PURPOSE: This SOP applies to the use of WHO International Standard for Plasmodium vivax lactate dehydrogenase (PvLDH), 19-116 (NIBSC, Hertfordshire, UK), in nucleic acid amplification technology and antigen detection assays. It combines preparation of the standards as dilution series for molecular testing and rapid diagnostic testing. The purpose for preparation of molecular testing standards is to standardize results across different PCR methods. Creating the dilution series of the standards followed by amplification will determine the limit of detection of the assay. The purpose of preparation of standards for antigen testing is to test WHO-prequalified or investigational malaria rapid tests for detection of *Plasmodium vivax* antigen *Plasmodium vivax* lactate dehydrogenase *(PvLDH)* for quality checking of new lots and at specific study timepoints, training, and proficiency testing. Creating the dilution series of the standards followed by aliquoting and freezing will preserve the standards at relevant concentrations to be used as needed.

RESPONSIBILITIES:

1. The Project lead has the authority to establish this procedure.
2. The Scientific lead is responsible for the control of SOP documentation.
3. Laboratory staff are responsible for the implementation of this procedure and for ensuring that all appropriate personnel are trained.

MATERIALS REQUIRED:

- Cryovials suitable to hold 50-500 µL volume. (minimum of 72)
- Titer tubes, microcentrifuge tubes, or cryotubes capable of holding volumes up to 1 mL, minimum of 15, for preparing primary dilutions.
- 15 mL vial for freezing of donor whole blood.
- Refrigerator (4°C) for storing dilutions, or wet ice if refrigerator not available.
- P-200 and P-1000 calibrated pipettors and pipette tips. Low-retention tips should be used, if available.
- Labels and labeling printer or markers.
- Whole blood diluent*: Plasmodium*-negative healthy universal (O+) donor whole blood, venous draw of K_2EDTA (see preliminary procedures), minimum volume of 9.5 mL for

preparation of dilutions and additional sufficient volume to screen by microscopy and extract for PCR confirmation of negativity.

- Materials to conduct microscopy screening and PCR of donor whole blood diluent.

1. Specimen Handling

1.1 Consider all human specimens as capable of transmitting infectious agents. Use Blood borne pathogen precautions for all samples. Personal Protective Equipment (PPE) must be used for handling specimens and reagents. PPE includes:

1.1.1 Laboratory coat or gown

1.1.2 Eye protection

1.1.3 Latex or nitrile gloves, non–powdered preferred

1.2 Dispose of all specimens and used materials in accordance with local applicable guidelines and/or regulations.

2. Specimen Rejection

2.1 Quality of specimens must be evaluated at the point of delivery.

2.2 Unlabeled or mislabeled specimens must be rejected.

2.3 Clotted specimens must be rejected.

***WHO standards*:** *Plasmodium vivax 19/116* standard is available at NIBSC for procurement. Shipment requires import permit.

WHO standard *NIBSC* material	Description	Intended Use	Unit	Link
19/116	Lyophilized red blood cell (RBC) lysates from *P. vivax* infected donors from Peru.	Standardization, and evaluation of performance and sensitivity of *P. vivax* antigen detection tests that detect *P. vivax* lactate dehydrogenase (PvLHDH).	1000 IU of PvLDH per ampoule	NIBSC 19-116 https://www.nibsc.org/documents/ifu/19-116.pdf

3. Preliminary procedures to be completed before preparing standards set:

a. Whole blood diluent is prepared from a venous draw of K2EDTA whole blood from a healthy, universal donor (O+), negative for any *Plasmodium* infection. Ideally, the donor can be confirmed negative by microscopy upon day of draw when fresh, and by PCR to confirm negativity, prior to use to prepare standards. Following any confirmation by microscopy, the full volume (minimum 9.5 mL) blood should be frozen, reserving approximately 150 uL for PCR to confirm Plasmodium negative.

PROCEDURES:

Preparation of the dilution series

Two dilution series will be created: The primary dilution series will be to support preparation of the standards for molecular testing. The secondary dilution series will be used to prepare frozen aliquots for use with RDT quality assessment. The dilution series should be made on the same day, using the same donor blood.

1. Primary Dilution Series for Molecular Testing.

Concentration of the *PvLDH19/116* standard: 1000 IU of PvLDH per vial.

Reconstitute the lyophilized product within the vial by adding 250 ul of whole blood diluent. The same whole blood diluent will be used to prepare both the primary and the secondary dilution series. Concentration of reconstituted vial: 1000 IU/250 µL or 4.00 IU/ µL or 4000 IU/mL.

- Gently tap down the vial before opening, to ensure all lyophilized material is near the bottom of the vial.
- After adding the 250 µL of whole blood diluent to the lyophilized material, wait for 5 minutes before mixing and pipetting. Mix the reconstituted stock thoroughly through pipetting and stirring with pipette tip, but mix carefully, avoiding bubbles.
- Pipette slowly since whole blood may be viscous. Try to avoid bubbles when pipetting.
- Mix each dilution gently, but thoroughly. Each dilution step should be mixed at least 20 times by gently pipetting up and down throughout the full volume, before proceeding to next dilution.

- Reconstituted stock and dilutions should be placed in the refrigerator (4°C) or on wet ice until aliquoted and frozen. Dilutions should be aliquoted and frozen within approximately 2 hours of reconstituting the stock.

Table 1: Primary Dilution Series

At least 100 µL of concentrations D0, D1-D6 (highlighted) should also be reserved and kept cold for preparing the secondary dilution series for antigen detection, before extraction of D1- D14.

Label ID	Volume stock or previous dilution, µL	Diluent blood, µL	Concentration PvLDH IU/µl	Total volume (µL)	Remaining volume, µL
Reconstitute - PvLDH19/116	entire contents of lyophilized vial	250	4.00E+00	250	150
Diluted PvLDH19/116_D0	100 µL of reconstituted material	400	8.00E-01	500	150
2-fold dilution series					
PvLDH19/116_D1	350 µL PvLDH19/116_D0	350	4.00E-01	700	350
PvLDH19/116_D2	350 µL PvLDH19/116_D1	350	2.00E-01	700	350
PvLDH19/116_D3	350 µL PvLDH19/116_D2	350	1.00E-01	700	350
PvLDH19/116_D4	350 µL PvLDH19/116_D3	350	5.00E-02	700	350
PvLDH19/116_D5	350 µL PvLDH19/116_D4	350	2.50E-02	700	350
PvLDH19/116_D6	350 µL PvLDH19/116_D5	350	1.25E-02	700	350
PvLDH19/116_D7	350 µL PvLDH19/116_D6	350	6.25E-03	700	350
PvLDH19/116_D8	350 µL PvLDH19/116_D7	350	3.13E-03	700	350
PvLDH19/116_D9	350 µL PvLDH19/116_D8	350	1.56E-03	700	350
PvLDH19/116_D10	350 µL PvLDH19/116_D9	350	7.81E-04	700	350
PvLDH19/116_D11	350 µL PvLDH19/116_D10	350	3.91E-04	700	350
PvLDH19/116_D12	350 µL PvLDH19/116_D11	350	1.95E-04	700	350
PvLDH19/116_D13	350 µL PvLDH19/116_D12	350	9.77E-05	700	350
PvLDH19/116_D14	350 µL PvLDH19/116_D13	350	4.88E-05	700	700

2. Secondary Dilution Series for antigen detection using RDTs

Immediately after preparing the primary dilution series, prepare the antigen detection panel using the following dilutions to prepare a larger volume secondary dilution series. Each selected primary dilution series will be further diluted by adding 100 µL of the primary dilution series to 300 µL of whole blood diluent. Aliquot the resulting dilutions PvLDH19/116 - RDT A-G into 7 to 8 labeled cryovials, each with 50 µL. Freeze immediately after aliquoting at -20°C or lower.

- Label tubes with Label ID and date prepared.
- Mix each dilution thoroughly with at least 20 times pipetting gently through the full volume before aliquoting.
- Mark any last aliquot tubes which are over or under 50 µL volume.

Table 2: Secondary Dilution Series#2

Label ID	100 uL of primary dilution series	Diluent blood, µL	Concentration PvLDH IU/mL	Total volume (µL)	Aliquot size (µL)	number of aliquots
PvLDH19/116 - RDT A	PvLDH19/116_D0	300	200	400	50	7 or 8
PvLDH19/116_RDT B	PvLDH19/116_D1	300	100	400	50	7 or 8
PvLDH19/116_RDT C	PvLDH19/116_D2	300	50	400	50	7 or 8
PvLDH19/116_RDT D	PvLDH19/116_D3	300	25	400	50	7 or 8
PvLDH19/116_RDT E	PvLDH19/116_D4	300	12.50	400	50	7 or 8
PvLDH19/116_RDT F	PvLDH19/116_D5	300	6.25	400	50	7 or 8
PvLDH19/116_RDT G	n/a	400	0	400	50	7 or 8

3. For PCR testing:

DNA Extraction: Perform DNA extraction of the entire dilution series as per site protocol. Extraction should be conducted the same day as the primary dilution series is made.

PCR Amplification: Follow study site malaria PCR protocol. Each DNA sample of the series will be amplified in 5 replicates. Cutoff for CT values will be as per site protocol.
Results: All results to be recorded in tables 2 and 3.

Analysis: Greater than 50% positive rate or ≥3/5 replicates will be considered positive. A template of result form is below as Table 3.

Table 2: Site-specific information

SITE:	
USER:	
DATE:	
Extraction method used	
PCR method used	
Volume of NIBSC dilution standard used for each extraction	
Final elution volume of DNA	
Volume of extracted nucleic acid used per PCR reaction	
Real-time or gel electrophoresis PCR output?	
If using real-time PCR, what is the upper CT value for positive?	

Table 3: PCR results of Dilution series#1

If running real-time PCR enter Ct value, and negative for samples with Ct values greater than cut-off value. If running gel electrophoresis enter positive or negative.

Label ID	Pv IU/ul	PCR results of replicates in CT values or positive/negative	No. of positives/ No. of replicates	Comments
PvLDH19/116_D1	4.00E-01	1.		
		2.		
		3.		
		4.		
		5.		
PvLDH19/116_D2	2.00E-01	1.		
		2.		
		3.		
		4.		
		5.		
PvLDH19/116_D3	1.00E-01	1.		
		2.		
		3.		
		4.		
		5.		
PvLDH19/116_D4	5.00E-02	1.		
		2.		
		3.		
		4.		
		5.		
PvLDH19/116_D5	2.50E-02	1.		
		2.		
		3.		
		4.		
		5.		
PvLDH19/116_D6	1.25E-02	1.		
		2.		
		3.		

		4.		
		5.		
PvLDH19/116_D7	6.25E-03	1.		
		2.		
		3.		
		4.		
		5.		
PvLDH19/116_D8	3.13E-03	1.		
		2.		
		3.		
		4.		
		5.		
PvLDH19/116_D9	1.56E-03	1.		
		2.		
		3.		
		4.		
		5.		
PvLDH19/116_D810	7.81E-04	1.		
		2.		
		3.		
		4.		
		5.		
PvLDH19/116_D11	3.91E-04	1.		
		2.		
		3.		
		4.		
		5.		
PvLDH19/116_D12	1.95E-04	1.		
		2.		
		3.		
		4.		
		5.		
PvLDH19/116_D13	9.77E-05	1.		
		2.		
		3.		

		4.		
		5.		
PvLDH19/116_D14	4.88E-05	1.		
		2.		
		3.		
		4.		
		5.		

To demonstrate the limit of detection: If PCR results of PvLDH19/116_D14 is still positive then extend 2-fold dilution series and perform PCR to get the limit of detection. Record data appropriately in table 1 for additional dilutions and use table 3 for PCR results.

For RDT testing:

Malaria RDTs should be tested using the controls, according to instructions for use. Aliquots of the secondary dilutions are single-use only and should be used within 2 hours of thawing and be kept on ice until 10 minutes before use when they can be brought to room temperature and mixed before using. For new lot testing or quality checks at a specific study timepoint, it is recommended that each RDT is tested in duplicate with each of the control dilutions, including the negative, for a total of 14 RDTs per testing. Each aliquot can be used with up to 4 different RDTs, tested in duplicate. If disagreement in result, error in run, or other problem is noted, then an additional replicate should be run as needed and volume may be limited for all 4 RDTs. Expected positivity with each control dilution may depend on which RDT is used, although some dilutions must have specific results for the lot to pass.

Data Collection:

Site information should be completed with each RDT testing to record location, user, date, purpose of testing, and test information. Results for each RDT should be recorded, as shown in the table below. If replicate 1 and 2 have disagreement in test line results or if there is an invalid or error/unreadable, a repeat test should be run with the same aliquot of control. If repeat due to invalid, error, unreadable, and repeat result should be used to determine passing. For repeats due to disagreement in result, should be noted as partial positivity. These will not meet criteria for pass unless control is listed with criteria of "Positivity depends on RDT".

Result choices:

Overall:

☐ Valid: Control line present and result area is readable
☐ Invalid (no control line): Present/absent test line, NO control line visible.
☐ Unreadable/error: unreadable, severe background with control line, user error or other problem with test

Test line for PvLDH

☐ Positive for PvLDH: test line visible and control line visible
☐ Negative for PvLDH: NO test line visible and control line visible

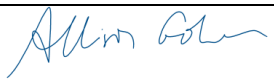
Other test lines (for non-Pv targets, if present). Note the test line target in results: HRP2, PfLDH, or N/A if not present.

☐ Positive: test line visible and control line visible
☐ Negative: NO test line visible and control line visible

SITE:	
USER:	
DATE:	
Purpose of testing	☐ New lot ☐ Proficiency ☐ Training ☐ Time point quality check
Test Name	
Reference and Lot Number	
Test Line(s)	

Control Dilution	**Results for Replicate 1**	**Results for Replicate 2**	**Repeat Test Result note reason**	**Criteria for PvLDH test line**	**Criteria for any non-Pv test lines**	**Pass/ Fail**
EXAMPLE	**Overall:** *valid* **PvLDH:** *positive* **Other:** *negative*	**Overall:** *Error-forgot buffer, invalid* **PvLDH:** *n/a - invalid* **Other:** *n/a invalid*	**Reason:** *error* **Overall:** *valid* **PvLDH:** *positive* **Other:** *negative*	Positive	Negative	*Pass*
PvLDH19/116 - RDT A	**Overall:** **PvLDH:** **Other:**	**Overall:** **PvLDH:** **Other:**	**Reason** **Overall:** **PvLDH:** **Other:**	Positive	Negative	
PvLDH19/116_RDT B	**Overall:** **PvLDH:** **Other:**	**Overall:** **PvLDH:** **Other:**	**Reason** **Overall:** **PvLDH:** **Other:**	Positive	Negative	
PvLDH19/116_RDT C	**Overall:** **PvLDH:** **Other:**	**Overall:** **PvLDH:** **Other:**	**Reason** **Overall:** **PvLDH:** **Other:**	Positive	Negative	
PvLDH19/116_RDT D	**Overall:** **PvLDH:** **Other:**	**Overall:** **PvLDH:** **Other:**	**Reason** **Overall:** **PvLDH:** **Other:**	Positivity depends on RDT	Negative	
PvLDH19/116_RDT E	**Overall:** **PvLDH:** **Other:**	**Overall:** **PvLDH:** **Other:**	**Reason** **Overall:** **PvLDH:** **Other:**	Positivity depends on RDT	Negative	
PvLDH19/116_RDT F	**Overall:** **PvLDH:** **Other:**	**Overall:** **PvLDH:** **Other:**	**Reason** **Overall:** **PvLDH:** **Other:**	Positivity depends on RDT	Negative	
PvLDH19/116_RDT G	**Overall:** **PvLDH:** **Other:**	**Overall:** **PvLDH:** **Other:**	**Reason** **Overall:** **PvLDH:** **Other:**	Negative	Negative	

4. Approval

Author	Signature	Date
Sampa Pal		09/09/2021
Allison Golden		4 May 2022