	12	16384	8192	192	64	64	1024	0
12	15	384	256	32	16	32	16	0
15	18	128	128	16	8	16	1	0
17	13	512	512	32	16	32	192	0
18 *	13	2048	2048	128	64	128	1024	0

*RDT 18 was a developmental product that has had a design change. It is no longer available

Table 7: Pan-band median end point titres

RDT	Lab	Median endpoint titres						
		IS-A	IS-B	A	B	C	pLDH	16/376
3	1	4096	16384	768	64	128	256	256
3	2	256	256	24	16	16	64	64
3	3	n/a	n/a	8	8	4	12	16
3	4	384	512	32	16	16	192	128
3	5	256	192	12	8	24	12	n/a
3	7	256	256	32	16	24	4	64
3	10	256	384	16	8	12	24	64
3	11	512	512	96	32	64	512	128
5	7	32	32	8	2	4	0.5	8
5	11	64	64	4	2	4	32	8
5	14	128	128	8	4	8	32	16
7	10	48	96	4	2	2	4	8
7	18	128	128	16	8	16	2	128
8	12	8192	4096	256	128	128	4096	512
11	13	128	64	4	3	6	16	32
13	15	384	256	32	16	48	16	10
14	17	768	1024	128	64	96	0	64
16	12	8192	8192	256	128	256	2048	0

Pvom specific line								
RDT	Lab	IS-A	IS-B	A	B	C	pLDH	16/376
9	5	64	64	8	6	4	8	0

Table 8: Variance is reduced when reporting potency relative to the candidate IS

[A] Pv-LDH bands

Type	Sample	Variance Components			Variance Components (as GCV)		
		Intra-RDT	Inter-RDT	Total	Intra-RDT	Inter-RDT	Total
Titre	IS-B	0.387	0.728	1.115	319%	613%	1037%
	A	0.212	0.923	1.135	189%	814%	1063%
	B	0.129	0.717	0.846	129%	602%	731%
	C	0.147	0.796	0.943	142%	680%	836%
	pLDH	0.760	0.538	1.298	644%	441%	1278%
Titre vs IS-A	IS-B	0.056	0	0.056	72%	0%	72%
	A	0.150	0	0.150	144%	0%	144%
	B	0.254	0	0.254	219%	0%	219%
	C	0.277	0	0.277	236%	0%	236%
	pLDH	0.667	0.283	0.951	556%	241%	844%

[B] Pan-LDH bands

Type	Sample	Variance Components			Variance Components (as GCV)		
		Intra-RDT	Inter-RDT	Total	Intra-RDT	Inter-RDT	Total
Titre	IS-B	0.376	0.135	0.511	311%	133%	418%
	A	0.351	0.165	0.516	291%	155%	423%
	B	0.115	0.171	0.286	118%	159%	242%
	C	0.176	0.188	0.364	163%	171%	301%
	pLDH	0.513	0.682	1.195	420%	569%	1139%
	16/376	0.171	0.069	0.240	159%	83%	209%
Titre vs IS-A	IS-B	0.055	0	0.055	71%	0%	71%
	A	0.047	0	0.047	65%	0%	65%
	B	0.045	0	0.045	63%	0%	63%
	C	0.058	0	0.058	74%	0%	74%
	pLDH	0.372	0.029	0.401	307%	48%	330%
	16/376	0.106	0.007	0.114	112%	22%	117%

Table 9 Accelerated thermal degradation assessment of trial fill preparation

[A] Potencies relative to the sample stored at -70 ℃ determined by ELISA.

Storage temp	**-70 ℃**	**-20 ℃**	**4 ℃**	**20 ℃**	**37 ℃**	**45 ℃**
0.25 mL fill	1	0.79	0.84	0.60	0.76	0.60
0.5 mL fill	1	1.29	1.19	1.24	1.30	1.44

Table 10 Accelerated thermal degradation assessment of candidate IS preparation.

[A] Potencies relative to the sample stored at -70 ℃ determined by ELISA.

Storage temp	**-70 ℃**	**-20 ℃**	**4 ℃**	**20 ℃**	**37 ℃**	**45 ℃**
PvLDH potency at 9 months	1	1.10	1.68	0.90	0.62	0.48
PvLDH potency at 10 months	1	1.10	1.22	1.09	1.03	0.72

[B] The percentage PvLDH loss per year for 19/116 predicted using the Arrhenius model.

Temperature (℃)	**% pLDH Loss per Year**	**95% Upper Confidence Limit (% pLDH Loss)**
-70	0	0
-20	0.001	0.001
4	0.103	0.213
20	1.536	2.472
37	18.385	21.358

Table 11 In-use stability after reconstitution

Samples stored at the stated conditions for 48 hours were compared to a freshly reconstituted sample

Storage temp	**-70 ℃**	**-20 ℃**	**4 ℃**	**Ambient temperature**
PvLDH potency after 48 hours	0.90	1.04	1.16	1.17

Table 12 Pf-band median end point titres

RDT	Lab	Median endpoint titres						
		IS-A	IS-B	A	B	C	pLDH	16/376
1	01	0	0	0	0	0	0	1024
1	02	0	0	0	0	0	0	256
1	03	0	0	0	0	0	0	n/a
1	04	0	0	0	0	0	0	2048
1	05	0	0	0	0	0	0	48
1	07	0	0	0	0	0	0	256
1	11	0	0	0	0	0	0	1024
1	16	0	0	0	0	0	2	2048
2	01	0	0	0	0	0	0	256
2	02	0	0	0	0	0	0	64
2	03	0	0	0	0	0	0	8
2	04	0	0	0	0	0	0	192
2	05	0	0	0	0	0	0	3
2	07	0	0	0	0	0	0	64
2	10	.	.	.	.	.	.	64
2	11	0	0	0	0	0	0	256
2	13	0	0	0	0	0	0	128
3	01	0	0	0	0	0	32	512
3	02	0	0	0	0	0	2	64
3	03	0	0	0	0	0	0	16
3	04	0	0	0	0	0	16	128
3	05	0	0	0	0	0	0	16
3	07	0	0	0	0	0	0	64
3	10	.	.	.	.	.	4	64
3	11	0	0	0	0	0	0	128
4	03	0	0	0	0	0	0	8
4	04	0	0	0	0	0	0	64
4	15	0	0	0	0	0	0	64
5	07	0	0	0	0	0	0	8
5	11	0	0	0	0	0	0	16
5	14	0	0	0	0	0	0	8
6	01	0	0	0	0	0	128	64
6	02	0	0	0	0	0	32	64
6	17	0	0	0	0	0	0	n/a
7	10	.	.	.	.	.	.	24
7	18	0	0	0	0	0	0	256
8	12	0	0	0	0	0	0	768
9	05	0	0	0	0	0	0	n/a
10	12	0	0	0	0	0	0	256
11	13	0	0	0	0	0	0	128
13	15	0	0	0	0	0	0	64
14	17	0	0	0	0	0	0	512
15	18	0	0	0	0	0	0	128
16	12	0	0	0	0	0	0	1024
17a	13	0	0	0	0	0	0	384
17b	13	0	0	0	0	0	0	128
18*	13	0	0	0	0	0	0	1024

* This was a developmental product that has had a design change since the testing and as such is not available

Table 13 Qualisa ED_{50} estimates for each laboratory and assay run.

Lab	Run	Sample				
		IS-B	A	B	C	D
2	1	10.77	6.05	3.11	6.04	5.07
	2	7.26	4.14	1.82	3.83	3.53
	3	14.60	8.47	3.58	7.86	6.26
	4	10.19	5.90	2.85	5.56	4.20
3	1	15.64	8.94	4.85	8.34	9.95
	2	12.20	5.85	3.09	5.81	6.56
	3*	70.49	12.56	2.73	9.26	11.12
	4	13.84	NP	NP	NP	NP
4	1*	6.92	NP	2.32	4.45	1.02
	2	NP	NP	NP	NP	NP
	3	Cannot fit model (no convergence)				
	4	Cannot fit model (no convergence)				
5	2	11.70	6.48	3.66	5.36	
	3	3.35	1.94	1.32	0.28	NP
	4	Cannot fit model (no convergence)				
7	1	30.69	21.41	9.16	18.28	
	2	51.66	15.82	9.12	17.62	
	3	9.77	14.91	8.40	20.48	
	4	12.03	13.56	9.29	21.73	NP
8	1	3.30	1.23	NP	0.94	NP
	2	NP	1.85	NP	NP	0.90
	3	NP	NP	NP	NP	0.13
	4	NP	NP	NP	NP	NP
10	1	0.00	0.00	4.00	0.00	0.00
	2	40.72	27.79	8.16	20.40	19.19
	3	0.00	0.00	5.61	12.33	0.00
	4	38.33	0.00	10.36	31.79	27.23

*excluded from further calculations due to result obtained for IS-B; NP = non-parallel

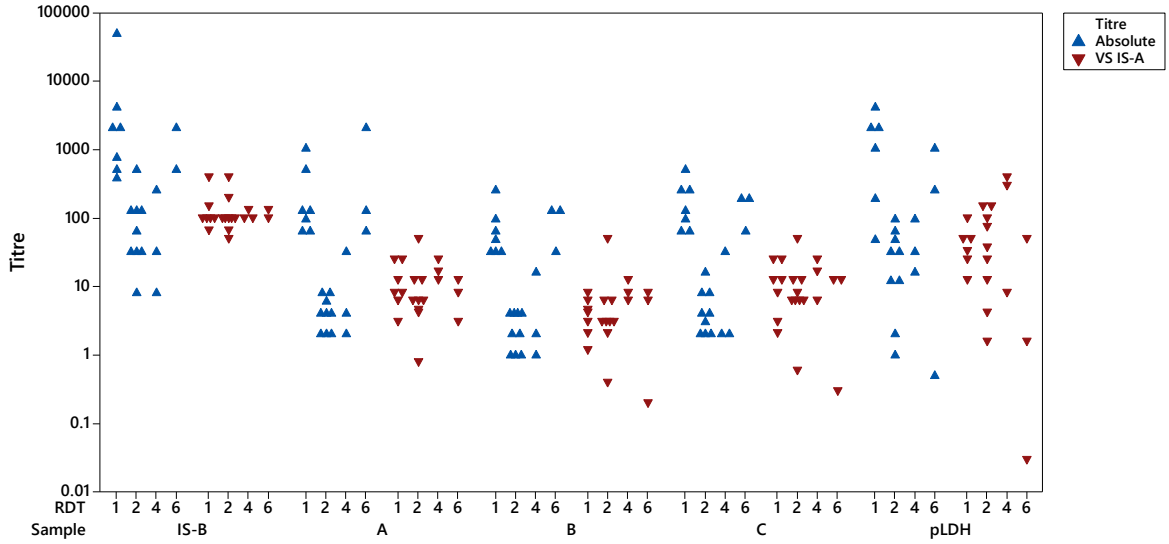
Table 14 Qualisa potency estimates as % relative to IS-A for each laboratory and assay run.

Lab	Run	Sample				
		IS-B	A	B	C	D
2	1	129.6	67.8	35.5	71.2	57.9
	2	101.3	63.4	26.3	56.3	50.5
	3	96.9	57.7	24.0	53.9	43.4
	4	99.4	59.6	28.1	56.8	42.0
3	1	60.6	42.9	19.2	34.8	40.5
	2	92.1	54.7	22.1	45.9	53.4
	3*	50.3	41.6	8.9	27.5	29.3
	4	126.0	NP	NP	NP	NP
4	1*	191.2	NP	36.0	85.1	432.7
	2	NP	NP	NP	NP	NP
	3	Cannot fit model (no convergence)				
	4	Cannot fit model (no convergence)				
5	2	76.5	44.3	21.4	36.2	
	3	69.7	30.4	16.9	20.2	NP
	4	Cannot fit model (no convergence)				
7	1	82.3	56.9	22.5	46.2	
	2	85.4	23.9	13.2	25.6	
	3	101.9	53.3	29.8	72.6	
	4	100.8	38.7	26.9	62.7	NP
8	1	80.6	61.8	NP	35.7	NP
	2	NP	31.1	NP	NP	15.5
	3	NP	NP	NP	NP	6.1
	4	NP	NP	NP	NP	NP
10	1	NP	NP	104.8	NP	NP
	2	101.4	64.6	17.3	47.9	33.4
	3	NP	NP	10.3	23.4	NP
	4	88.1	NP	19.2	66.4	52.7

*excluded from further calculations due to result obtained for IS-B; NP = non-parallel

Figure 1 Reporting LOD-titre relative to IS-A reduces GCV on the RDT Pv-LDH line

[A] Titre for limit of detection (LOD) either absolute (blue) or as % relative to IS-A (red) for each laboratory analysing RDTs 1,2,4,6 against each of the biosamples.



[B] Total variance components as GCV for LOD-titre either absolute (blue) or relative to IS-A (red).

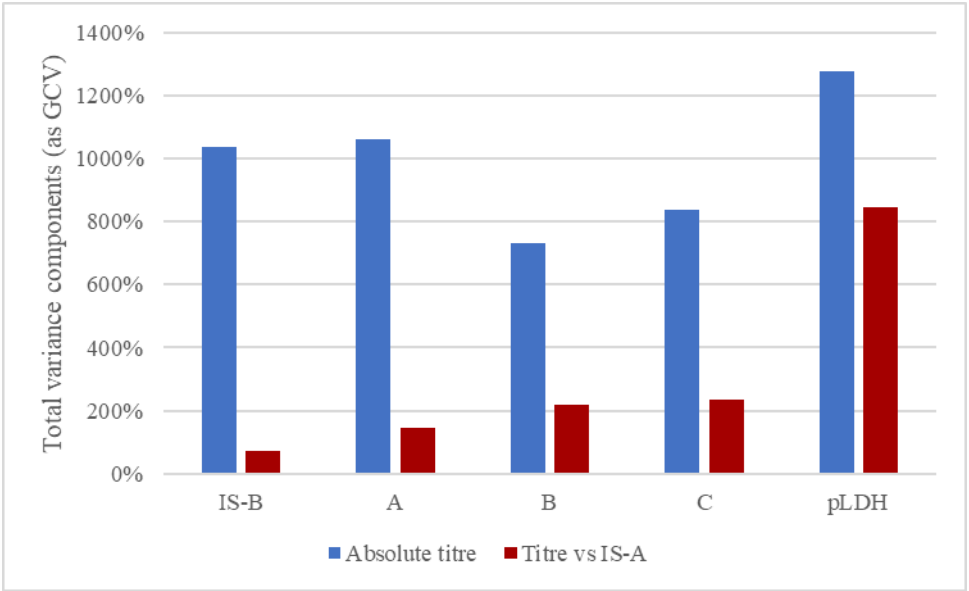
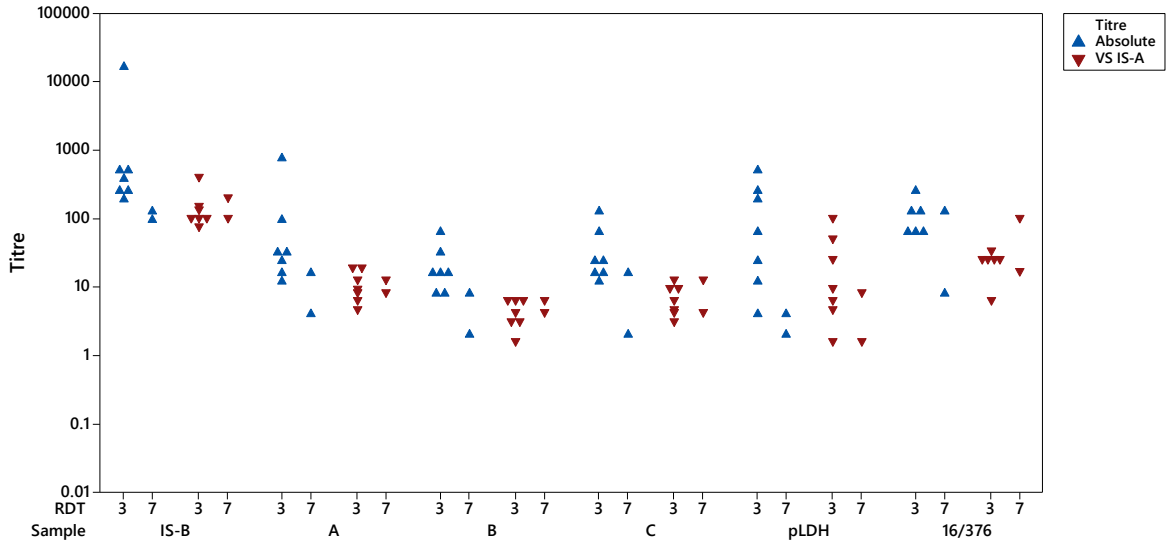


Figure 2 Reporting LOD-titre relative to IS-A reduces GCV on the RDT Pan-LDH line

[A] Titre for limit of detection (LOD) either absolute (blue) or as % relative to IS-A (red) for each laboratory analysing RDTs 3,7 against each of the biosamples.



[B] Total variance components as GCV for LOD-titre either absolute (blue) or relative to IS-A (red).

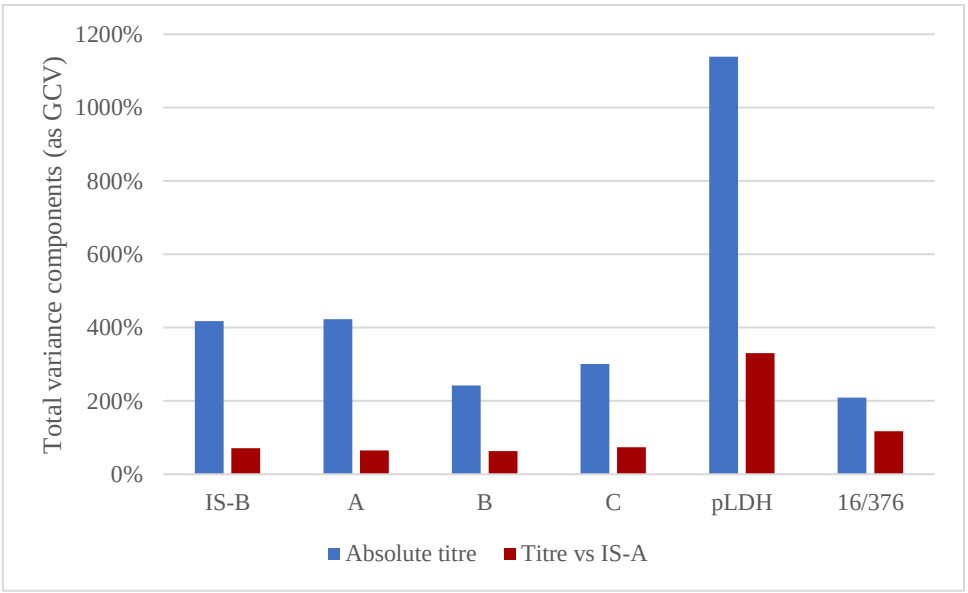
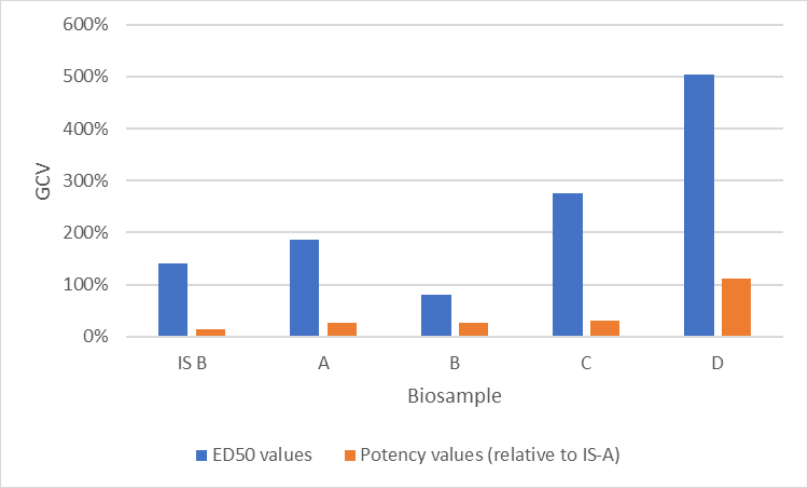


Figure 3 Expressing potency relative to IS-A reduces GCV in the ELISA

[A] Plot of GCV for each of the biosamples when consider insolation (ED_{50}, blue) or relative to IS-A (orange)



[B] Geometric mean and GCV derived from ED_{50} values

Lab	Sample GM (from ED_{50} values)				
	IS B	A	B	C	D
2	10.39	5.95	2.76	5.64	4.66
3	13.82	8.69	3.45	7.66	8.99
5	6.26	3.55	2.2	1.23	
7	20.77	16.18	8.99	19.46	
8	3.3	1.51		0.94	0.35
10	39.51	27.79	6.6	20	22.86
Overall GM	11.6	7.06	4.16	5.18	4.26
GCV	140.20%	185.30%	81.30%	274.70%	503.70%

[C] Geometric mean and GCV derived from potency relative to IS-A

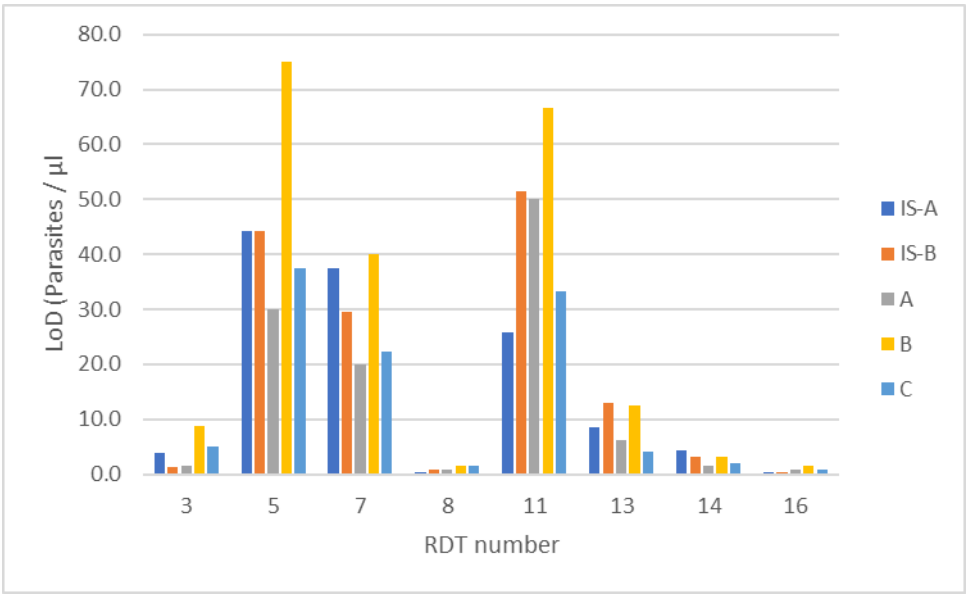
Lab	Sample GM (from potency relative to IS-A)				
	IS B	A	B	C	pLDH
2	106.1	62	28.2	59.2	48.1
3	88.9	46.1	15.5	35.3	39.9
5	73	36.7	19	27.1	
7	92.2	40.9	22.1	48.2	
8	80.6	43.9		35.7	9.8
10	94.5	64.6	24.5	42.1	42
Overall GM	88.6	47.9	21.4	40	29.8
GCV	13.90%	25.70%	25.80%	31.30%	111.00%

Appendix 1 Sensitivity limits of RDTs expressed in parasites per µL

[A] Limit of detection for devices with a Pan-line

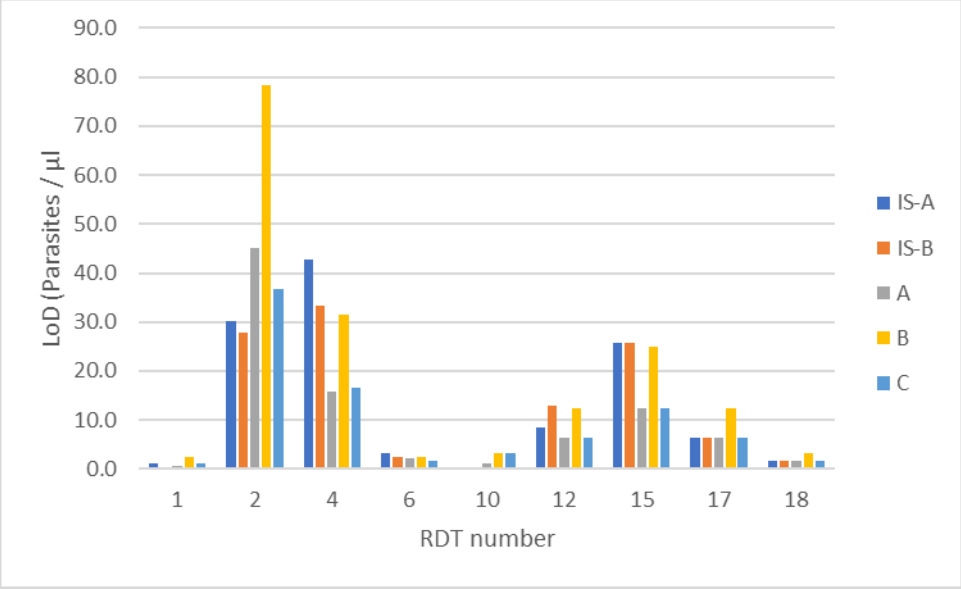
	LOD (Parasites / µL) for each biosample				
RDT	IS-A	IS-B	A	B	C
3	3.8	1.2	1.4	8.8	4.9
5*	44.2	44.2	30.0	75.0	37.5
7	37.5	29.5	20.0	40.0	22.2
8	0.4	0.8	0.8	1.6	1.6
11	25.8	51.6	50.0	66.7	33.3
13	8.6	12.9	6.3	12.5	4.2
14	4.3	3.2	1.6	3.1	2.1
16	0.4	0.4	0.8	1.6	0.8
Correlation with IS-A		0.88	0.76	0.91	0.93

*This device detects aldolase not PvLDH



[B] Limit of detection for devices with a PvLDH-line

	LOD (Parasites / µL) for each biosample				
RDT	IS-A	IS-B	A	B	C
1	1.0	0.4	0.7	2.5	1.0
2	30.2	27.9	45.0	78.3	36.7
4	42.7	33.4	15.8	31.6	16.7
6	3.2	2.6	2.1	2.5	1.6
10	0.2	0.4	1.0	3.1	3.1
12	8.6	12.9	6.3	12.5	6.3
15	25.8	25.8	12.5	25.0	12.5
17	6.4	6.4	6.3	12.5	6.3
18	1.6	1.6	1.6	3.1	1.6
Correlation with IS-A		0.71	0.74	0.75	0.77



[C] Limit of detection for device with a Pvom line

	LOD (Parasites / µL) for each biosample				
RDT	IS-A	IS-B	A	B	C
9	51.6	51.6	25.0	33.3	50.0

Appendix 2 Collaborative study participants

(In alphabetical order)

Institution	Country	Organisation type	Participants
Abbott	Global	RDT Manufacturer	Guarav Singh
			Yujin Kim
Access Bio Inc.	USA	RDT Manufacturer	Young Woo Kim
ARKRAY Healthcare Pvt Ltd	India	RDT Manufacturer	Sarang Selote
CDC	USA	Government	Michael Aidoo
Cellabs Pty Ltd	Australia	Manufacturer	Diane Dogcio-Hall
Guangzhou Wondfo Biotech Co., Ltd.	China	RDT Manufacturer	Junxing Huang
			Ms. Amy
Interactive Research and Development Global	Pakistan	Research	Mah Talat
			Sabiha Anis
IS Global	Spain	Research	Alfredo Mayor
			Alfons Jimenez
Jiangsu Bioperfectus Biotech	China	RDT Manufacturer	Wei Jin
			Tong Guoquan
National Institute for Biological Standards and Control	UK	Government	Paul Bowyer
			Lynne Harris
			Charles Olomu
National Institute for Malaria Research	India	Government	Anupkumar Anvikar
			Bina Srivastva
			Paras Mahale
Premier Medical Corporation Private Ltd.	India	RDT Manufacturer	ajeshkumar patel
Research Institute for Tropical Medicine	The Philippines	Research	Jennifer Luchavez
			Christian Anthony Luna
SD Biosensor	Korea	RDT Manufacturer	Cedric Jo
Span Diagnostics	France	Manufacturer	Daniela Balvay Walid Yakoub Maria Haupt
University of Lagos	Nigeria	Research	Wellington Oyibo

Appendix 3 Instructions for use

Medicines & Healthcare products Regulatory Agency

NIBSC Confidence in Biological Medicines

WHO International Standard
1st WHO International Standard for Plasmodium vivax antigen (LDH)
NIBSC code: 19/116
Instructions for use
(Version 1.00, Dated)

1. INTENDED USE

This preparation contains red blood cell (RBC) lysates from P. vivax-infected donors from Peru. The intended use for is for the standardisation, and evaluation of performance and sensitivity of P. vivax antigen detection tests that detect P. vivax lactate dehydrogenase (PvLHDH) as well as for the calibration of secondary reference materials.

2. CAUTION

This preparation is not for administration to humans or animals in the human food chain.

The preparation contains material of human origin, and either the final product or the source materials, from which it is derived, have been tested and found negative for HBsAg, anti-HIV and HCV RNA. As with all materials of biological origin, this preparation should be regarded as potentially hazardous to health. It should be used and discarded according to your own laboratory's safety procedures. Such safety procedures should include the wearing of protective gloves and avoiding the generation of aerosols. Care should be exercised in opening ampoules or vials, to avoid cuts.

3. UNITAGE

This material has an assigned unitage of 1000 International Units of PvLDH per ampoule.

4. CONTENTS

Country of origin of biological material: Peru.
Each ampoule is a lyophilate of a preparation that contained 0.5 ml of lysed red blood cells from P. vivax infected donors diluted 1:4 into 11.43 mM Tris pH 7.4, 6.43 % trehalose, 1.14 mM EDTA buffer.

5. STORAGE

This preparation should be stored at -20°C or below on receipt. If the material is to be stored for more than 3 months prior to use storage at -70°C is recommended.
Please note: because of the inherent stability of lyophilized material, NIBSC may ship these materials at ambient temperature.

6. DIRECTIONS FOR OPENING

DIN ampoules have an 'easy-open' coloured stress point, where the narrow ampoule stem joins the wider ampoule body. Various types of ampoule breaker are available commercially. To open the ampoule, tap the ampoule gently to collect material at the bottom (labelled) end and follow manufactures instructions provided with the ampoule breaker.

7. USE OF MATERIAL

No attempt should be made to weigh out any portion of the freeze-dried material prior to reconstitution
This material is supplied lyophilised and before use should be reconstituted in 0.25 mL of whole blood. Reconstituted material should be used on the day of reconstitution.

8. STABILITY

Reference materials are held at NIBSC within assured, temperature-controlled storage facilities. Reference Materials should be stored on receipt as indicated on the label.

NIBSC follows the policy of WHO with respect to its reference materials.

9. REFERENCES

Charles Olomu, Lynne M. Harris, Peter Rigsby, Eleanor Atkinson, Seda Yerlikaya, Xavier Ding, Paul W. Bowyer,* and the Collaborative Study Group (2020). Collaborative study to evaluate the proposed First World Health Organization International Standard for Plasmodium vivax antigens. WHO/BS/2020.xxxx

10. ACKNOWLEDGEMENTS

This material was developed in collaboration with the Foundation for Innovative New Diagnostics (FIND). We gratefully acknowledge the significant contributions of the collaborative study group in the development of this IS. We would like to thank Professor Dionicia Gamboa and Dr. Katherine Torres from Malaria Laboratory in UPCH (Lima, Peru) and the study staff in Iquitos, Peru for undertaking the specimen collection study, Further acknowledgment is extended to the patients who kindly donated samples for this project. We also thank the WHO malaria specimen bank at the US CDC for providing clinical isolates for the collaborative study.

11. FURTHER INFORMATION

Further information can be obtained as follows;
This material: enquiries@nibsc.org
WHO Biological Standards:
http://www.who.int/biologicals/en/
JCTLM Higher order reference materials:
http://www.bipm.org/en/committees/jc/jctlm/
Derivation of International Units:
http://www.nibsc.org/standardisation/international_standards.aspx
Ordering standards from NIBSC:
http://www.nibsc.org/products/ordering.aspx
NIBSC Terms & Conditions:
http://www.nibsc.org/terms_and_conditions.aspx

12. CUSTOMER FEEDBACK

Customers are encouraged to provide feedback on the suitability or use of the material provided or other aspects of our service. Please send any comments to enquiries@nibsc.org

13. CITATION

In all publications, including data sheets, in which this material is referenced, it is important that the preparation's title, its status, the NIBSC code number, and the name and address of NIBSC are cited and cited correctly.

14. MATERIAL SAFETY SHEET

Classification in accordance with Directive 2000/54/EC, Regulation (EC) No 1272/2008: Not applicable or not classified

Physical and Chemical properties			
Physical appearance: Freeze-dried		Corrosive:	No
Stable:	Yes	Oxidising:	No
Hygroscopic:	No	Irritant:	No
Flammable:	No	Handling:	See caution, Section 2
Other (specify):	Contains material of human origin		

Toxicological properties	
Effects of inhalation:	Not established, avoid inhalation
Effects of ingestion:	Not established, avoid ingestion
Effects of skin absorption:	Not established, avoid contact with skin

National Institute for Biological Standards and Control,
Potters Bar, Hertfordshire, EN6 3QG. T +44 (0)1707 641000, nibsc.org
WHO International Laboratory for Biological Standards,
UK Official Medicines Control Laboratory

World Health Organization

Medicines & Healthcare products Regulatory Agency

NIBSC Confidence in Biological Medicines

Suggested First Aid	
Inhalation:	Seek medical advice
Ingestion:	Seek medical advice
Contact with eyes:	Wash with copious amounts of water. Seek medical advice
Contact with skin:	Wash thoroughly with water.

Action on Spillage and Method of Disposal
Spillage of ampoule contents should be taken up with absorbent material wetted with an appropriate disinfectant. Rinse area with an appropriate disinfectant followed by water. Absorbent materials used to treat spillage should be treated as biological waste.

15. LIABILITY AND LOSS

In the event that this document is translated into another language, the English language version shall prevail in the event of any inconsistencies between the documents.

Unless expressly stated otherwise by NIBSC, NIBSC's Standard Terms and Conditions for the Supply of Materials (available at http://www.nibsc.org/About_Us/Terms_and_Conditions.aspx or upon request by the Recipient) ("Conditions") apply to the exclusion of all other terms and are hereby incorporated into this document by reference. The Recipient's attention is drawn in particular to the provisions of clause 11 of the Conditions.

16. INFORMATION FOR CUSTOMS USE ONLY

Country of origin for customs purposes*: United Kingdom * Defined as the country where the goods have been produced and/or sufficiently processed to be classed as originating from the country of supply, for example a change of state such as freeze-drying.
Net weight: 0.523 g
Toxicity Statement: Non-toxic
Veterinary certificate or other statement if applicable. **Attached:** No

17. CERTIFICATE OF ANALYSIS

NIBSC does not provide a Certificate of Analysis for WHO Biological Reference Materials because they are internationally recognised primary reference materials fully described in the instructions for use. The reference materials are established according to the WHO Recommendations for the preparation, characterization and establishment of international and other biological reference standards http://www.who.int/bloodproducts/publications/TRS932Annex2_Inter_biolefstandardsrev2004.pdf (revised 2004). They are officially endorsed by the WHO Expert Committee on Biological Standardization (ECBS) based on the report of the international collaborative study which established their suitability for the intended use.

Appendix 4

PvLDH ELISA (QUALISA) Protocol

COLLABORATIVE STUDY PROTOCOL

First WHO International Standard for *Plasmodium vivax* antigens

pLDH ELISA (QUALISA)

Version: V1.2
Date: 5th August 2019

Project Manager at NIBSC: Lynne Harris
E-mail: lynne.harris@nibsc.org


FIND
Because diagnosis matters
NIBSC

CONFIDENTIALITY STATEMENT

VERSION HISTORY

Authors: Lynne Harris, NIBSC
Paul Bowyer, NIBSC
Seda Yerlikaya, FIND

Initial Release Version: 1.0
Initial Release Date: 20th March 2019

Current Version: 1.2
Current Version Date: 5th August 2019

Protocol revision summary

Date of Revision	Initial version	Summary of revision	Updated version
	1.0		
4th June 2019	1.0	Protocol revised following protocol review by all study participants	1.1
5th August 2019	1.1	Initial biosample dilutions added. Volume of biosamples added. Number of serial dilutions updated. Instructions for addition of samples to ELISA plate updated. Figure 1 updated.	1.2

TABLE OF CONTENTS

ABBREVIATIONS

ELISA	Enzyme Linked Immunosorbent Assay
FIND	Foundation for Innovative New Diagnostics
HRP2	Histidine-Rich Protein II
IS	International Standard
NIBSC	National Institute for Biological Standards and Control
Pf	*Plasmodium falciparum*
pLDH	*Plasmodium* lactate dehydrogenase
Pv	*Plasmodium vivax*
SOP	Standard Operating Procedure
TMB	3,3',5,5'-Tetramethylbenzidine
WHO	World Health Organization

First WHO International Standard for *Plasmodium vivax* antigens

Study Protocol

1. BACKGROUND

In order to prevent the unnecessary use of antimalarial drugs the World Health Organization (WHO) recommends that all suspected cases of malaria are confirmed by a parasitological diagnosis prior to treatment. Immunochromatographic rapid diagnostic tests (RDTs), that detect *Plasmodium*-specific antigens in patient blood samples, offer a convenient means of diagnosis. RDTs are based on the detection of two *Plasmodium* antigens – histidine-rich protein II (HRP2) and *Plasmodium* lactate dehydrogenase (pLDH). HRP2 is specific to *P. falciparum* whereas pLDH is expressed in all human-infecting *Plasmodium* species. Species-specific isoforms of pLDH have enabled the development of antibodies specific to *P. falciparum* pLDH (Pf-pLDH) or *P. vivax* pLDH (PvLDH). Hence, the main *Plasmodium vivax* antigen targeted by RDTs is PvLDH.

In 2017 the 1st WHO International Standard (IS) for *P. falciparum* antigens was established. However, there is currently no validated international reference standard for use as a quality control and calibrator material in PvLDH antigen detection assays. To this end, the Foundation for Innovative New Diagnostics (FIND) is collaborating with the National Institute for Biological Standards and Control (NIBSC) to establish the first WHO IS for *P. vivax* antigens. It is intended that the development of this IS will provide a standardised material that can be used in the quality control and standardisation of RDTs worldwide, for monitoring the development of more sensitive diagnostic tests, and for the calibration of other reference materials and controls to be used on a larger scale.

2. AIM OF THE COLLABORATIVE STUDY

To assess the suitability of lyophilized preparations of *P. vivax* parasites to serve as the first WHO International Standard for *P. vivax* antigens.

3. APPROACH

NIBSC and FIND will distribute materials to participant laboratories where the experiments are to be performed. The raw data should be returned to NIBSC in the form described in the template associated with each protocol. The combined data from all participants will be analysed by NIBSC and FIND and used in the construction of a report describing all aspects of the establishment of the International Standard. This report will be submitted to the WHO Expert Committee on Biological Standardization (ECBS). A subsequent report for submission to a scientific journal may also be written.

Participants are requested to measure pLDH levels in the samples provided by carrying out an ELISA. **The ELISA study protocol detailed herein should be repeated four times with each repeat carried out on a different day and starting with fresh, unopened sample vials**.

4. SAFETY CONSIDERATIONS

Prior to initiating the study, please read this document carefully. Note all the statements regarding safety and that these materials are not for human use. Please note that blood samples pose a potential risk of infection. Use universal precautions to minimize biohazards.

It is the responsibility of the laboratory supervisor (the person who has immediate responsibility for the laboratory) to ensure the development and adoption of a biosafety management plan and a safety operations manual. The laboratory supervisor should ensure that regular training in laboratory safety is provided. Personnel should be required to read the local standard operation procedures manual on safety and a copy of this manual should be available in the laboratory.

5. STUDY TIMELINES

	MAR		APR		MAY		JUN		JUL		AUG		SEP		OCT		NOV	
ACTIVITIES																		
1. Protocol review by participants																		
2. Where applicable, participants apply for import permits and/or local ethical approval																		
3. Shipment of study materials to participating centres																		
4. Laboratory testing																		
5. Deadline for data submission to NIBSC																		

6. SOP. QUALISA pLDH ELISA for malaria antigen in blood

6.1. Purpose

This SOP describes the materials, equipment, and procedures required to correctly and safely use the Qualpro Diagnostics Qualisa malaria antigen pLDH ELISA kit.

6.2. Scope

This SOP has been developed in the context of the collaborative study to establish the first WHO International Standard for *Plasmodium vivax* antigens. This procedure is an adaptation of part of the methodology described in the "Methods Manual for laboratory quality control testing of malaria rapid diagnostic tests" (SOP 3.10 and SOP 5.01), as well as the SOP used at the Hospital for Tropical Diseases (UK) for the training of laboratory personnel using the Qualisa malaria antigen pLDH ELISA kit in research settings. The protocol was originally designed to describe the use of this assay to assess pLDH antigen content within patient blood samples.

6.3. Principle of test

Qualisa malaria antigen ELISA is suitable for the detection, in a blood sample, of the four species of malaria infecting humans. After whole blood is lysed, pLDH in the blood specimen is bound to immobilised Pan-specific monoclonal antibodies. Unbound material is then removed with a rigorous wash step and bound pLDH recognised by addition of a biotinylated antibody Pan-specific for pLDH. After removal of unbound biotinylated antibody with additional rigorous washing, streptavidin-peroxidase is added. Peroxidase activity is measured using a substrate solution of TMB and the reaction product is subsequently quenched using an acid stop solution. The colour intensity of the resulting product is directly proportional to the pLDH concentration and is measured as ΔOD 450/620 nm.

6.4. Materials

6.4.1. Biosamples

Participants will be sent a total of 6 biosamples (5 aliquots of each biosample – allowing 1 aliquot for each of the four independent experimental runs + 1 spare) including:

- Two candidate International Standard preparations (ISA and ISB): lyophilised pools of *P. vivax* clinical isolates.
- Three *P. vivax* clinical isolates CI-A, CI-B and CI-C (liquid). Each aliquot contains 30 µL.
- One recombinant PvLDH protein (liquid). Each aliquot contains 10 µL.

Please store all biosamples at -20 °C or below upon receipt.

Participants are requested to provide parasite-free whole blood (of any blood type) which should be used for the following:

- To reconstitute the lyophilised IS materials
- As a negative control
- For sample dilutions.

The required parasite-free blood volume for the whole ELISA study in each centre is 27 mL. The parasite-free blood should be stored at -70 °C prior to use in order to lyse red blood cells. This avoids any clumping issues. Sub-aliquot the parasite-free blood prior to starting the study such that one vial is thawed per experimental run.

6.4.2. Equipment

- Sarstedt tubes
- Rack for dilutions
- Micropipettes (1-20 µL, 20 – 200 µL and 100-1000 µL)
- Multichannel micropipette (50 – 250 µL)
- Pipette tips (1-20 µL, 20 – 200 µL and 100-1000 µL capacity)
- Rack for samples and dilutions
- Timer
- Vortex
- Anti-pLDH coated test plate*
- Sample diluent*
- Antibody reagent*
- Enzyme conjugate*
- Conjugate diluent*
- TMB substrate solution*
- Stop solution*
- 1 X PBST (20 x is supplied with the test kit)
- Plate lid
- Automated plate washer
- 37 °C incubator
- -70ºC freezer
- +4ºC refrigerator
- Incubator thermometer (range: 0ºC to +100ºC)
- Spectrophotometer
- Marker pen
- Waste bin for biological samples
- Gloves

**Contained within individual Qualisa pLDH kit boxes.* ELISA reagents are allowed to equilibrate to room temperature for 15 min prior to use.

6.5. Procedure

6.5.1. Reconstitution of candidate IS materials and dilution of samples for testing

A fresh tube/ampoule of each biosample should be used for each of the four independent experimental runs. In the case of the lyophilised samples this will require reconstitution of a fresh ampoule prior to the start of each experimental run. Dilutions of all preparations should be tested at the same time in order to simultaneously determine and compare the pLDH content.

- Remove one aliquot of each of the two international standard candidates, one aliquot of each of the three clinical isolates, one aliquot of the recombinant protein and parasite-free blood from the freezer. Thaw out on the bench at room temperature. Samples should be used immediately once completely thawed.
- Reconstitute each of the candidate IS materials in 250 µL of parasite-free blood. Allow the material in the vial to reconstitute for 15-20 minutes at room temperature with gentle agitation (e.g. a rocker platform or rotation by hand). Avoid vigorous shaking that can cause foaming and protein denaturation.
- Biosample dilutions should be prepared using parasite-free human whole blood as a diluent. The blood should have gone through a minimum of one freeze-thaw cycle prior to use as a diluent. The blood will also be used as a negative control. **Keep all dilutions on ice** until the end of the experiment.
- Each clinical isolate aliquot contains 30 µL. Perform a 1:5 dilution of each clinical isolate by adding 120 µL parasite-free blood **directly** to each aliquot. 2-fold serial dilutions should be prepared from this initial 1:5 dilution (Table 1).
- Prepare **seven (7) 2- fold serial dilutions** to obtain a total of eight (8) dilution points (see Table 1). Samples should be pipetted up and down at least three times and mixed on a vortex before transferring to the next tube. A fresh pipette tip should be used for each transfer.

Table 1. Dilution of the clinical isolates

Dilution stock	1 (1:5)	1/2	1/4	1/8	1/16	1/32	1/64	1/128
Working stock	Stock aliquot	1	1/2	1/4	1/8	1/16	1/32	1/64
Volume stock (µL)	30	75	75	75	75	75	75	75
Volume diluent (µL)	120	75	75	75	75	75	75	75
Total volume (µL)	150	150	150	150	150	150	150	150

- Each of the two candidate IS ampoules contains 250 µL once reconstituted. Perform a 1:3 dilution of each candidate IS sample by adding 100 µL parasite-free blood to 50 µL of the reconstituted candidate IS sample. 2-fold serial dilutions should be prepared from this initial 1:3 dilution (Table 2).
- Prepare **seven (7) 2-fold, serial dilutions** to obtain a total of eight (8) dilution points as detailed in Table 2. Samples should be pipetted up and down at least three times and mixed on a vortex before transferring to the next tube. A fresh pipette tip should be used for each transfer.

Table 2. Dilution of the candidate IS materials

Dilution stock	1 (1:3)	1/2	1/4	1/8	1/16	1/32	1/64	1/128
Working stock	Reconstituted ampoule	1	1/2	1/4	1/8	1/16	1/32	1/64
Volume stock (µL)	50	75	75	75	75	75	75	75
Volume diluent (µL)	100	75	75	75	75	75	75	75
Total volume (µL)	150	150	150	150	150	150	150	150

- Aliquots of recombinant PvLDH contain 10 µL. 2-fold serial dilutions should be prepared from an initial 1:200 dilution of the aliquot supplied. It is recommended that this initial 1:200 dilution is performed in two steps to prevent pipetting small volumes.
- Prepare six **(6) 2-fold serial dilutions** to obtain a total of seven (7) dilution points (See Table 3). Samples should be pipetted up and down at least three times and mixed on a vortex before transferring to the next tube. A fresh pipette tip should be used for each transfer.

Table 3. Dilution of the recombinant protein

Dilution stock	1 (1:200)	1/2	1/4	1/8	1/16	1/32	1/64
Working stock	Stock aliquot	1	1/2	1/4	1/8	1/16	1/32
Volume stock (µL)	?	75	75	75	75	75	75
Volume diluent (µL)	?	75	75	75	75	75	75
Total volume (µL)	150	150	150	150	150	150	150

- Sample aliquots and dilutions should be used **only once** (do not re-freeze unused and/or left-over samples). Discard left-over samples according to local laboratory safety procedures.

6.5.2. Preparation of the coated plate

Take out the required number of well strips from packaging and fix into the plate carcass.

Using a multichannel pipette, add 100 µL sample diluent to all wells of the anti-pLDH coated microplate.

Add 25 µL of each of the pre-prepared dilutions, in duplicate, into the wells using a standard micropipette. Add 25 µL of blood diluent only to negative control wells.

The columns that each set of biosample dilutions are added to should be randomised for each of the 4 experimental runs. Dilutions of a particular biosample should always run down the plate (rows A-H) but the columns the biosamples are allocated to should be randomised for each experimental run. An example of this is shown in figure 1, runs 1-4. This example is for illustrative purposes only and should not be followed as an exact template.

When all samples have been added to the coated plate gently shake the plate to mix contents. Apply plate sealer and incubate at 37°C for 30 mins

Figure 1. ELISA sample layout (example)

Run 1

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	ISA_1	ISA_1	A_1	A_1	B_1	B_1	C_1	C_1	D_1	D_1	ISB_1	ISB_1
B	ISA_2	ISA_2	A_2	A_2	B_2	B_2	C_2	C_2	D_2	D_2	ISB_2	ISB_2
C	ISA_3	ISA_3	A_3	A_3	B_3	B_3	C_3	C_3	D_3	D_3	ISB_3	ISB_3
D	ISA_4	ISA_4	A_4	A_4	B_4	B_4	C_4	C_4	D_4	D_4	ISB_4	ISB_4
E	ISA_5	ISA_5	A_5	A_5	B_5	B_5	C_5	C_5	D_5	D_5	ISB_5	ISB_5
F	ISA_6	ISA_6	A_6	A_6	B_6	B_6	C_6	C_6	D_6	D_6	ISB_6	ISB_6
G	ISA_7	ISA_7	A_7	A_7	B_7	B_7	C_7	C_7	D_7	D_7	ISB_7	ISB_7
H	ISA_8	ISA_8	A_8	A_8	B_8	B_8	C_8	C_8	**Neg CTL**	**Neg CTL**	ISB_8	ISB_8

Run 2

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	ISB_1	ISB_1	ISA_1	ISA_1	A_1	A_1	B_1	B_1	C_1	C_1	D_1	D_1
B	ISB_2	ISB_2	ISA_2	ISA_2	A_2	A_2	B_2	B_2	C_2	C_2	D_2	D_2
C	ISB_3	ISB_3	ISA_3	ISA_3	A_3	A_3	B_3	B_3	C_3	C_3	D_3	D_3
D	ISB_4	ISB_4	ISA_4	ISA_4	A_4	A_4	B_4	B_4	C_4	C_4	D_4	D_4
E	ISB_5	ISB_5	ISA_5	ISA_5	A_5	A_5	B_5	B_5	C_5	C_5	D_5	D_5
F	ISB_6	ISB_6	ISA_6	ISA_6	A_6	A_6	B_6	B_6	C_6	C_6	D_6	D_6
G	ISB_7	ISB_7	ISA_7	ISA_7	A_7	A_7	B_7	B_7	C_7	C_7	D_7	D_7
H	ISB_8	ISB_8	ISA_8	ISA_8	A_8	A_8	B_8	B_8	C_8	C_8	**Neg CTL**	**Neg CTL**

Run 3

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	D_1	D_1	ISB_1	ISB_1	ISA_1	ISA_1	A_1	A_1	B_1	B_1	C_1	C_1
B	D_2	D_2	ISB_2	ISB_2	ISA_2	ISA_2	A_2	A_2	B_2	B_2	C_2	C_2
C	D_3	D_3	ISB_3	ISB_3	ISA_3	ISA_3	A_3	A_3	B_3	B_3	C_3	C_3
D	D_4	D_4	ISB_4	ISB_4	ISA_4	ISA_4	A_4	A_4	B_4	B_4	C_4	C_4
E	D_5	D_5	ISB_5	ISB_5	ISA_5	ISA_5	A_5	A_5	B_5	B_5	C_5	C_5
F	D_6	D_6	ISB_6	ISB_6	ISA_6	ISA_6	A_6	A_6	B_6	B_6	C_6	C_6
G	D_7	D_7	ISB_7	ISB_7	ISA_7	ISA_7	A_7	A_7	B_7	B_7	C_7	C_7
H	**Neg CTL**	**Neg CTL**	ISB_8	ISB_8	ISA_8	ISA_8	A_8	A_8	B_8	B_8	C_8	C_8

Run 4

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	C_1	C_1	D_1	D_1	ISB_1	ISB_1	ISA_1	ISA_1	A_1	A_1	B_1	B_1

B	C_2	C_2	D_2	D_2	ISB_2	ISB_2	ISA_2	ISA_2	A_2	A_2	B_2	B_2
C	C_3	C_3	D_3	D_3	ISB_3	ISB_3	ISA_3	ISA_3	A_3	A_3	B_3	B_3
D	C_4	C_4	D_4	D_4	ISB_4	ISB_4	ISA_4	ISA_4	A_4	A_4	B_4	B_4
E	C_5	C_5	D_5	D_5	ISB_5	ISB_5	ISA_5	ISA_5	A_5	A_5	B_5	B_5
F	C_6	C_6	D_6	D_6	ISB_6	ISB_6	ISA_6	ISA_6	A_6	A_6	B_6	B_6
G	C_7	C_7	D_7	D_7	ISB_7	ISB_7	ISA_7	ISA_7	A_7	A_7	B_7	B_7
H	C_8	C_8	**Neg CTL**	**Neg CTL**	ISB_8	ISB_8	ISA_8	ISA_8	A_8	A_8	B_8	B_8

ISA and ISB: candidate international standard materials.
Neg. CTL: negative control, consisting of 25 µL of parasite-free blood.
A, B, C: clinical isolates
D: recombinant PvLDH protein

6.5.3. Wash steps

The wash solution contained within the assay kit is 20x PBST. A 1 Litre stock of 1x PBST should be made up and used to wash the wells of the coated plate x 6 with an automatic plate washer set to fill the wells with 350 µL solution.

6.5.4.Addition of detection antibody

Working strength antibody reagent should be made up fresh. For each plate, 220 µL antibody reagent stock solution (50x) should be diluted in 11 mL conjugate diluent and mixed thoroughly. Using a multichannel pipette, 100 µL working strength antibody reagent should be dispensed into all test wells. The plate should be covered with a plastic lid and incubated for 30 mins at 37 ºC.

Plates should then be washed rigorously again as described in 6.5.3.

6.5.5.Addition of enzyme conjugate

Working strength enzyme conjugate should be made up fresh. For each plate, 220 µL enzyme conjugate stock solution (50x) should be diluted in 11 mL conjugate diluent and mixed thoroughly. Using a multichannel pipette, 100 µL working strength antibody reagent should be dispensed into all test wells. The plate should be covered with a plastic lid and incubated for 30 mins at room temperature.

Plates should then be washed rigorously again as described in 6.5.3.

6.5.6.Development of substrate

The TMB substrate contained within the kits is already at working strength and 100 µL of working substrate should be dispensed into each well using the multichannel pipette.

The plate should then be covered with a plastic lid and incubated at room temperature for ~ 10 minutes in the dark.

The acid stop solution is contained within the assay kit and 100µL should be then dispensed in all wells. The endpoint absorbance of the wells should be read at 450 nm with a reference wavelength of 620 nm within 10 mins of adding stop solution.

Repeat the procedure in order to perform a total of FOUR independent tests on separate days.

6.6. Information to be supplied and presentation of results

An excel template ("CS644 Result form_ELISA") is provided so that all relevant information can be recorded as indicated. A separate data table should be completed for each independent run. Please **PROVIDE ALL RAW DATA** to enable consistent handling and analysis of data from all participants. Also use the excel file to give brief information regarding:

- dilutions used (stock and working solutions)
- assay (type, when it was performed, results)
- Plasmodium-free blood used

Participants should follow the protocol. ALL protocol deviations, however minor, must be recorded in the additional comments/relevant information section of the excel template for each run.
Participants are requested to return all data and any information relating to the assays electronically in the requested format to:

Contact: Paul Bowyer
Tel: +44 (0)1707 64 1474
E-mail: paul.bowyer@nibsc.org

Contact: Charles Olomu
Tel: +44 (0)1707 64 1432
E-mail: charles.olomu@nibsc.org

Contact: Seda Yerlikaya
Tel: +41 (0) 22 749 19 21
E-mail: Seda.Yerlikaya@finddx.org
Deadline for data submission: November 30th 2019.

6.7. Health and safety

6.7.1. Hazardous reagents

Table 3. Hazardous chemicals used in the QUALISA malaria antigen pLDH ELISA

Product	Fire hazard	Health hazard	Toxicity	Storage requirements
Hydrogen peroxide (TMB)	Explosive under heat	Irritant to eyes/skin/nasal passage	Moderately toxic	Easily decomposes 2-8 ^{0}C

Sulphuric acid (stop solution)	Flammable	Irritating to eyes/skin. Burns. Harmful by ingestion.	Toxic	Keep in a locked store
TMB	Flammable	Harmful swallowed/inhaled/absorbed by skin	Toxic	Store solutions in light proof container at 4 -8 °C

6.7.2.Safety precautions

Disposable latex or nitrile gloves must be worn while handling clinical specimens and reagents. All clinical material i.e. all components containing blood must be autoclaved before disposal. The assay stop solution contains sulphuric acid a corrosive and hazardous substance. Avoid eye and skin contact by wearing protective clothing and eye protection.

Hands must be washed once work has been completed. Please follow local laboratory health and safety practices at all times.

6.7.3. Technical precautions

- Components must not be used after their expiry date.
- Different batches/lots of reagents should never be interchanged.
- Storage of reagents must be at the recommended conditions.
- Contamination of reagents should be avoided by changing pipette tips where necessary.

7. FINAL REPORTING

A draft report will be prepared and circulated to all participants for comment prior to submission to the WHO Expert Committee on Biological Standardization. In the report, participating laboratories will be identified by a laboratory number only and any request to treat information in confidence will be respected. The report will contain the following sections:

- Summary
- Introduction
- Collaborative Study (Aims of study; Participants; Organisation of the collaborative study)
- Materials
- Study design and assay methods
- Stability studies (Accelerated degradation study; In use stability after reconstitution)
- Statistical Analysis
- Results (Data received; Stability Studies)
- Discussion
- Proposal

NOTE: Participants in the collaborative study are asked to note that:

- They participate in the study with the understanding that they agree not to publish or circulate information concerning the materials sent to them without the prior consent of the organizers; and
- Their results may be shared anonymously with not-for-profit public health bodies in the interests of global harmonization.

Appendix 5:

PvLDH ELISA (CELISA) ProtocolPvLDH ELISA (CELISA) Protocol

Collaborative Study Protocol
Protocol for the WHO collaborative study to assess the suitability of an International Standard for *P. vivax* antigens

Please follow this protocol below if your laboratory is measuring PvLDH levels using an in-house ELISA method or a commercial kit other than the Qualisa malaria kit.

Background
In order to prevent the unnecessary use of antimalarial drugs the World Health Organization (WHO) recommends that all suspected cases of malaria are confirmed by a parasitological diagnosis prior to treatment. Immunochromatographic rapid diagnostic tests (RDTs), that detect *Plasmodium*-specific antigens in patient blood samples, offer a convenient means of diagnosis. RDTs are based on the detection of two *Plasmodium* antigens – histidine-rich protein II (HRP2) and *Plasmodium* lactate dehydrogenase (pLDH). HRP2 is specific to *P. falciparum* whereas pLDH is expressed in all human-infecting *Plasmodium* species. Species-specific isoforms of pLDH have enabled the development of antibodies specific to *P. falciparum* pLDH (Pf-pLDH) or *P. vivax* pLDH (PvLDH). Hence, the main *Plasmodium vivax* antigen targeted by RDTs is PvLDH.

In 2017 the 1st WHO International Standard (IS) for *P. falciparum* antigens was established. However, there is currently no validated international reference standard for use as a quality control and calibrator material in PvLDH antigen detection assays. To this end, the Foundation for Innovative New Diagnostics (FIND) is collaborating with the National Institute for Biological Standards and Control (NIBSC) to establish the first WHO IS for *P. vivax* antigens. It is intended that the development of this IS will provide a standardised material that can subsequently be used in the quality control and standardisation of RDTs worldwide, for monitoring the development of more sensitive diagnostic tests, and for the calibration of other reference materials and controls that can then be used on a larger scale.

Aims
To assess the suitability of lyophilized preparations of *P. vivax* parasites to serve as the first WHO International Standard for *P. vivax* antigens.

Study samples
Participants will be sent a total of 6 biosamples (5 aliquots of each biosample) including:

- Two candidate International Standard preparations (IS-A and IS-B): lyophilised pools of *P. vivax* clinical isolates. Please resuspend in 250 µL whole blood prior to use.
- Three frozen *P. vivax* clinical isolates (liquid). Each aliquot contains 30 µL at a concentration of 2000 parasites/ µL.

- One recombinant PvLDH protein (liquid). Each aliquot contains 10 µL at a concentration of 91 µg/mL.

This will allow 4 independent assays by one method plus 1 spare.

Please store all biosamples at -20 °C or below upon receipt.

Participants are requested to provide parasite-free whole blood to reconstitute the candidate IS preparation (IS-A and IS-B).

The parasite-free blood should be stored at -70 °C prior to use in order to lyse red blood cells. This avoids any clumping issues.

The required parasite-free blood volume for the whole ELISA study in each centre will depend upon the ELISA method being used to detect PvLDH.

Assay Methods

Study participants are requested to use a range of different ELISA methods to detect PvLDH antigen. It is requested that your laboratory use the following ELISA method:

1. Quantimal pLDH Malaria CELISA (Cellabs) following the manufacturer's instructions.

Design of study

Participants are requested to:

- Perform 4 independent assays on separate days.
- Use a freshly opened/reconstituted study sample for each assay. To prepare the lyophilised samples for assay, bring the samples to ambient temperature and resuspend in 250 µL parasite-free blood.
- For each independent assay and test serial dilutions of each study sample in order to establish the limit of detection of each sample.
- Include all study samples in each assay run

Study timelines

	MAR		APR		MAY		JUN		JUL		AUG		SEP		OCT		NOV	
ACTIVITIES																		
1. Protocol review by participants																		
2. Where applicable, participants apply for import permits and/or local ethical approval																		
3. Shipment of study materials to participating centres																		
4. Laboratory testing																		
5. Deadline for data submission to NIBSC																		

Information to be supplied and presentation of results

Please send the assay results in an excel sheet. A separate data table should be completed for each independent run. Please **PROVIDE ALL RAW DATA** to enable consistent handling and analysis of data from all participants. Also use the excel file to give brief information regarding:

- dilutions used (stock and working solutions)
- assay (type, when it was performed, results)

- Plasmodium-free blood used

Participants are requested to return all data and any information relating to the assays electronically in the requested format to:

Contact: Paul Bowyer
Tel: +44 (0)1707 64 1474
E-mail: paul.bowyer@nibsc.org

Contact: Charles Olomu
Tel: +44 (0)1707 64 1432
E-mail: charles.olomu@nibsc.org

Contact: Seda Yerlikaya
Tel: +41 (0) 22 749 19 21
E-mail: Seda.Yerlikaya@finddx.org
Deadline for data submission: November 30th 2019.

Appendix 6:

PvLDH ELISA (apDia and DRG diagnostics) Protocol

Collaborative Study Protocol

Protocol for the WHO collaborative study to assess the suitability of an International Standard for *P. vivax* antigens

Please follow this protocol below if your laboratory is measuring PvLDH levels using an in-house ELISA method or a commercial kit other than the Qualisa malaria kit.

Background

In order to prevent the unnecessary use of antimalarial drugs the World Health Organization (WHO) recommends that all suspected cases of malaria are confirmed by a parasitological diagnosis prior to treatment. Immunochromatographic rapid diagnostic tests (RDTs), that detect *Plasmodium*-specific antigens in patient blood samples, offer a convenient means of diagnosis. RDTs are based on the detection of two *Plasmodium* antigens – histidine-rich protein II (HRP2) and *Plasmodium* lactate dehydrogenase (pLDH). HRP2 is specific to *P. falciparum* whereas pLDH is expressed in all human-infecting *Plasmodium* species. Species-specific isoforms of pLDH have enabled the development of antibodies specific to *P. falciparum* pLDH (Pf-pLDH) or *P. vivax* pLDH (PvLDH). Hence, the main *Plasmodium vivax* antigen targeted by RDTs is PvLDH.

In 2017 the 1st WHO International Standard (IS) for *P. falciparum* antigens was established. However, there is currently no validated international reference standard for use as a quality control and calibrator material in PvLDH antigen detection assays. To this end, the Foundation for Innovative New Diagnostics (FIND) is collaborating with the National Institute for Biological Standards and Control (NIBSC) to establish the first WHO IS for *P. vivax* antigens. It is intended that the development of this IS will provide a standardised material that can subsequently be used in the quality control and standardisation of RDTs worldwide, for monitoring the development of more sensitive diagnostic tests, and for the calibration of other reference materials and controls that can then be used on a larger scale.

Aims

To assess the suitability of lyophilized preparations of *P. vivax* parasites to serve as the first WHO International Standard for *P. vivax* antigens.

Study samples

Participants will be sent a total of 6 biosamples (5 aliquots of each biosample) including:

- Two candidate International Standard preparations (IS-A and IS-B): lyophilised pools of *P. vivax* clinical isolates. Please resuspend in 250 µL whole blood prior to use.
- Three frozen *P. vivax* clinical isolates (liquid). Each aliquot contains 30 µL at a concentration of 2000 parasites/ µL.
- One recombinant PvLDH protein (liquid). Each aliquot contains 10 µL at a concentration of 91 µg/mL.

This will allow 4 independent assays by one method plus 1 spare.

Please store all biosamples at -20 °C or below upon receipt.

Participants are requested to provide parasite-free whole blood to reconstitute the candidate IS preparation (IS-A and IS-B).

The parasite-free blood should be stored at -70 °C prior to use in order to lyse red blood cells. This avoids any clumping issues.

The required parasite-free blood volume for the whole ELISA study in each centre will depend upon the ELISA method being used to detect PvLDH.

Assay Methods

Study participants are requested to use a range of different ELISA methods to detect PvLDH antigen. It is requested that your laboratory use the following ELISA method:

1. Malaria AG ELISA (apDIA) following the manufacturer's instructions.
2. Malaria AG ELISA (DRG Diagnostics) following the manufacturer's instructions.
3. Quantimal pLDH Malaria CELISA (Cellabs) following the manufacturer's instructions.

Design of study

Participants are requested to:

- Perform 4 independent assays on separate days.
- Use a freshly opened/reconstituted study sample for each assay. To prepare the lyophilised samples for assay, bring the samples to ambient temperature and resuspend in 250 µL parasite-free blood.
- For each independent assay and test serial dilutions of each study sample in order to establish the limit of detection of each sample.
- Include all study samples in each assay run

Study timelines

	MAR		APR		MAY		JUN		JUL		AUG		SEP		OCT		NOV	
ACTIVITIES																		
1. Protocol review by participants																		
2. Where applicable, participants apply for import permits and/or local ethical approval																		
3. Shipment of study materials to participating centres																		
4. Laboratory testing																		
5. Deadline for data submission to NIBSC																		

Information to be supplied and presentation of results

Please send the assay results in an excel sheet. A separate data table should be completed for each independent run. Please **PROVIDE ALL RAW DATA** to enable consistent handling and analysis of data from all participants. Also use the excel file to give brief information regarding:

- dilutions used (stock and working solutions)
- assay (type, when it was performed, results)
- Plasmodium-free blood used

Participants are requested to return all data and any information relating to the assays electronically in the requested format to:

Contact: Paul Bowyer
Tel: +44 (0)1707 64 1474
E-mail: paul.bowyer@nibsc.org

Contact: Charles Olomu
Tel: +44 (0)1707 64 1432
E-mail: charles.olomu@nibsc.org

Contact: Seda Yerlikaya
Tel: +41 (0) 22 749 19 21
E-mail: Seda.Yerlikaya@finddx.org
Deadline for data submission: November 30th 2019.

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Appendix 7:

Rapid Diagnostic Tests Protocol

COLLABORATIVE STUDY PROTOCOL

First WHO International Standard for *Plasmodium vivax* antigens

Rapid Diagnostic Tests

Version: 1.2
Date: 31st July 2019

E-mail: seda.yerlikaya@finddx.org

Project Manager at NIBSC: Lynne Harris
E-mail: lynne.harris@nibsc.org

FIND
Because diagnosis matters

NIBSC

CONFIDENTIALITY STATEMENT

VERSION HISTORY

Authors: Lynne Harris, NIBSC
Paul Bowyer, NIBSC
Seda Yerlikaya, FIND

Initial Release Version: 1.0
Initial Release Date: 20th March 2019

Current Version: 1.2
Current Version Date: 31st July 2019

Protocol revision summary

Date of Revision	Initial version	Summary of revision	Updated version
	1.0		
4th June 2019	1.0	Protocol revised following protocol review by all study participants	1.1
31st July 2019	1.1	Initial biosample dilutions added. Volume of biosamples added. Volume of biosample dilutions updated.	1.2

TABLE OF CONTENTS

ABBREVIATIONS

FIND	Foundation for Innovative New Diagnostics
HRP2	Histidine-Rich Protein II
IS	International Standard
NIBSC	National Institute for Biological Standards and Control
Pf	*Plasmodium falciparum*
pLDH	*Plasmodium* lactate dehydrogenase
Pv	*Plasmodium vivax*
RDT	Rapid Diagnostic Test
SOP	Standard Operating Procedure
WHO	World Health Organization

First WHO International Standard for *Plasmodium vivax* antigens

Study Protocol

8. BACKGROUND

In order to prevent the unnecessary use of antimalarial drugs the World Health Organization (WHO) recommends that all suspected cases of malaria are confirmed by a parasitological diagnosis prior to treatment. Immunochromatographic rapid diagnostic tests (RDTs), that detect *Plasmodium*-specific antigens in patient blood samples, offer a convenient means of diagnosis. RDTs are based on the detection of two *Plasmodium* antigens – histidine-rich protein II (HRP2) and *Plasmodium* lactate dehydrogenase (pLDH). HRP2 is specific to *P. falciparum* whereas pLDH is expressed in all human-infecting *Plasmodium* species. Species-specific isoforms of pLDH have enabled the development of antibodies specific to *P. falciparum* pLDH (Pf-pLDH) or *P. vivax* pLDH (PvLDH). Hence, the main *Plasmodium vivax* antigen targeted by RDTs is PvLDH.

In 2017 the 1st WHO International Standard (IS) for *P. falciparum* antigens was established. However, there is currently no validated international reference standard for use as a quality control and calibrator material in PvLDH antigen detection assays. To this end, the Foundation for Innovative New Diagnostics (FIND) is collaborating with the National Institute for Biological Standards and Control (NIBSC) to establish the first WHO IS for *P. vivax* antigens. It is intended that the development of this IS will provide a standardised material that can subsequently be used in the quality control and standardisation of RDTs worldwide, for monitoring the development of more sensitive diagnostic tests, and for the calibration of other reference materials and controls that can then be used on a larger scale.

9. AIM OF THE COLLABORATIVE STUDY

To assess the suitability of lyophilized preparations of *P. vivax* parasites to serve as the first WHO International Standard for *P. vivax* antigens.

10. APPROACH

NIBSC and FIND will distribute materials to participant laboratories where the experiments are to be performed. The raw data should be returned to NIBSC in the form described in the template associated with each protocol. The combined data from all participants will be analysed by NIBSC and FIND and used in the construction of a report describing all aspects of the establishment of the International Standard. This report will be submitted to the WHO Expert Committee on Biological Standardization (ECBS). A subsequent report for submission to a scientific journal may also be written.

Participants are requested to detect PvLDH in the samples provided. **The RDT study protocol detailed herein should be performed a total of four times with each run carried out on a different day and starting with fresh unopened sample vials**. The first run will be used to determine preliminary end-point titres of all samples on each RDT. The three subsequent runs will test a limited number of dilutions on either side of the preliminary end-point titre in order to obtain accurate end-point titres.

11. SAFETY CONSIDERATIONS

Prior to initiating the study, please read this document carefully. Note all the statements regarding safety and that these materials are not for human use. Please note that blood samples pose a potential risk of infection. Use universal precautions to minimize biohazards.

It is the responsibility of the laboratory supervisor (the person who has immediate responsibility for the laboratory) to ensure the development and adoption of a biosafety management plan and a safety operations manual. The laboratory supervisor should ensure that regular training in laboratory safety is provided. Personnel should be required to read the local standard operation procedures manual on safety and a copy of this manual should be available in the laboratory.

12. STUDY TIMELINES

	MAR		APR		MAY		JUN		JUL		AUG		SEP		OCT		NOV	
ACTIVITIES																		
1. Protocol review by participants																		
2. Where applicable, participants apply for import permits and/or local ethical approval																		
3. Shipment of study materials to participating centres																		
4. Laboratory testing																		
5. Deadline for data submission to NIBSC																		

13. SOP. RDT testing

13.1. Purpose

This SOP describes the procedure (including materials) for performing RDTs, using the candidate International Standard preparations, clinical isolates and recombinant proteins.

13.2. Scope

This SOP has been developed in the context of the collaborative study to establish the first WHO IS for *Plasmodium vivax* antigens. This procedure is an adaptation of part of the methodology described in the "Methods Manual for laboratory quality control testing of malaria rapid diagnostic tests" (SOP 2.02, SOP 2.04, SOP 2.05, SOP 3.12, SOP 3.10 and 5.05).

13.3. Materials

13.3.1. Biosamples

Participants will be sent a total of 7 biosamples (5 aliquots of each biosample) including:

- Two candidate International Standard preparations (IS-A and IS-B): lyophilised pools of *P. vivax* clinical isolates.
- Three *P. vivax* clinical isolates CI-A, CI-B and CI-C (liquid). Each aliquot contains 15 µL.
- One recombinant PvLDH protein (liquid). Each aliquot contains 10 µL.
- The WHO International Standard for *P. falciparum* antigens 16/376 (lyophilised).

Upon receipt please store all biosamples at -20 °C or below.

Participants are requested to provide parasite-free whole blood (of any blood type) which should be used for the following:

- To reconstitute the lyophilised IS materials
- As a negative control
- For sample dilutions

The required volume of parasite-free blood for the whole RDT study in each centre is 35 mL. The parasite-free blood should be stored frozen at -70 °C prior to use in order to lyse red blood cells. This avoids any clumping issues. Sub-aliquot the parasite-free blood prior to starting the study such that one vial is thawed per experimental run.

13.3.2. Equipment

- Microtubes
- Micropipettes (1-20 µL, 20 – 200 µL and 100-1000 µL)
- Pipette tips (1-20 µL, 20 – 200 µL and 100-1000 µL capacity)
- Rack for samples and dilutions
- RDTs for testing (see Appendix 1)
- Timer
- Vortex mixer
- -70ºC freezer
- +4ºC refrigerator
- Marker pen
- Waste bin for biological samples
- Gloves

Upon receipt please store RDTs according to the manufacturer's instructions. Label all RDT boxes with the date of receipt. Inspect the RDT boxes for damage, traces of humidity, etc., and record any relevant information in the excel file provided ("CS644 Result form_RDT").

13.4. Procedure

13.4.1. Reconstitution of candidate IS/IS materials and preparation of sample dilutions for testing

A fresh tube/ampoule of each biosample should be used for each of the four independent experimental runs. In the case of the candidate IS and IS samples, this will require reconstitution of a fresh ampoule prior to the start of each experimental run. Dilutions of all biosamples should be tested at the same time in all RDTs in order to simultaneously determine and compare the pLDH content. Please, note that one independent experimental run constitutes testing all the biosamples (and resulting dilutions) on all the RDT products listed in Appendix I.

Remove one ampoule of each of the two candidate IS samples, one aliquot of each of the three clinical isolates, one aliquot of the recombinant PvLDH protein, one ampoule of the IS for *Pf* antigens and parasite-free blood from the freezer. Thaw out on the bench at room temperature. Samples should be used immediately once completely thawed.

- Reconstitute **each of the candidate IS samples in 250 µL** of parasite-free blood. Reconstitute **the IS for *P. falciparum* antigens in 500 µL** of parasite-free blood. Allow the material in the vials to reconstitute for 15-20 minutes at room temperature with gentle agitation (e.g. a rocker platform or rotation by hand). Avoid vigorous shaking which can cause foaming and protein denaturation.
- Biosample dilutions should be prepared using parasite-free human whole blood as a diluent. The blood should have gone through a minimum of one freeze-thaw cycle prior to use as a diluent. The blood will also be used as a negative control. **Keep all dilutions on ice** until the end of the experiment.
- Each clinical isolate aliquot contains 15 µL. Perform a 1:10 dilution of each clinical isolate by adding 135 µL parasite-free blood **directly** to each aliquot. 2-fold serial dilutions should be prepared from this initial 1:10 dilution (Table 1).
- **For the first experimental run** prepare 2- fold serial dilutions **until three (3) consecutive no-band readings are observed in each RDT and for each band** (see example in Table 1). Keep all samples and dilution vials on ice, in case further dilutions need to be prepared and tested in order to obtain three (3), consecutive no-band readings. Samples should be pipetted up and down at least three times and mixed on a vortex before transferring to the next tube. A fresh pipette tip should be used for each transfer.

Table 1. Dilution of the clinical isolates

Dilution stock	1 (1:10)	1/2	1/4	1/8	1/16	1/32	1/64	**1/128**	1/256	1/512	1/1024
Working stock	Stock aliquot	1	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512
Volume stock (µL)	15	75	75	75	75	75	75	75	75	75	75
Volume diluent (µL)	135	75	75	75	75	75	75	75	75	75	75
Total volume (µL)	150	150	150	150	150	150	150	150	150	150	150

Scoring	4	4	3	3	2	2	1	1	**0**	**0**	**0**

NB: If the last three dilutions also give a positive score, prepare further 2-fold dilutions from the 1/1024 (the last dilution) until three (3), consecutive no-band readings are observed. If the RDT has more than one test line, this applies to all test lines.

- **For the first experimental run**, prepare 2-fold serial dilutions of the reconstituted candidate Pv IS materials, and IS for Pf antigens, **until three (3) consecutive no-band readings are observed in each RDT and for each band** (see example in Table 2). Keep all samples and dilution vials on ice, in case further dilutions need to be prepared and tested in order to obtain three (3), consecutive non-band readings.
- Samples should be pipetted up and down at least three times and mixed on a vortex before transferring to the next tube. A fresh pipette tip should be used for each transfer.

Table 2. Dilution of the candidate IS (Pv) and IS (Pf) materials

Dilution stock	1	1/2	1/4	1/8	1/16	1/32	1/64	1/128	**1/256**	1/512	1/1024	1/2048
Working stock	Stock vial	1	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1024
Volume stock (µL)	150	75	75	75	75	75	75	75	75	75	75	75
Volume diluent (µL)	0	75	75	75	75	75	75	75	75	75	75	75
Total volume (µL)	150	150	150	150	150	150	150	150	150	150	150	150
Scoring	4	4	4	3	3	3	2	1	1	**0**	**0**	**0**

NB: If the last three dilutions also give a positive score, prepare further 2-fold dilutions from the 1/1024 (the last dilution) until 3, consecutive no-band readings are observed. *If the RDT has more than one test line, this applies to all test lines.*

- Aliquots of recombinant PvLDH contain 10 µL. Perform an initial 1:50 dilution by adding 3 µL recombinant protein to 147 µL parasite-free blood. 2-fold serial dilutions should be prepared form this initial 1:50 dilution (Table 3).
- **For the first experimental run**, prepare 2- fold serial dilutions of the recombinant PvLDH protein **until three (3) consecutive no-band readings are observed in each RDT and for each band** (see example in Table 3). Keep all samples and dilution vials on ice, in case further dilutions need to be prepared and tested in order to obtain three (3), consecutive non-band readings.
- Samples should be pipetted up and down at least three times and mixed on a vortex before transferring to the next tube. A fresh pipette tip should be used for each transfer.

Table 3. Dilution of the recombinant PvLDH protein

Dilution stock	1 (1:50)	1/2	1/4	1/8	1/16	1/32	1/64	1/128	**1/256**	1/512	1/1024	1/2048
Working stock	Stock aliquot	1	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1024
Volume stock (µL)	3	75	75	75	75	75	75	75	75	75	75	75
Volume diluent (µL)	147	75	75	75	75	75	75	75	75	75	75	75
Total volume (µL)	150	150	150	150	150	150	150	150	150	150	150	150
Scoring	4	4	4	3	3	3	2	1	1	**0**	**0**	**0**

NB: If the last three dilutions also give a positive score, prepare further 2-fold dilutions from the 1/1024 (the last dilution) until 3, consecutive no-band readings are observed. *If the RDT has more than one test line, this applies to all test lines.*

- **For the next three test runs, for all biosamples, only three dilutions above and below the limit of detection established in the first run (dilutions 1/128, 1/256 and 1/256 in the examples provided in tables 1, 2 and 3 respectively) should be tested.**
- Put aside 75 µL whole blood diluent. This will be used for negative control tests. For each experimental run, and for each RDT product, please test whole blood only, in duplicate as a negative control.
- Sample aliquots and dilutions should be used **only once** (do not re-freeze unused and/or left-over samples). Discard left-over samples according to local laboratory safety procedures.

13.4.2. RDT testing

- Before performing the RDT testing, study the RDT manufacturer's instruction sheet.
- Approximately 30 minutes before testing, bring RDTs to room temperature (~25ºC) BEFORE OPENING the package. This applies only to RDTs stored under conditions other than room temperature (e.g. fridge).
- Remove the RDT packaging.
- Check integrity of RDT packaging when opening. If signs of moisture are present, DO NOT use the RDT.
- Check desiccant for any colour changes (e.g. blue to white). If present, discard RDT and use another kit for testing.
- Take out the required number of RDTs and label them. Note that in each run dilutions should be tested in DUPLICATE.
- Label the RDT with sample ID (e.g. A), dilution (e.g. ½), and date of test (DD/MM/YY), using a marker pen.
- Mix the sample dilution vigorously (flick or use vortex) prior to opening and pipetting the blood.

- Test the RDTs with the sample as per the manufacturer's instructions, BUT use a micropipette to transfer the specified blood volume to the RDT.
- Change the pipette tip for testing of each sample and each dilution.
- Use a timer to record all steps exactly as per the manufacturer's instructions.
- Read RDT results within the manufacturer recommended time. Results should be read by TWO people independently. If an invalid test is returned (e.g. no control line, band does not clear) then repeat on a fresh RDT.
- Refer to the standard colour chart provided by WHO-FIND for rating the band intensity from 0 (negative) to +4.
- Record the results as well as any relevant information in the results excel sheet as explained in section 7.5 below.
- If needed, perform further dilution of the biosamples **until 3 consecutive no-band readings are observed in each RDT** (see section 8.4.1), and continue with the RDT testing.
- Discard left-over samples according to standard laboratory safety procedures.

Repeat the procedure in order to perform a total of FOUR independent tests on separate days.

13.5. Information to be supplied and presentation of results

An excel template is provided ("Result form_RDT") so that all relevant information can be recorded as indicated. A separate data sheet should be completed for each independent run. Please, **PROVIDE ALL RAW DATA** to enable consistent handling and analysis of data from all participants. Also use the excel file to give brief information regarding:

- dilutions used (stock and working solutions)
- assay (type, when it was performed, results)
- Plasmodium-free blood used

Participants should follow the protocol. ALL protocol deviations, however minor, must be recorded in the Additional comments/relevant information section of the excel template for each run.

Participants are requested to return all data and any information relating to the assays electronically in the requested format to:

Contact: Paul Bowyer
Tel: +44 (0)1707 64 1474
E-mail: paul.bowyer@nibsc.org

Contact: Charles Olomu
Tel: +44 (0)1707 64 1432
E-mail: charles.olomu@nibsc.org

Contact: Seda Yerlikaya
Tel: +41 (0) 22 749 19 21
E-mail: Seda.Yerlikaya@finddx.org
Deadline for data submission: November 30th 2019.