PURPOSE: This SOP applies to the use of WHO International Standard for Plasmodium falciparum histidine-rich protein 2 (HRP2) and *Plasmodium falciparum* lactate dehydrogenase (PfLDH), 16/376 (NIBSC, Hertfordshire, UK), for antigen detection assays. The purpose of preparation of standards for antigen testing is to test WHO-prequalified or investigational malaria rapid tests for detection of *Plasmodium falciparum* antigens HRP2 and PfLDH for quality checking of new lots and specific timepoints during the study, training, and proficiency testing.

SCOPE: This SOP applies to the use of WHO International Standard for *Plasmodium falciparum* antigens HRP2 and PfLDH (NIBSC, Hertfordshire, UK) for checking malaria rapid diagnostic test quality. 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 135)
- 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 falciparum 16/376* standard is available at NIBSC for procurement. Shipment requires import permit.

WHO standard *NIBSC* material	Description	Intended Use	Unit	Link
16/376	freeze-dried preparation of culture-derived P. falciparum parasites of the W2 strain	Standardization, and evaluation of performance and sensitivity of *P. falciparum* antigen detection tests.	1000 IU of HRP2 and 1000 IU of PfLDH per ampoule	NIBSC 16/376 https://www.nibsc.org/documents/ifu/16-376.pdf

1000 International Units of HRP2 per ampoule and 1000 International Units of pLDH per ampoule

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 7.0 mL) blood should be frozen, reserving approximately 150 µL for PCR to confirm it is *Plasmodium* negative.

PROCEDURES:

Preparation of the dilution series Concentration of the *Pf 16/376* standard: 1000 IU of HRP2 and 1000 IU of PfLDH per vial. Reconstitute the lyophilized product within the vial by adding 500 µL of whole blood diluent. The whole blood diluent will be used to prepare both the primary and

the secondary dilution series. Concentration of antigens in reconstituted vial: 1000 IU/500 µL or 2000 IU/mL.

- Gently tap down the vial before opening, to ensure all lyophilized material is near the bottom of the vial.
- After adding the 500 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: Dilution Series

Label ID	*Volume Stock or Previous concentration, µL*	*Diluent blood, µL*	*Total Volume*	*Remaining Volume*	*Concentration IU/mL*	*Aliquots, 50 µL*
Stock	Vial	500	500	340	2000	6-7
Pf 16/376 - RDT A	160	1440	1600	800	*200*	15-16
Pf 16/376 - RDT B	800	800	1600	800	100	15-16
Pf 16/376 - RDT C	800	800	1600	800	*50*	15-16
Pf 16/376 - RDT D	800	1200	2000	800	*20*	15-16
Pf 16/376 - RDT E	1200	400	1600	800	*15*	15-16
Pf 16/376 - RDT F	800	400	1200	800	*10*	15-16
Pf 16/376 - RDT G	400	400	800	800	*5*	15-16
Pf 16/376 – RDT H	0	800	800	800	*0*	15-16

Aliquot the remaining stock into 6 or 7 aliquots of 50 µL and aliquot dilutions Pf 16/376-RDT A-G into 15 or 16 labeled cryovials, each with 50 µL. Freeze immediately after aliquoting at -20°C or lower.

- Label tubes with Label ID and date prepared.
- Mark any last aliquot tubes which are over or under 50 µL volume.

For RDT testing:

Malaria RDTs should be tested using the controls, according to instructions for use. Aliquots are single-use only and should be used within 2 hours of thawing and be kept cold 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, for a total of 14 RDTs of each type 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 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 HRP2

- ☐ Positive for HRP2: test line visible and control line visible
- ☐ Negative for HRP2: NO test line visible and control line visible

Test line for PfLDH

- ☐ Positive for PfLDH: test line visible and control line visible
- ☐ Negative PfLDH: NO test line visible and control line visible

If a test contains a combination test line, such as detection of HRP2 and PfLDH together, then this should be noted and criteria for HRP2 test line should be used. Both test line criteria should pass for RDT overall to pass. If tests do not meet passing criteria, consult supervisor for further troubleshooting.

NIBSC *P. falciparum* 16/376 control dilutions for Pf rapid antigen testing

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 HRP2 test line**	**Criteria for PfLDH test line**	**Pass/Fail**
EXAMPLE	**Overall:** *valid* **HRP2:** *positive* **PfLDH:** *negative*	**Overall:** *Error-forgot buffer, invalid* **HRP2:** *n/a - invalid* **PfLDH:** *n/a invalid*	**Reason:** *error* **Overall:** *valid* **HRP2:** *positive* **PfLDH:** *negative*	Positive	Positivity depends on RDT	*Pass*
Pf 16/376 - RDT A	**Overall:** **HRP2:** **PfLDH:**	**Overall:** **HRP2:** **PfLDH:**	**Reason:** **Overall:** **HRP2:** **PfLDH:**	Positive	Positive	
Pf 16/376 - RDT B	**Overall:** **HRP2:** **PfLDH:**	**Overall:** **HRP2:** **PfLDH:**	**Reason:** **Overall:** **HRP2:** **PfLDH:**	Positive	Positivity depends on RDT	
Pf 16/376 - RDT C	**Overall:** **HRP2:** **PfLDH:**	**Overall:** **HRP2:** **PfLDH:**	**Reason:** **Overall:** **HRP2:** **PfLDH:**	Positive	Positivity depends on RDT	
Pf 16/376 - RDT D	**Overall:** **HRP2:** **PfLDH:**	**Overall:** **HRP2:** **PfLDH:**	**Reason:** **Overall:** **HRP2:** **PfLDH:**	Positive	Positivity depends on RDT	
Pf 16/376 - RDT E	**Overall:** **HRP2:** **PfLDH:**	**Overall:** **HRP2:** **PfLDH:**	**Reason:** **Overall:** **HRP2:** **PfLDH:**	Positivity depends on RDT	Positivity depends on RDT	
Pf 16/376 - RDT F	**Overall:** **HRP2:** **PfLDH:**	**Overall:** **HRP2:** **PfLDH:**	**Reason:** **Overall:** **HRP2:** **PfLDH:**	Positivity depends on RDT	Positivity depends on RDT	
Pf 16/376 - RDT G	**Overall:** **HRP2:** **PfLDH:**	**Overall:** **HRP2:** **PfLDH:**	**Reason:** **Overall:** **HRP2:** **PfLDH:**	Positivity depends on RDT	Positivity depends on RDT	
Pf 16/376 – RDT H	**Overall:** **HRP2:** **PfLDH:**	**Overall:** **HRP2:** **PfLDH:**	**Reason:** **Overall:** **HRP2:** **PfLDH:**	Negative	Negative	

4. Approval

Author	Signature	Date
Sampa Pal		04/07/2022
Allison Golden		3 May 2022