PURPOSE: Aim is to harmonize malaria Nucleic acid amplification technique (NAAT) results generated through different methods, by using WHO international standards for *P. falciparum.*

SCOPE: This SOP applies to the use of WHO International Standard for *Plasmodium falciparum* DNA nucleic acid amplification technology assays (NIBSC, Hertfordshire, UK) to standardize results across different PCR methods. Creating the dilution series of the standard followed by amplification will determine the limit of detection of the assay.

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 is responsible for the implementation of this procedure and for ensuring that all appropriate personnel are trained.

PROCEDURES:

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 Unacceptable specimen criteria

2.3 Unlabeled or mislabeled specimens must be rejected.

2.4 Clotted specimens must be rejected.

***WHO standards*:** *Plasmodium falciparum* 04/176 standard is available at NIBSC for procurement. Shipment requires import permit.

WHO standard *NIBSC* material	Description	Intended Use	Unit	Link
04/176	Lyophilized whole blood infected with *P. falciparum*	The WHO International Standard for *Plasmodium falciparum* DNA nucleic acid amplification technology (NAAT) assays	5 x 10^8 IU per vial.	NIBSC 04-176

3. Preparation of the dilution series

Diluent preparation: Collect whole blood in K_2EDTA from a healthy, universal donor (O+) and confirm negative for any plasmodium infection by microscopy or PCR. Freeze the blood within the same day of collection. For dilution, thaw this O+ blood specimen (diluent) and use to prepare the NIBSC control dilution series.

Total two dilution series will be created separately from two individual vials. Both dilution series will be made as described in the table 1 below. Freshly made dilution series will be used for DNA extraction. Other dilution series will be saved at -80^0C for future testing with RDT.

Concentration of the Pf 04/176 standard: 5 x 10^8 IU per vial. Reconstitute the lyophilized product within the vial by adding 500 ul of sterile nuclease free water. Concentration of reconstituted vial: 5 x 10^8 IU /500 ul or 1.00E+06/ul. Proceed with dilution as shown in Table 1.

Table 1: Dilution Series

Label ID	Dilution	Concentration Pf 04/176 IU/ul	Comments
Reconstitute - Pf04/176	500 ul of Nuclease-free water to the vial	1.00E+06	Reconstitution

Pf04/176-D0	30 µL of reconstituted material + 270µL Blood	1.00E+05	**10 fold dilution**
Pf04/176-D1	30 µL of Pf04/176-D0 + 270µL Blood	1.00E+04	
Pf04/176-D2	30 µL of Pf04/176-D1 + 270µL Blood	1.00E+03	
Pf04/176-D3	30 µL of Pf04/176-D2 + 270µL Blood	1.00E+02	
Pf04/176_D4	30 µL of Pf04/176-D3 + 270µL Blood	1.00E+01	
Pf04/176_D5	300ul Pf04/176_D4 + 300 ul Blood	5.00E+00	**2 fold dilution**
Pf04/176_D6	300ul Pf04/176_D5 + 300 ul Blood	2.50E+00	
Pf04/176_D7	300ul Pf04/176_D6 + 300 ul Blood	1.25E+00	
Pf04/176_D8	300ul Pf04/176_D7 + 300 ul Blood	6.25E-01	
Pf04/176_D9	300ul Pf04/176_D8 + 300 ul Blood	3.13E-01	
Pf04/176_D10	300ul Pf04/176_D9 + 300 ul Blood	1.56E-01	
Pf04/176_D11	300ul Pf04/176_D10 + 300 ul Blood	7.81E-02	
Pf04/176_D12	300ul Pf04/176_D11 + 300 ul Blood	3.91E-02	

DNA Extraction: Perform DNA extraction of the entire dilution series as per site protocol.

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

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	Pf IU/ul	PCR results of replicates in CT values or positive/negative	No. of positives/ No. of replicates	Comments
Pf04/176_D1	1.00E+04	1.		
		2.		
		3.		
		4.		
		5.		

Pf04/176_D2	1.00E+03	1.		
		2.		
		3.		
		4.		
		5.		
Pf04/176_D3	1.00E+02	1.		
		2.		
		3.		
		4.		
		5.		
Pf04/176_D4	1.00E+01	1.		
		2.		
		3.		
		4.		
		5.		
Pf04/176_D5	5.00E+00	1.		
		2.		
		3.		
		4.		
		5.		
Pf04/176_D6	2.50E+00	1.		
		2.		
		3.		
		4.		
		5.		
Pf04/176_D7	1.25E+00	1.		
		2.		
		3.		
		4.		
		5.		
Pf04/176_D8	6.25E-01	1.		
		2.		
		3.		
		4.		
		5.		

Pf04/176_D9	3.13E-01	1.		
		2.		
		3.		
		4.		
		5.		
Pf04/176_D810	1.56E-01	1.		
		2.		
		3.		
		4.		
		5.		
Pf04/176_D11	7.81E-02	1.		
		2.		
		3.		
		4.		
		5.		
Pf04/176_D12	3.91E-02	1.		
		2.		
		3.		
		4.		
		5.		

To demonstrate the limit of detection: If PCR results of Pf04176_D8 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.

4. Approval

Author	Signature	Date
Sampa Pal		11/04/2021