Method validation for dried blood spot *Plasmodium* 18S rRNA RT-PCR on Roche cobas automated molecular diagnostic platform

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Abstract

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Malaria, caused by *Plasmodium* spp., is a major health burden worldwide. *Plasmodium falciparum* is responsible for the most severe cases and most human mortality in sub-Saharan Africa. Conventional diagnostic approaches like microscopy and rapid diagnostic tests (RDTs) provide point-of-care screening but lack sufficient sensitivity for low-density infections and surveillance in endemic regions. Molecular diagnostic platforms, particularly nucleic acid tests (NATs), which target highly abundant *Plasmodium* 18S ribosomal RNA (rRNA), have emerged as sensitive and specific platforms for malaria detection and quantification. The FDA approved Roche cobas® 6800 system, which is a fully automated, high-throughput molecular platform assay for the qualitative detection of *Plasmodium* spp. from whole blood samples by targeting the 18S rRNA.

This study aimed (1) to adapt the cobas® malaria assay for off-label use with non-standard sample types, including 50 µL of whole blood and dried blood spot (DBS) samples, and (2) to

generate a standard curve for absolute quantification of the parasites. To achieve this, asexual-stage *P. falciparum* cultures were established and diluted to generate a full panel of validation samples across clinically relevant densities (1×10^8–1×10^1 parasites/mL). Fifty- microliter blood samples were spotted onto DBS cards. Together, whole blood liquid and DBS samples were processed then tested using the cobas® 6800 system. *Plasmodium* Armored RNA calibrators were used to generate standard curves for quantification. The study evaluated performance metrics parameters, that included pilot feasibility, standard curve assessment, linearity, limit of detection (LoD), precision, accuracy, carryover, DBS RNA stability under different storage conditions, calibrators-sample matrix matched assessment and extraction buffer elution assessment.

The modified FDA-approved Roche cobas® 6800 malaria assay demonstrated successful adaptation for detecting and quantifying *P. falciparum* 18S rRNA in 50 µL whole blood samples. However, performance was reduced for DBS samples. Both whole blood and DBS sample formats achieved ≥95% detection at the 100 parasites/mL threshold. A matrix mismatch using liquid calibrators to quantify DBS samples resulted in systematic underestimation bias of +0.79 $\log_{10}$ parasites/mL due to poor RNA recovery from DBS samples. The use of DBS-specific calibrators corrected the loss with a reduced bias of -0.07 $\log_{10}$ parasites/mL, which enabled for the reliable quantification of *P. falciparum* concentration in the DBS samples. Low (100 parasites/mL) density DBS samples stored at room temperature (22–25 °C) and -80°C consistently maintained a 100% positivity hit rate across all the two timepoints (weeks 1 and 6). All MID (2000 parasites/mL) parasite density DBS samples exhibited 100% positivity across both time points and temperature storage conditions. The stability of DBS samples indicated

reliable qualitative detection of *P. falciparum* RNA for DBS samples stored at 22-25°C or -80°C conditions, which supports their use in decentralized sample collection.

Overall, these results suggest that the modified FDA- approved Roche cobas® 6800 malaria assay performs well with 50 µL liquid whole blood samples but improvements are needed to achieve optimal results for 50 µL DBS samples.

Table of Contents

1 Introduction 3

1.1 Malaria burden and the need for improved diagnostics 3

1.2 Molecular diagnostics and *Plasmodium* 18S rRNA testing 4

1.3 Prospect of molecular platforms for *Plasmodium* detection 5

1.4 Context of use: expanding the Roche cobas® assay to field-compatible formats ... 6

1.5 Comparative assay landscape and rationale for modification 7

2 Materials and methods 8

2.1 Cultivation of parent sample material and production of validation sample stocks 8

2.2 Aliquoting and storage of test sample sets from validation study stock dilutions 10

2.3 Production of Armored RNA standard curve in whole blood 11

2.4 Off-label DBS modified assay based on the FDA-approved Roche cobas® malaria assay 11

2.5 cobas® malaria assay and system overview 13

2.5.1 Reagents and controls 13

2.5.2 Sample processing 13

2.5.3 Amplification and detection 14

2.5.4 Pilot studies of small volume whole blood samples on the Roche cobas malaria assay 14

2.6 Intended method validation performance characteristics to be tested 16

2.6.1 Assessment of standard curve quantitative performance 16

2.6.2 Assessment of assay linearity 18

2.6.3 Analytical sensitivity (LoD) 18

2.6.4 Precision 19

2.6.5 Accuracy 20

2.6.6 Carryover 20

2.6.7 DBS stability 21

2.7 Statistical analysis 21

3 Results 23

3.1 Pilot study of small whole blood samples on the Roche cobas malaria assay 23

3.2 Assessment of Armored RNA standard curve in a liquid whole blood matrix on the Roche cobas malaria assay 26

3.3 Linearity and bias for liquid and DBS samples 30

3.4 Analytical sensitivity (LoD) 32

3.4.1 Detection performance by concentration 33

3.4.2 Probit analysis and LoD cutoff 34

3.4.3 Sensitivity of *P. falciparum* Armored RNA controls 35

3.5 Precision 36

3.6 Accuracy 38

3.6.1 Bias assessment for liquid samples vs DBS samples with Matrix Matched calibrators 41

3.7 Carryover 42

3.8 DBS stability 43

3.9 Further optimization opportunities 45

3.9.1 Improving DBS RNA elution 45

4 Discussion 47

4.1 Feasibility of low blood-volume and DBS sampling 47

4.2 Standard curve-based quantification 47

4.3 Linearity and bias for liquid and DBS samples 48

4.4 Analytical sensitivity (LoD) 49

4.5 Precision 50

4.6 Carryover contamination and assay workflow integrity 50

4.7 DBS RNA stability under different storage conditions 51

4.8 Improving DBS RNA elution 51

5 Conclusions 53

6 Acknowledgements 55

7 Funding 55

8 References 56

1 Introduction

1.1 Malaria burden and the need for improved diagnostics

Malaria is a parasitic disease caused by *Plasmodium* species and transmitted via the bite of an infected female *Anopheles* mosquitoes **(Figure 1)**. Human malaria is primarily caused by six species of *Plasmodium*, of which *P. falciparum* and *P. vivax* account for the greatest number of cases. Of the others, *P. malariae, P. ovale curtisi* and *P. ovale wallikeri* are human-only species. The natural host of *P. knowlesi* are macaque monkeys in Southeast Asia but it can also cause fatal zoonotic cases in humans [1]. Despite decades of control efforts, malaria continues to pose a significant public health challenge. In 2023, the World Health Organization (WHO) estimated over 263 million malaria cases and 597,000 deaths globally in 83 countries, with sub-Saharan Africa bearing about 94% (246 million) of all malaria cases and 95% (569,000) of deaths. Children under 5 years of age account for about 76% of all malaria deaths in the region [2].

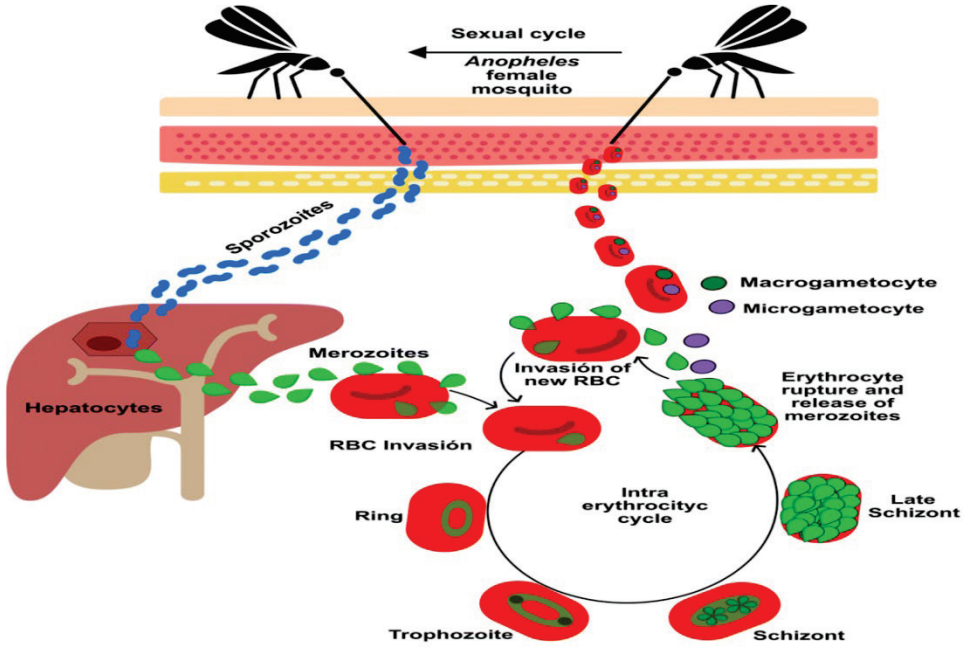

Figure 1: The *P. falciparum* life cycle. An infected female *Anopheles* mosquito inoculates sporozoites as it bites a human host, the sporozoites then migrates in the host's bloodstream and infect the hepatocytes. Merozoites are released and then invade erythrocytes, where they mature through various stages (ring, trophozoite and schizont stages) and undergo asexual multiplication (~ 10 new merozoites) every 48 h, releasing new merozoites which perpetuate the asexual cycle. Some of them enter the sexual cycle by becoming female and male gametocytes which are ingested by the mosquito when it bites an infected host, thereby starting the cycle all over again. Adapted from Molina-Franky, J *et al* [3].

Effective malaria control relies heavily on timely and accurate diagnosis. Traditional diagnostic methods, including microscopy and rapid diagnostic tests (RDTs), are widely used but have critical limitations. Microscopy is labor-intensive and operator-dependent, while RDTs may lack sensitivity for low-density infections and are prone to false positives due to antigen persistence. These limitations underscore the need for highly sensitive molecular platforms capable of detecting asymptomatic or sub-patent infections, especially in elimination settings and surveillance programs.

1.2 Molecular diagnostics and *Plasmodium* 18S rRNA testing

Molecular diagnostics, particularly nucleic acid tests (NATs) which is a highly sensitive testing technology that directly detects the genetic material (DNA or RNA) of various organisms [4]. It provides enhanced sensitivity and specificity over conventional methods. Over the past 20 years, sensitive NATs that afford earlier detection at lower parasite densities have been developed [5]. Real-time quantitative reverse transcription–PCR (qRT-PCR) with probe-based detection [6]. Quantitative reverse transcription polymerase chain reaction (RT-qPCR) is used to detect and quantify RNA, the total RNA is first transcribed into complementary DNA (cDNA) and finally the cDNA is then used as the template for the quantitative PCR or real-time PCR reaction (qPCR) [7].

The most common NAT targets of *Plasmodium* are its conserved 18S rRNA [8] with asexual (A)-type 18S rRNAs more highly expressed in erythrocyte-stage parasites and sexual (S)-type 18S rRNAs more abundant in mosquito stages [9]. Each *Plasmodium* parasite expresses thousands of 18S rRNAs from a few coding genes [10], making it even possible to detect single parasites in a 0.05–1mL blood sample by qRT-PCR [11]. Controlled human malaria infection (CHMI) involves the intentional infection of healthy individuals with malaria parasites, close observation of the volunteers, and clearance of the parasite at a predetermined endpoint [12]. Initially, malaria NATs were used retrospectively in trials, but as the techniques matured, such methods have been increasingly used as primary endpoint assays in CHMI trials in non-endemic sites [13].

1.3 Prospect of molecular platforms for *Plasmodium* detection

Molecular diagnostic platforms have specifically been developed or adapted to strengthen malaria diagnostics. The University of Washington Malaria Molecular Diagnostic Laboratory (UW-MMDL) pioneered a laboratory developed test (LDT) that targets the *Plasmodium* 18S rRNA using the Abbott m2000sp/rt instruments. The Abbott m2000sp platform is the front-end extraction apparatus for the integrated, semi-automated, m2000 system. The m2000sp uses silica-based extraction following off-board cell lysis and DNase and RNase inactivation by guanidinium thiocyanate-based lysis buffer. m2000sp extraction leads to recovery of high-quality RNA and DNA across a range of sample types, including whole blood.

The Abbott m2000 system which is a semi-automated RT-PCR platform validated for *P. falciparum* detection using 18S rRNA, has shown excellent performance with dried blood spot (DBS) samples in field studies [14].Although LDTs, such as the assay developed at the UW-MMDL, they offer excellent design and performance, their complex multi-step workflows and dependence on expert personnel may limit their use in resource-limited, malaria endemic regions.

A new addition to the portfolio of available molecular assays is the cobas® Malaria RT-PCR assay which is based on a fully automated cobas®6800 sample preparation (nucleic acid extraction and purification) followed by RT-PCR amplification and detection. The whole system consists of the sample supply module, the transfer module, the processing module, and the analytic module, reducing operator variability and increasing throughput.

Automated data management is performed by the cobas® 6800 systems software, which assigns test results for all tests as either non-reactive, reactive or invalid. Results can be reviewed directly on the system screen and printed as a report [15]. The cobas® Malaria assay is FDA-approved for the qualitative detection of *Plasmodium* from EDTA whole blood samples, targeting the 18S rRNA sequence. However, the assay is not FDA-approved for alternative sample types such as DBS or smaller volume blood specimens critical for surveillance, pediatric testing, and remote field collection.

1.4 Context of use: expanding the Roche cobas® assay to field-compatible formats

The approved use of the cobas® Malaria assay is to screen the blood supply. Here, our intended use is different. The modified cobas® Malaria assay for whole blood and DBS will be used as a biomarker to indicate the presence of malaria parasites in blood samples. Such samples may come from controlled human malaria infection (CHMI) studies, field studies, epidemiology studies and national surveys. The modified cobas® Malaria assay is intended to perform similarly to the existing Gen3.5 *Plasmodium* 18S rRNA RT-PCR LDT, with the limitation that the cobas® Malaria assay does not provide species identification of *P. falciparum*.

To address the gaps of the semi-automated RT-PCR platform, this study primarily sought to adapt the cobas® Malaria assay for off-label use with 50 µL of whole blood and DBS samples. These

sample type formats are well-suited for field-based and pediatric sampling where venous draws may be impractical. Secondly, a standard curve was developed to allow for the absolute quantification of *P. falciparum* 18S rRNA copies

1.5 Comparative assay landscape and rationale for modification

The prospect for the adaptation of the modified FDA approved Roche cobas® 6800 malaria assay was initiated through benchmarking against the Abbott m2000 Gen 3.5 RT-PCR LDT, which is a validated reference diagnostic method for *Plasmodium* 18S rRNA detection and absolute quantification of the targets in malaria molecular diagnostic. The successful validation of the LDT for compatibility with small blood volume of 50 µL and DBS samples.

This was the cornerstone for malaria molecular diagnostics technological advancement for better methods. The difference between the two molecular platforms are automation levels, throughput, reagent management, and intended use (qualitative vs quantitative). All these factors have potential effects on their performance and application. Concurrently both systems target *Plasmodium* 18S rRNA, distinctions in sample processing, extraction buffer contents, and calibration design plan approaches are more likely to have effects on the quantitative performance of the platforms.

Consequently, it was of much importance to perform same matrix-matched calibration and method validation across multiple performance parameters, including calibration curve assessment, linearity, analytical sensitivity, precision, accuracy, carryover, DBS stability, calibration matrix assessment, and extraction buffer elution assessment. The defined successful performance of the established parameters formed the baseline to ensure the adapted cobas® malaria assay meets the validation acceptability threshold for the platform that reliably supports surveillance and clinical research.

2 Materials and methods

2.1 Cultivation of parent sample material and production of validation sample stocks

Asexual culture of *P. falciparum* (NF54 strain) was established from frozen stock at Seattle Children's Research Institute (SCRI) using a three-step protocol following standard procedures [16]. Culture was maintained at 5% hematocrit O+ human red blood cell corpuscles (BioIVT-Westbury, NY), in RPMI 1640 25 µM HEPES, 2 µM L-glutamine (Alkali Scientific, Fort Lauderdale, FL, USA) supplemented with 50µM hypoxanthine and 5% AlbuMAX II (ThermoFisher Scientific Inc. USA), in an atmosphere of 5% CO_2, 5% O_2, 90% N_2 and 37°C. Culture media was changed every 24 hours by gently aspirating the old medium and replacing with fresh medium. The culture was gently rocked for the 12-24 hours prior to harvest to facilitate single infections **(Figure 2)**. Cultured parasite density was estimated by Giemsa (Sigma-Aldrich)-stained thin blood smears with a goal of 2% parasitemia for general maintenance. Ring stage parasites were periodically synchronized using 5% sorbitol (Sigma life science # S3889,USA) following the standard protocol [17].

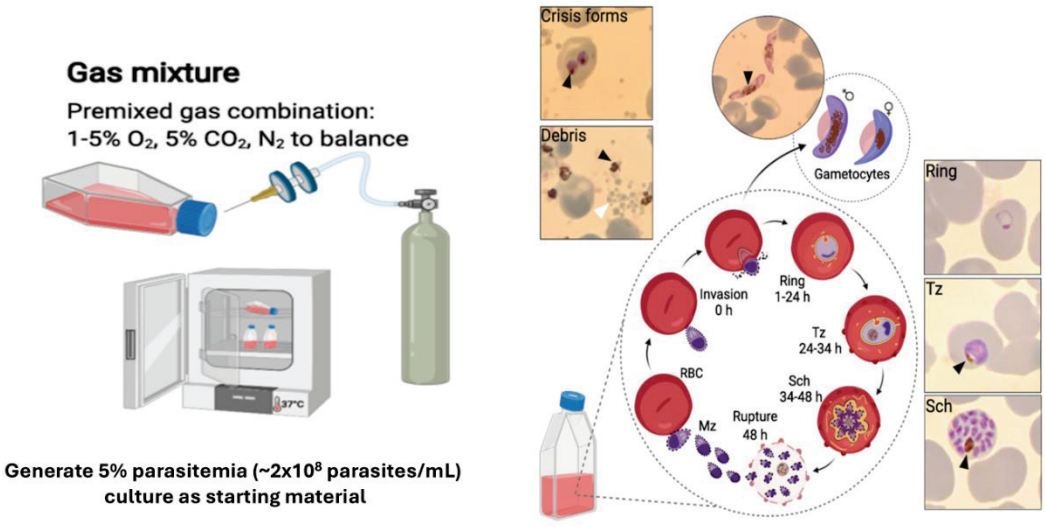

Figure 2: *In vitro* culture method of asexual blood stages of *P. falciparum* adapted from [18].

On the day of validation sample set preparation, the parent *P. falciparum* culture was sampled for thin blood smears, sorbitol treated to eliminate residual mature erythrocyte stage parasites, washed free of sorbitol, resuspended in fresh RPMI and adjusted to 5.4 % ring-stage parasitemia at 45 % hematocrit using freshly collected whole human blood. The final RBC count was determined using a hemocytometer to establish an infected RBCs/mL value in order to calculate parasites/mL with a target of 1-2 x 10^8 parasites/mL in whole human blood. From the highest *P. falciparum* concentration sample in whole blood, dilutions series were prepared using malaria negative human whole blood to generate densities ranging from 1 x 10^7, 1 x 10^6, 1 x 10^5, 1 x 10^4, 2 x 10^3, 1 x 10^3, 2.5 x 10^2, 1 x 10^2, 5 x 10^1, 2 x 10^1 to 1 x 10^1 **(Figure 3).** Additional aliquots were made for precision studies at density of 4x10^5parasites/mL.

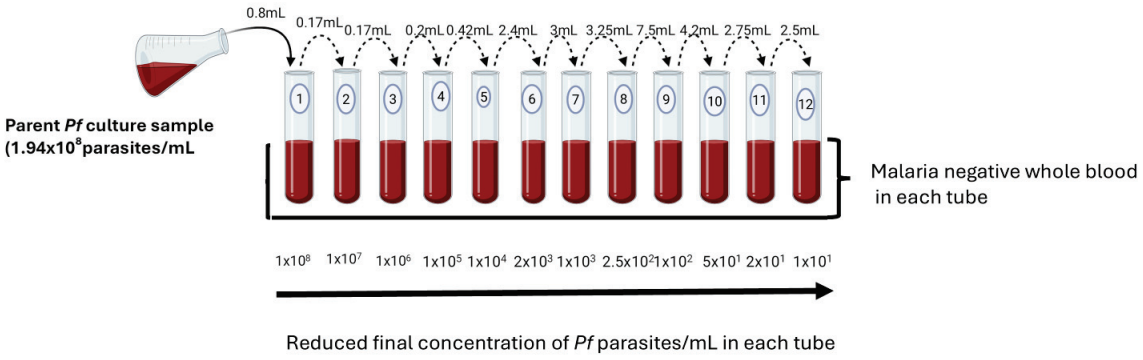

Figure 3: Serial dilution to generate samples for method validation studies. Image created in Biorender.com.

2.2 Aliquoting and storage of test sample sets from validation study stock dilutions

The blood volume of 50 µL of the prepared *P. falciparum* parasite densities ranging from 1 x 10^8, 1 x 10^7, 1 x 10^6,4 x 10^5,1 x 10^5,1 x 10^4,2 x 10^3,1 x 10^3,2.5 x 10^2,1 x 10^2,5 x 10^1,2 x 10^1,1 x 10^1 to malaria negative whole blood were spotted on each circle of the labeled Whatman Protein Saver 903 cards (Cytiva Life Sciences #10534612) **(Figure 4).**

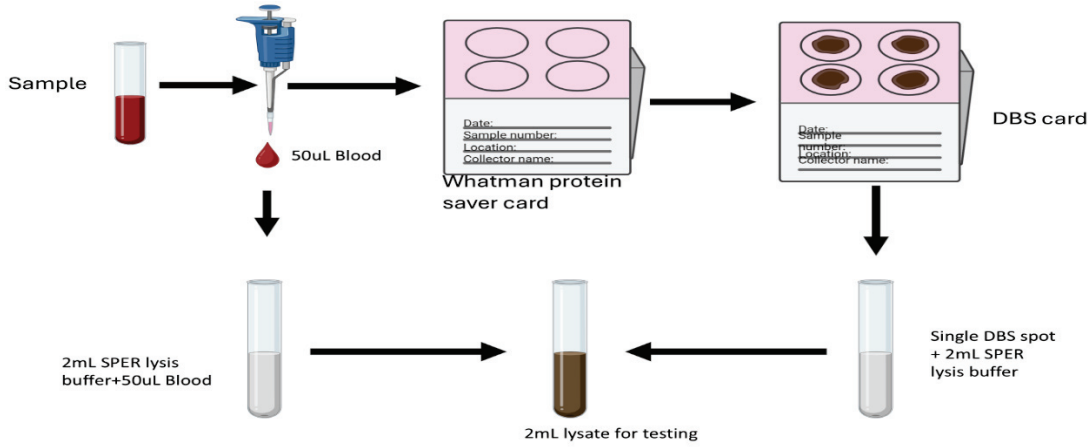

Figure 4: DBS and Whole blood samples preparation. Image created in Biorender.com.

The cards were allowed to air-dry in the biosafety cabinet class 2 for 8-12 hours and packed into gas-impermeable bags (Cytiva Life Sciences #10534321) with desiccants and humidity indicator cards. Packed cards were stored at room temperature 22-25°C and in the freezer at -80°C until use. To test the DBS samples, individual spots were excised with a laser cutter [19] into barcode labeled tubes then added 2 mL of cobas® omni–lysis reagent (Roche Part No. 06997538190), composed of 42.56% guanidine thiocyanate, 5% polydocanol, 2% dithiothreitol, and dihydro sodium citrate [20]. DBS spots in buffer tubes were then incubated in a water bath at 55°C for 30 minutes with shaking at 10-minute intervals. The processed DBS samples were stored at 2-8°C until use. As a comparator, 50 µL of whole blood of each of the listed parasite densities were pipetted into individual labeled tubes along with 2 mL of pre-dispensed cobas® omni–lysis reagent followed by

vortexing for 30 seconds. Whole blood processed samples were stored in the freezer at -80°C until use.

2.3 Production of Armored RNA standard curve in whole blood

To produce standard curve sample material, a custom manufactured *P. falciparum* full-length 18S rRNA Armored RNA (Asuragen, catalog #49594, stock 5 x 10^{11} copies/mL) was added to malaria negative human whole blood to obtain a ten-fold serial dilution series from 2.65 x 10^{7}, 2.65 x 10^{6}, 2.65 x 10^{5}, 2.65 x 10^{4}, to 2.65 x 10^{3} and a four-fold dilution for 6.63 x 10^{2} copies/mL **(Figure 5).**

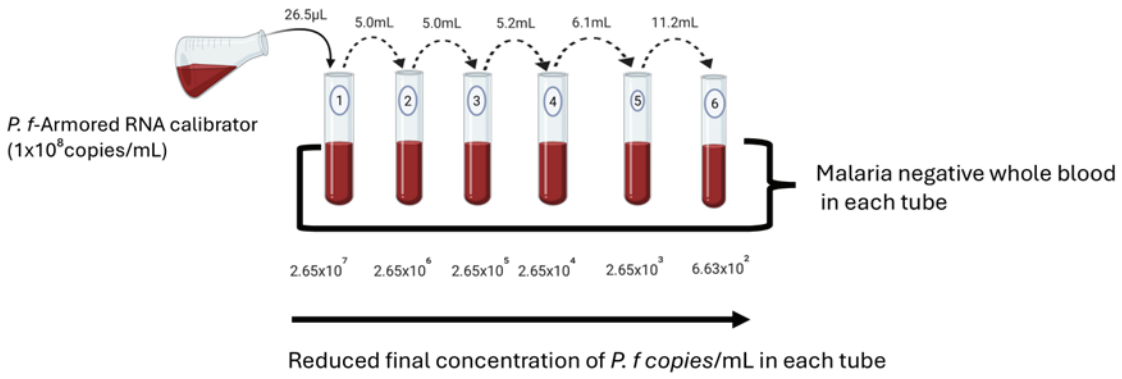

Figure 5: Serial dilution to generate samples for standard curve establishment. Image created with Biorender.com

2.4 Off-label DBS modified assay based on the FDA-approved Roche cobas® malaria assay

The approved Roche cobas® malaria assay combines automated silica-based nucleic acid extraction followed by RT-PCR for *Plasmodium* 18S rRNA **(Figure 6A).** For the intended DBS context of use herein, the Roche cobas® assay was modified in the following ways as shown in **Figure 6B.** First, use of 50 µL blood volumes as liquid samples or DBS. Second, addition of a standard curve to allow for target quantification.

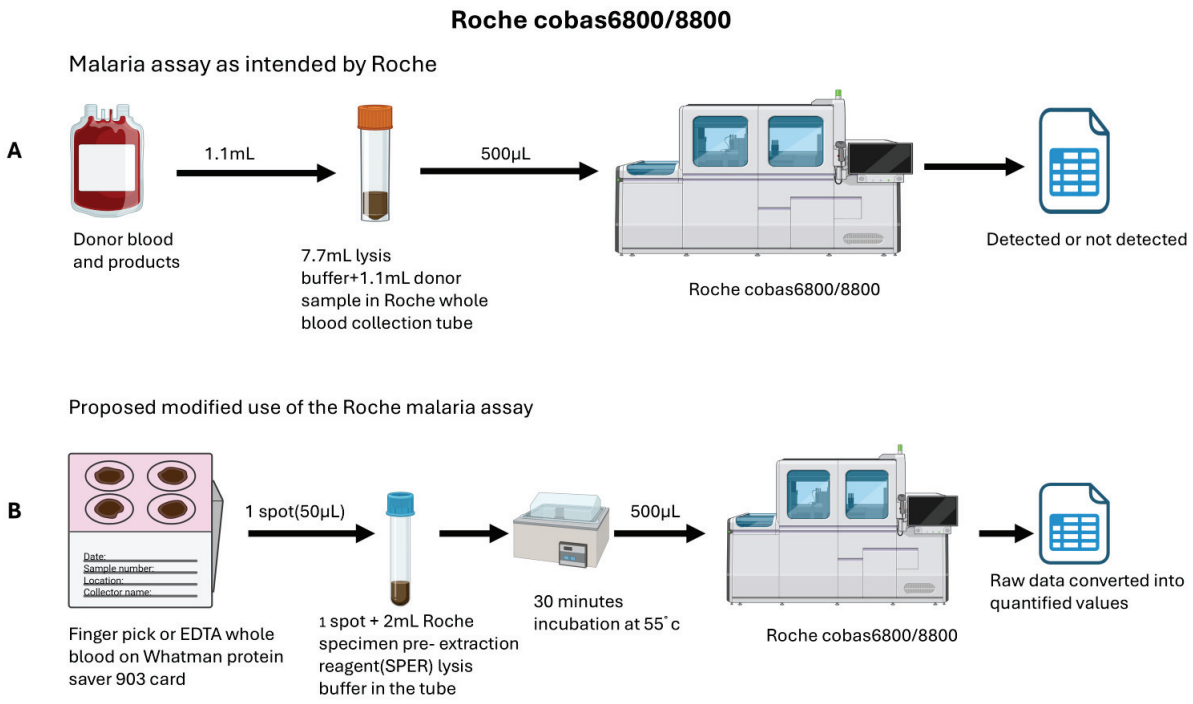

Figure 6: **Comparison of the Roche cobas® malaria assay's FDA-approved Roche and the modified workflows for *P. falciparum* 18S rRNA detection. A**: The Roche cobas® 6800 system as originally intended by the manufacturer processes 1.1 mL of liquid donor whole blood, mixed with 7.7 mL of lysis buffer in a Roche whole blood collection tube. A 500 µL aliquot of this lysate is analyzed by the cobas® system, which provides a qualitative result (detected or not detected). **B:** The proposed modified cobas® workflow for use with DBS samples, a 50 µL spot of whole blood collected on a Whatman 903 protein saver card is incubated with 2 mL of SPER buffer for 30 minutes at 55°C to facilitate RNA extraction. A 500 µL aliquot of the resulting lysate is then processed on the cobas® 6800 system. In the modified version, raw data are exported and subjected to a standard curve for parasite quantification. Image created in Biorender.com.

Frozen samples at -80°C for respective validation parameters as indicated in the validation plan, were retrieved from the freezer, allowed to thaw for 30-45 minutes at 22-25°C room temperature in the biosafety cabinet class 2. The DBS samples were centrifuged at 1,724 x g relative centrifugal force using Thermo Scientific X Pro centrifuge for 2 minutes to deposit the spot at the bottom of the tubes to enable smooth pipetting and prevent blockage during sample aspiration by the analyzer. The malaria assigned racks (blue coded) were slotted to the tray first, followed by placing the sample tubes in the rack slots with barcodes clearly visible. The cobas® 6800 system was

initialized to ready mode. The reagents and supplies were uploaded in the analyzer as instructed by the operation manual [21].

2.5 cobas® malaria assay and system overview

Detection of *Plasmodium* was performed using the cobas® Malaria assay on the cobas® 6800 system (Roche Molecular Systems, Branchburg, NJ, USA; distributed by Roche Diagnostics, Indianapolis, IN, USA). This is a fully automated, closed-system platform that integrates specimen preparation, nucleic acid extraction, amplification, and real-time polymerase chain reaction (RT-PCR) detection.

2.5.1 Reagents and controls

The assay employed the cobas® Malaria reagent cassette (Part No. 09352511190), which includes all necessary reagents pre-loaded for automated processing. The cassette contained proteinase solution, internal control, elution buffer, master mix reagent 1 and master mix reagent 2. Each assay run included internal and external controls. The positive control Kit (Part No. 09352520190) and the negative control kit (Part No. 09051554190).

2.5.2 Sample processing

All specimen processing was performed by the cobas® 6800 system using reagents from the cobas® omni line, including: magnetic glass particle reagent (Part No. 06997546190), specimen diluent (Part No. 06997511190), lysis reagent (Part No. 06997538190) and wash reagent (Part No. 06997503190). Additional consumables loaded into the instrument included processing and amplification plates, pipette tips, and solid/liquid waste containers. All reagents were stored as per manufacturer recommendations (typically 2–8°C; wash reagent at 15–30°C). No manual pipetting,

reagent preparation, or handling was performed, as all processes were fully automated and integrated within the instrument.

2.5.3 Amplification and detection

Specimens were lysed and nucleic acids were extracted using magnetic particle technology, followed by RT-PCR amplification and detection of *Plasmodium* 18S rRNA. The cobas® 6800 system software automatically interpreted the results as reactive, non-reactive, or invalid based on pre-set threshold criteria. Results were reviewed on-screen, printed, and digitally exported for further analysis.

2.5.4 Pilot studies of small volume whole blood samples on the Roche cobas malaria assay

To evaluate the feasibility of using small-volume whole blood samples for *P. falciparum* 18S rRNA detection on the cobas® 6800 System, pilot studies were first performed to establish that 50 µL of input volume would be sufficient for reliable detection and quantification. This evaluation was critical given that field-collected samples, especially those obtained from pediatric populations or through fingerstick sampling, are often limited in volume. A ten-fold serial dilution series was prepared using *P. falciparum* Armored RNA spiked into *Plasmodium*-negative whole blood to produce samples at target densities of 2.65×10^7, 2.65×10^6, 2.65×10^5, 2.65×10^4, 2.65×10^3, and 2.65×10^2 copies/mL, along with a four-fold dilution to yield 6.63×10^2 copies/mL. For each density, 50 µL samples were pipetted into pre-labeled tubes containing 2 mL of cobas® omni–lysis reagent. Tubes were vortexed for 30 seconds to ensure homogenization. Samples were prepared in duplicate and stored at –80°C until testing.

Parallel pilot testing was conducted using dried blood spot (DBS) samples to replicate field conditions. The malaria negative whole blood was spiked with *P. falciparum* positive blood and diluted in ten-fold and four-fold steps to match the same parasite densities. 50 µL of each diluted sample was spotted onto pre-labeled Whatman Protein Saver 903 cards, allowed to air dry in a Class 2 biosafety cabinet for 8–12 hours, and subsequently sealed in gas-impermeable bags with desiccant and humidity indicators. DBS cards were stored at ambient temperature (22–25°C) until use. For testing, DBS spots were laser-cut into barcode-labeled tubes, then added 2 mL of cobas® omni–lysis reagent. The tubes were then incubated for 30 minutes with intermittent shaking every 10 minutes at 55°C. Duplicate samples for each concentration were processed and stored at 2–8°C until analysis. Prior to testing, frozen liquid samples were thawed for 30–45 minutes at room temperature (22–25°C) in a Class 2 biosafety cabinet. DBS samples were centrifuged at 1,724 × g using a Thermo Scientific X Pro centrifuge for 2 minutes to settle the sample and facilitate efficient aspiration by the analyzer. All processed samples were then tested on the cobas® 6800 System to assess compatibility of the 50 µL sample volume with the assay workflow and detection sensitivity.

2.6 Intended method validation performance characteristics to be tested

Since this is an FDA-modified assay intended as a quantitative molecular assay, we evaluated standard curve, linearity, analytical sensitivity (limit of detection), precision, accuracy, carryover and dried blood spot stability (**Table 1**).

Table 1: Integrated validation parameters and sample characteristics.

Validation parameter	Sample type	Number of samples (# replicates x # densities)	Densities (parasites/mL)	Number of test runs
Linearity	DBS	3 x 8	1×10^8 to 1×10^1	3
	Whole blood	3 x 8	1×10^8 to 1×10^1	1
Analytical sensitivity (Limit of Detection)	DBS	5 x 5		4
	Whole blood	5 x 5	2.5×10^2, 1×10^2, 5×10^1, 2×10^1, 1×10^1	4
Precision	DBS	2 x 3	4×10^5, 2×10^3, 1×10^2	10
Carryover	DBS	2 X 2	4×10^5, negative	6
DBS stability	DBS (22-25°C)	5 X 2	2×10^3 and 1×10^2	3
	DBS (-80°C)	5 X 2	2×10^3 and 1×10^2	3
	Whole blood (-80°C)	5 X2	2×10^3 and 1×10^2	3

2.6.1 Assessment of standard curve quantitative performance

To enable absolute quantification of *P. falciparum* 18S rRNA in tested samples using the cobas® 6800/8800 System, standard curves were established and evaluated using negative malaria whole blood samples spiked with *P. falciparum* Armored RNA. Six target concentrations were prepared: 2.65×10^7, 2.65×10^6, 2.65×10^5, 2.65×10^4, 2.65×10^3, and 6.63×10^2 copies/mL **(Table 2)**. Two replicates of each concentration were tested per run, with a total of 11 independent assay runs

performed. For each run, the cycle threshold (Ct) values obtained were plotted against the corresponding nominal concentrations to generate standard curves. From these curves, key performance metrics including the slope, y-intercept, and coefficient of determination (r^2) were calculated to evaluate the assay's linearity and quantitative capability. The r^2 value was used as the primary indicator of linear correlation between Ct values and input RNA concentration, with an r^2 of ≥ 0.970 considered acceptable for quantitative assays.

Table 2: Standard curve whole blood *P. falciparum* Armored RNA sample densities.

Validation parameter	Sample type	Number of samples (# replicates x # densities)	Densities (*P. falciparum* RNA copies/mL)	Number of test runs
Standard curve quantitative performance	P. falciparum Armored RNA	2 x 6	2.65×10^7 2.65×10^6 2.65×10^5 2.65×10^4 2.65×10^3 6.63×10^2	11

Additionally, the lowest standard concentration (6.63×10^2 copies/mL) was specifically analyzed to assess the analytical sensitivity of the assay when detecting *P. falciparum* Armored RNA in whole blood. The ability to consistently detect this concentration across all runs contributed to defining the lower limit of quantification and provided further evidence for the assay's robustness in detecting low-copy RNA targets.

2.6.2 Assessment of assay linearity

Triplicate aliquots of each of the seven liquid sample densities ranging from 1×10^8, 1×10^7, 1×10^6, 1×10^5, 1×10^4, 1×10^3, to 1×10^2 parasites/mL were retrieved from −80°C storage. Samples were thawed at room temperature (22–25°C) for 30 to 45 minutes inside a Class 2 biosafety cabinet to ensure sample integrity and prevent contamination. Similarly, triplicate aliquots of processed DBS samples of the same concentrations stored at 2-8°C were retrieved. Prior to analysis, the DBS samples were centrifuged at 1,724 × *g* for 2 minutes to sediment the filter paper at the bottom of the tubes to facilitate smooth sample aspiration for downstream test processing. Data obtained from the assay were analyzed by linear regression to evaluate assay performance across the dilution series. The slope and coefficient of determination (r^2) were calculated, with acceptable linearity performance of $r^2 \geq 0.970$.

2.6.3 Analytical sensitivity (LoD)

The limit of detection (LoD) studies were designed using the Clinical Laboratory Standards Institute (CLSI) document EP17-A2 "Protocols for determination of Limits of Detection and Limits of Quantitation" [22]. The LoD and limit of quantification (LOQ) for *P. falciparum* samples were determined by testing DBS and liquid samples with parasite densities of 2.5 x 10^2, 1 x 10^2, 5 x 10^1, 2 x 10^1 and 1 x 10^1 parasites per mL**.** For each density, five duplicate samples were tested per run day, and a total of four independent runs were conducted.

The parasite density of the parent sample material was quantified using thin blood smear microscopy. This was followed by serial dilutions to the specified parasite densities. This approach has consistently yielded reproducible data across multiple generations of the related laboratory

developed test (LDT) assay [23]. Because the assay is mostly ribosomal RNA based (rRNA), we cannot use the World Health Organization (WHO) international standard [WHOIS, [24]] since the RNA in the reagent is degraded (data not shown). Instead, for this study, intact cultured *P. falciparum* parasites were added to fresh malaria negative whole blood, which was then stabilized in lysis buffer, to preserve RNA integrity and simulate clinical sample conditions.

The LoD was defined as the lowest concentration of parasites, at which ≥95% of replicate samples yielded results detected as positive. Probit regression analysis was performed to statistically determine this threshold. Since each *Plasmodium* parasite contains thousands of rRNA copies, Probit analysis also considered the Poisson distribution effects associated with detecting intact parasites at low densities. In addition to the whole parasite detection, a separate series of tests used *P. falciparum* Armored RNA calibrators in whole blood sample format (CAL-F) to evaluate the cobas® assay performance at low RNA concentrations. CAL-F samples at the lowest testable density were run in duplicate across ten non-consecutive days.

2.6.4 Precision

To assess the precision of the cobas® assay, *P. falciparum* dried blood spot (DBS) samples from three parasite densities were evaluated: high positive (4×10^5 parasites/mL), moderate positive (2×10^3 parasites/mL), and low positive (1×10^2 parasites/mL). Duplicate samples for each density were tested during the ten non-consecutive run days to evaluate both within-run (repeatability) and within-lab (reproducibility) precision. The data obtained were analyzed to assess variability. The average variation was used to calculate the total assay standard deviation (SD) and the percentage geometric coefficient of variation (%GCV). Acceptability criteria were defined as follows: within-

run %GCV ≤100% and within-lab %GCV ≤100%, in accordance with established performance benchmarks [25].

2.6.5 Accuracy

The accuracy assessment of the cobas® assay was evaluated by using linearity data generated from seven parasite densities ranging from 1 x 10^8, 1 x 10^7, 1 x 10^6, 1 x 10^5, 1 x 10^4, 1 x 10^3, to 1 x 10^2 parasites/mL. The Bland–Altman difference plot was generated to evaluate the mean bias and assess percentage agreement between paired results. The 95% limits of agreement and ±0.55 $\log_{10}$ parasites/mL for bias acceptable criteria was applicable.

2.6.6 Carryover

Carryover was evaluated to determine the potential for cross-contamination between high-positive and negative samples during automated processing. For this study, twelve DBS samples containing *P. falciparum* at high concentration (4 × 10^5 parasites/mL), used in the precision study, were interspersed with twelve confirmed negative malaria DBS samples and tested within each daily run. A total of six assay runs were conducted. To assess evidence of carryover, the percentage of positive results among the negative DBS samples was calculated. The expected outcome was 0% positivity among negative samples, indicating no detectable cross-contamination during the cobas® assay workflow.

2.6.7 DBS stability

A stability study was conducted to evaluate the impact of storage conditions and duration on the integrity of *P. falciparum* RNA in DBS samples. Liquid whole blood *P. falciparum* samples from these two parasite densities (2×10^3 and 1×10^2 parasites/mL) were prepared to make ten replicate samples for each density. The liquid samples served as the control group. The five replicate samples from each parasite density stored at −80°C were tested at the elapse of week 1 and week 6.

In parallel, two *P. falciparum* sample densities (2×10^3 and 1×10^2 parasites/mL) were spotted onto DBS filter cards. The spotted DBS cards were stored unprocessed (without laser cutting) at room temperature (22–25°C) and at −80°C for 1 week and 6 weeks' time points. At each designated time point, the corresponding DBS cards were retrieved. For each storage condition and parasite density, five DBS spots were laser cut into individually labeled tubes and processed to generate five replicate samples. These samples were then tested using the cobas® 6800 system. The resulting data from all samples tested were analyzed to determine the percentage positivity rate for each storage condition and time point. Stability was assessed by evaluating whether the DBS samples maintained consistent detection performance over time.

2.7 Statistical analysis

The Statistical data analysis was performed by using Microsoft Excel. Linear regression analysis was applied to standard curve and linearity study to assess linear correlation reported as coefficient of determination (r^2). Probit regression analysis was used in Analytical sensitivity (LoD) study to determine the 95% limit of detection for whole blood and DBS sample matrices.

Descriptive statistics which include means, standard deviation (SD), percentage geometric coefficient of variation (%GCV) were calculated for replicate samples across densities to assess precision of the cobas® 6800 system. Calculated means and standard deviations were applied for DBS stability assessment. Bland Altman plots were constructed to visualize the agreement between measurements and illustrate the bias between paired measurements.

3 Results

3.1 Pilot study of small whole blood samples on the Roche cobas malaria assay

To assess the feasibility and analytical sensitivity of using small-volume (50 µL) whole blood and dried blood spot (DBS) sample formats for detecting *P. falciparum* 18S rRNA on the cobas® 6800 system, pilot studies were conducted using seven dilution series concentrations of samples ranging from 2.65×10^7 to 2.65×10^2 copies/mL. All processed samples were tested on the cobas® 6800 System. The results obtained from whole blood samples spiked with *P. falciparum* Armored RNA demonstrated 100% detection across all seven tested parasite densities **(samples A–G; Table 3A)**, with both replicates in each set yielding positive qRT-PCR results. This indicated that the 50 µL whole blood sample format was compatible with the cobas® workflow and maintained high analytical sensitivity, including at the lowest tested concentration of 2.65×10^2copies/mL.

In contrast, DBS samples prepared using *P. falciparum*-positive whole blood showed 100% detection at higher concentrations (samples A–D), but sensitivity decreased at lower densities **(Table 3B)**. The detection dropped to 50% in sample E and was undetectable in samples F and G, suggesting reduced assay sensitivity when using DBS at lower parasite densities.

Table 3: Pilot study analytical sensitivity of 50 µL whole blood and DBS samples across seven parasite densities. (A) Whole blood samples spiked with *P. falciparum* Armored RNA (DBS_MAL_A–G). (B) DBS samples made from *P. falciparum*–positive whole blood (samples DBS_*P. falciparum* _A–G).

A – Whole Blood			B - DBS		
Sample ID	**Number of replicates tested**	**Number detected (%) by *P. falciparum* qRT-PCR**	**Sample ID**	**Number of replicates tested**	**Number detected (%) by *P. falciparum* qRT-PCR**
DBS_MAL_A	2	2 (100%)	DBS_*P. falciparum*_A	2	2 (100%)
DBS_MAL_B	2	2 (100%)	DBS_*P. falciparum*_B	2	2 (100%)
DBS_MAL_C	2	2 (100%)	DBS_*P. falciparum*_C	2	2 (100%)
DBS_MAL_D	2	2 (100%)	DBS_*P. falciparum*_D	2	2 (100%)
DBS_MAL_E	2	2 (100%)	DBS_*P. falciparum*_E	2	1 (50%)
DBS_MAL_F	2	2 (100%)	DBS_*P. falciparum*_F	2	0 (0%)
DBS_MAL_G	2	2 (100%)	DBS_*P. falciparum*_G	2	0 (0%)

To evaluate the quantitative capability of the assay, 4 and 5 point standard curves were generated using serial dilutions of *P. falciparum* Armored RNA in *Plasmodium*-negative whole blood whole (MAL A–D and A–E respectively). Linear regression and efficiency of the standard curves were assessed by the evaluation of the slopes, y-intercepts, and coefficient of determination (r^2) **(Figure 7).**

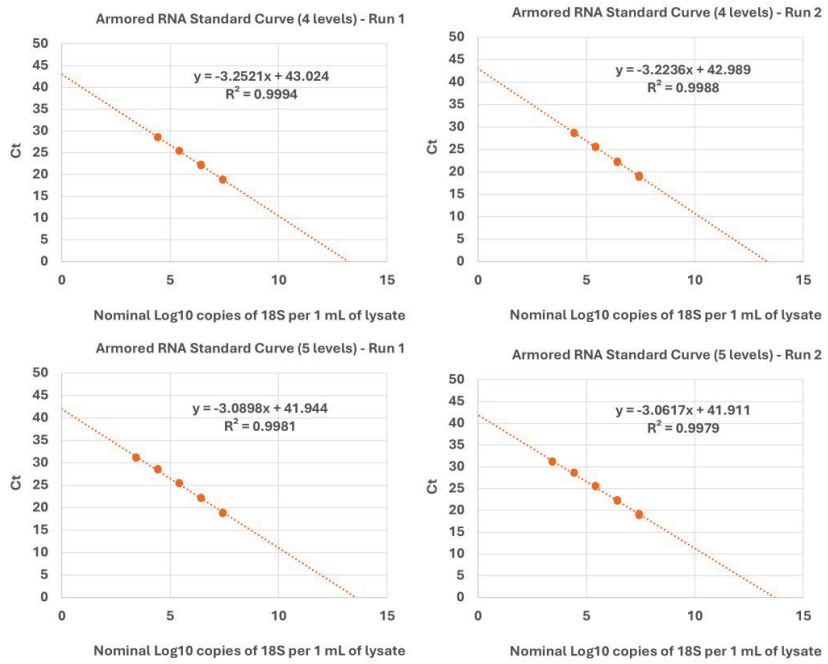

Figure 7: Pilot study standard curve linear regression performance of 4- and 5-point calibrators using *P. falciparum* Armored RNA, two runs for each calibration evaluation.

The average slopes ranged from –3.24 (4-point) to –3.08 (5-point), all were within the acceptable range (–3.65 to –2.95 cycles/$\log_{10}$ copies/mL). y-intercepts varied from 41.93 to 43.01 cycles and remained within the predefined acceptable range of 42–49 cycles. The R^2 was (≥0.998), which confirmed excellent linear correlation between Ct values and the *Plasmodium* RNA concentrations **(Table 4)**. All the standard curves assessed fell within the set performance acceptable criteria, which indicated that the assay exhibited strong quantitative linearity and reproducibility applicable to 50 µL whole blood samples.

Table 4: Pilot study standard curve assessment performance of the 4- and 5-point calibrators using *P. falciparum* Armored RNA.

4-point standard curve assessment (*P. falciparum* Armored RNA WB lysate CAL A-D)				
Parameter	**Mean**	**+/- 2 Standard deviation (SD)**	**Acceptability criteria**	**Performance**
Slope	-3.24 cycles/log10 copies RNA/mL lysate	-3.28 to -3.20 cycles/log10 copies RNA/mL lysate	-3.65 to 2.95 cycles/log10 copies RNA/mL lysate	Acceptable
Y intercept	43.01 cycles	42.97 to 43.05 cycles	42 to 49 cycles	Acceptable
r^2	0.999	0.998 to 0.999	≥ 0.970	Acceptable
5-point standard curve assessment (*P. falciparum* Armored RNA WB lysate CAL A-E)				
Slope	-3.08 cycles/log10 copies RNA/mL lysate	-3.12 to -3.04 cycles/log10 copies RNA/mL lysate	-3.65 to 2.95 cycles/log10 copies RNA/mL lysate	Acceptable
Y intercept	41.93 cycles	41.98 to 41.97 cycles	42 to 49 cycles	Acceptable
r^2	0.998	0.998 to 0.999	≥ 0.970	Acceptable

3.2 Assessment of Armored RNA standard curve in a liquid whole blood matrix on the Roche cobas malaria assay

The first objective was to generate and evaluate a standard curve that determined an accurate estimate of absolute quantification of the detected *P. falciparum* 18S rRNA in the tested samples by the cobas® 6800 system. While the cobas® 6800 system is highly automated and designed for high-throughput molecular testing, it typically provides qualitative results by default. Therefore, a custom standard curve was essential for quantification. The cobas® system detects target presence through fluorescence during RT-PCR amplification and reports a Ct value; the standard curve allows for the conversion of these Ct values into absolute RNA copy numbers.

To enable absolute quantification of *P. falciparum* 18S rRNA in tested samples using the cobas® 6800/8800 system, a ten-fold serial dilution series was performed using malaria negative whole blood samples spiked with *P. falciparum* Armored RNA to generate sample densities from $2.65x10^7$, $2.65x10^6$, $2.65x10^5$, $2.65x10^4$ to $2.65x10^3$ and a four-fold dilution for $6.63x10^2$ copies per mL sample density **(Figure 5).** Each dilution was tested in duplicate within a run day to assess repeatability. The 4 and 5 highest concentration dilutions were termed as 4-point and 5-point standard curves respectively **(Figure 8,9)**. Eleven independent runs were conducted to evaluate both the 4-point and the 5-point standard curves.

Importantly, these calibrators were prepared only using liquid whole blood samples**,** not DBS, based on precedent set by the Abbott m2000 laboratory-developed test (LDT) validation approach [26], which demonstrated that liquid calibrators yielded reliable, accurate quantification for both liquid samples and for DBS. The 4-point and 5-point standard curves had average coefficient of determination (r^2) of 0.999 and 0.998 respectively which were within the set threshold (≥0.970), they both demonstrated excellent linear regression **(Figures 8-9)**. This indicated a strong correlation between Ct values and *Plasmodium* 18S rRNA copy number target.

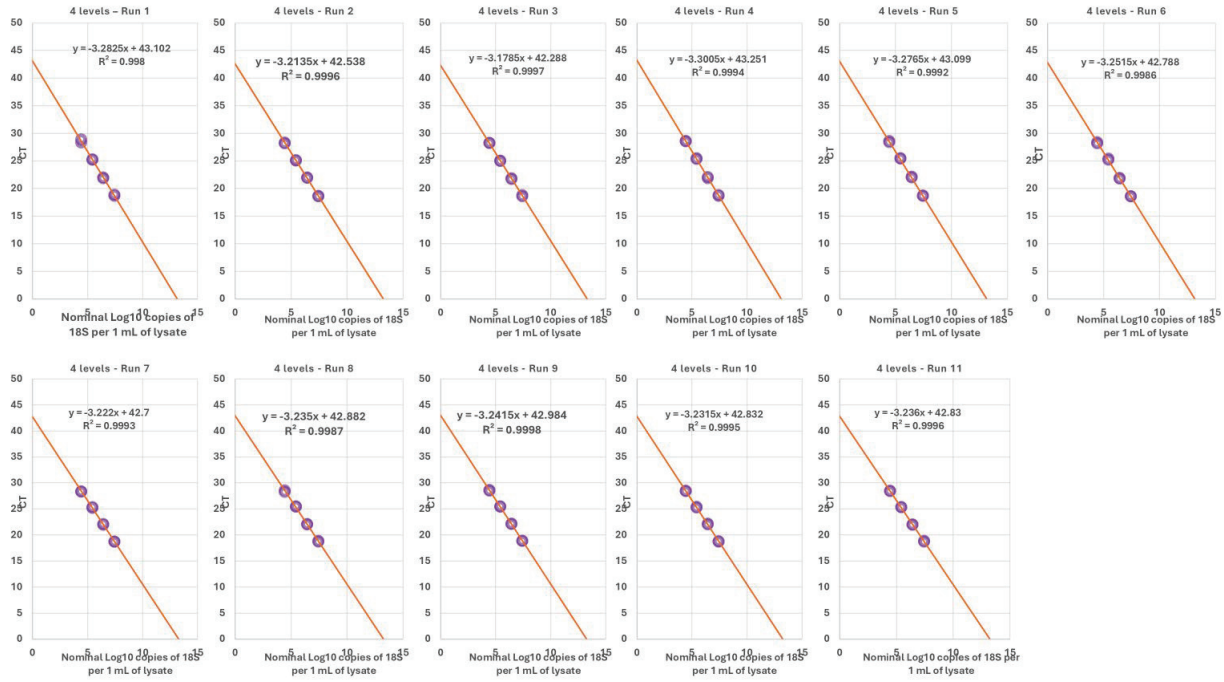

Figure 8: Representative 4-point standard curve plots across eleven independent runs using liquid whole blood calibrators spiked with *P. falciparum* Armored RNA.

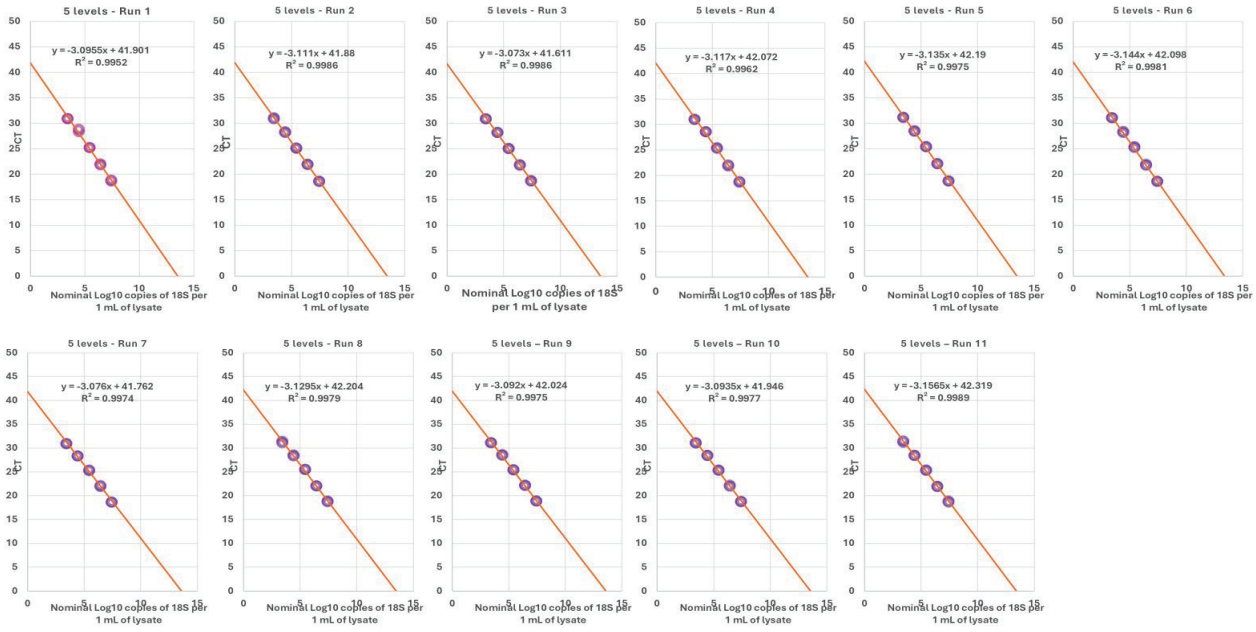

Figure 9: Representative 5-point standard curve plots across eleven independent runs using the same calibrator preparation and testing conditions as the 4-point curves.

The 4-point standard curve had a mean average slope of -3.24 cycles/log_{10} copies RNA per mL (range: -3.30 to -3.18), and the 5-point had -3.11 cycles/log_{10} copies RNA per mL (range: -3.17 to -3.05). Both standard curves were within the acceptable range of -3.65 to -2.95, which indicated optimal amplification efficiency **(Table 5)**. The average y-intercept values (42.85 for 4-point; 42.06 for 4-point) were within the acceptable range of 42–49 cycles, which showed consistent Ct results for the lowest concentration calibrators. Collectively all the slope, y-intercept, and r^2value for the eleven runs were within ±2 standard deviations, which demonstrated strong repeatability.

Table 5: Summary of performance characteristics for the 4-point (A) and 5-point (B) standard curve using liquid whole blood spiked with *P. falciparum* Armored RNA (n = 11 runs).

TABLE 5A*:* 4-point standard curve assessment (*P. falciparum* Armored RNA WB lysate CAL A-D)				
Parameter	**Mean**	**+/- 2 Standard deviation (SD)**	**Acceptability criteria**	**Performance**
Slope	-3.24 cycles/log10 copies RNA/mL lysate	-3.30 to -3.18 cycles/log10 copies RNA/mL lysate	-3.65 to 2.95 cycles/log10 copies RNA/mL lysate	Acceptable
Y intercept	42.85 cycles	42.30 to 43.38 cycles	42 to 49 cycles	Acceptable
r^2	0.999	0.998 to 0.999	≥ 0.970	Acceptable
TABLE 5B: 5-point standard curve assessment (*P. falciparum* Armored RNA WB lysate CAL A-E)				
Slope	-3.11 cycles/log10 copies RNA/mL lysate	-3.17 to -3.05 cycles/log10 copies RNA/mL lysate	-3.65 to 2.95 cycles/log10 copies RNA/mL lysate	Acceptable
Y intercept	42.06 cycles	41.70 to 42.42 cycles	42 to 49 cycles	Acceptable
r^2	0.998	0.995 to 0.999	≥ 0.970	Acceptable

High precision shown by the 4-point standard curve, made the basis for its use in the accurate calculation and estimation of absolute quantification of *P. falciparum* 18S rRNA copies in the validation study samples. The inclusion of the lowest calibrator level in the 5-point curve introduced greater variability, likely due to amplification noise near the limit of detection. By focusing on 4 levels ranging from 2.65×10^7 to 2.65×10^4 copies/mL, the 4-point standard curve preserved a broad dynamic range (four orders of magnitude) sufficient for the quantification of both high- and low-density *P. falciparum* infections. Limited number of calibrators optimized reagent usage and minimized intricacies encountered during high throughput testing.

3.3 Linearity and bias for liquid and DBS samples

Linearity refers to the ability of an assay to produce results that are directly proportional to the concentration of the analyte within a given range. It is a key performance characteristic for quantitative assays that ensure changes in signal correspond predictably to changes in target concentration. The dilution series of parasites in human blood were next tested as whole blood liquid and DBS samples in linearity and bias studies on the cobas® assay across concentrations densities ranging from 1×10^8, 1×10^7, 1×10^6, 1×10^5, 1×10^4, 1×10^3, to 1×10^2 parasites/mL representing orders of magnitude. The assay demonstrated reliable correlation with comparable whole blood linear regression ($r^2 = 0.997$) and DBS linear regression ($r^2=0.991$); both were within the acceptable range of $r^2 \geq 0.970$ **(Figure 10A-B and Table 6).**

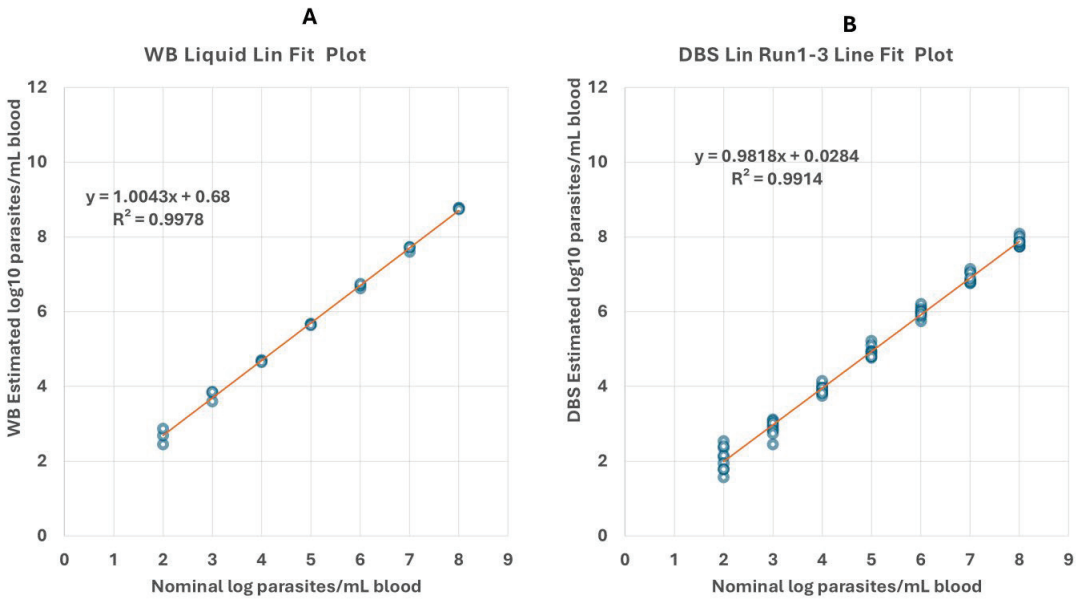

Figure 10: (A) Linearity plot of *P. falciparum* qRT-PCR assay using whole blood (single-run pilot). **(B)** Linearity plot of *P. falciparum* qRT-PCR assay using dried blood spot (DBS) samples (Run 1–3).

Table 6: Linear regression summary of *P. falciparum* qRT-PCR assay for whole blood and DBS sample types.

Sample type	r² values (*P. falciparum* qRT-PCR)	Acceptable criteria	Performance
Whole blood *P. falciparum* (Single run)	0.997	≥0.970 to 1.0	Acceptable
DBS *P. falciparum* (Run1-3)	0.991	≥0.970 to 1.0	Acceptable

Bias is the systematic difference between the measured value and the true value of an analyte. It reflects the degree to which the results deviate constantly in one direction and expressed as the mean difference between paired values. To compare the quantification performance between sample matrices, a Bland-Altman bias assessment was performed utilizing results from liquid whole blood calibrators tested on DBS samples.

The observed mean bias for whole blood samples was +0.70 log_{10} parasites/mL, with 95% limits of agreement from 0.51 to 0.89 log_{10} parasites/mL.

The plot in **Figure 11** showed the observed mean bias for DBS samples was +0.79 log_{10} parasites/mL, with 95% limits of agreement from +0.42 to +1.15 log_{10} parasites/mL. The DBS bias exceeded the acceptable bias of ±0.55 log_{10} parasites/mL. This indicated that DBS samples underestimated the parasite concentration relative to liquid samples, both sample types were calibrated with liquid calibrators.

WB liquid Calibrators on DBS samples		*P. falciparum* qRT-PCR	Acceptable criteria	Performance
Bias		0.79	Within +/- 0.55 log_{10} parasites/mL	Unacceptable
95% limits of agreement	From	0.42		
	To	1.15		

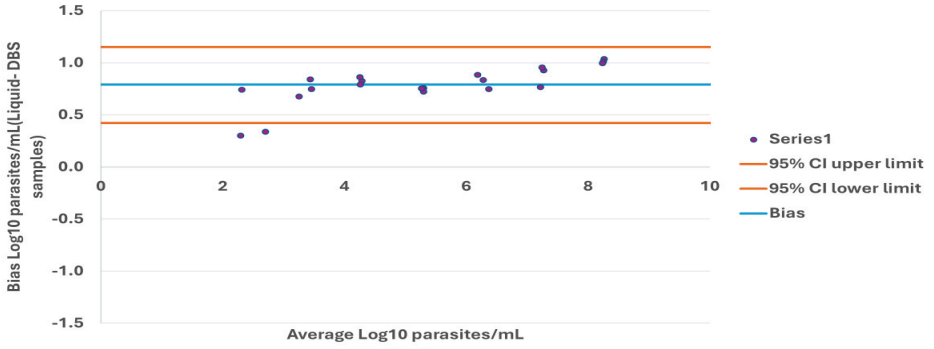

Figure 11: Bland-Altman bias analysis comparing liquid samples vs DBS samples, both sample types calibrated with whole blood liquid calibrators.

3.4 Analytical sensitivity (LoD)

The Limit of Detection (LoD) was defined as the lowest concentration of *P. falciparum* RNA that can reliably be detected in ≥95% of replicate samples. Twenty replicate liquid blood and DBS samples with *P. falciparum* nominal concentrations ranging from 2.5 x 10^2, 1 x 10^2, 5 x 10^1, 2 x 10^1, to 1 x 10^1 parasites per mL were tested using cobas® *Plasmodium* 18S rRNA qRT-PCR assay.

3.4.1 Detection performance by concentration

As shown in **Table 7**, detection performance across decreasing parasite densities demonstrated decline in assay sensitivity. Both matrices achieved ≥95% detection down to 100 parasites/mL, but detection decreased notably at lower concentrations. At 50 parasites/mL, DBS detected 85% and WB 90%. At 20 parasites/mL, detection fell to 75% for DBS and 70% for WB and at 10 parasites/mL, only 25% (5/20) of DBS samples and 30% (6/20) of WB samples were detected. The decrease in detection was consistent with limiting sample frequencies governed by Poisson statistics and cannot be overcome by conventional pipetting [27]. Nonetheless, these data were used to model the LoD statistically using probit regression analysis.

Table 7: Detection performance of *P. falciparum* 18S rRNA in whole blood and DBS samples at decreasing parasite concentrations.

Nominal parasites/mL	Nominal average parasites/50-µL blood	Number of replicates tested	Number detected (%) by *P. falciparum* qRT-PCR (WB lysate)	Number detected (%) by *P. falciparum* qRT-PCR (DBS)
250	12.5	20	20 (100%)	20 (100%)
100	5	20	20 (100%)	19 (95%)
50	2.5	20	18 (90%)	17 (85%)
20	1	20	14 (70%)	15 (75%)
10	0.5	20	6 (30%)	5 (25%)

3.4.2 Probit analysis and LoD cutoff

Probit evaluation was conducted to determine that the tested replicates were able to produce 95% positive results of the total replicate samples tested. The DBS samples generated a Log_{10} =1.96 LoD, which corresponded to 90 parasites/mL **(Figure 12A)**. Whole blood liquid samples produced a Log_{10} =1.81 LoD that corresponded to 64 parasites/mL **(Figure 12B)**. These values were further supported by the Probability prediction models (**Figures 13 and 14)**, were developed and confirmed the estimated LoD cutoffs at the 95% detection threshold that fell within lower and upper 95% confidence limits.

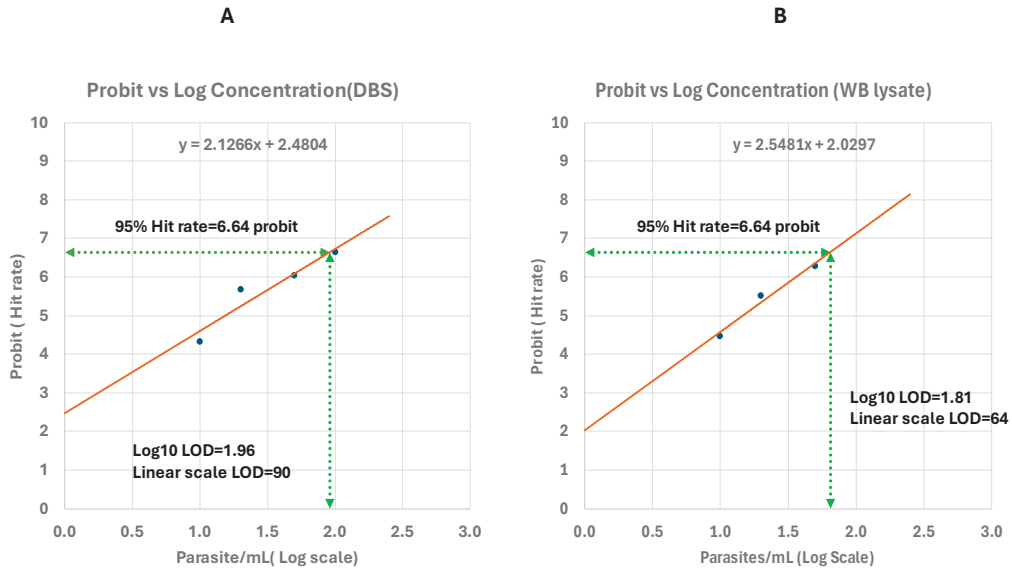

Figure 12: Probit regression analysis for DBS and whole blood samples. **(A)** The linear relationship between probit hit rate and log_{10} concentration produced a 95% detection rate of 90 parasites/mL. **(B)** The 95% detection threshold corresponded to 64 parasites/mL for whole blood.

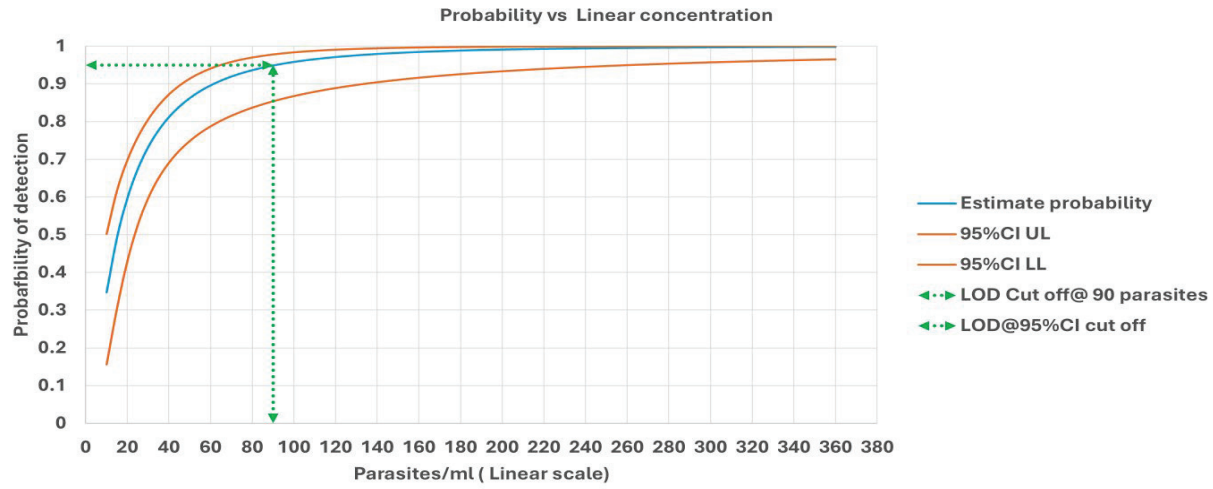

Figure 13: DBS probability prediction model showing estimated probability of detection and 95% CI limits with LoD cutoff at 90 parasites/mL.

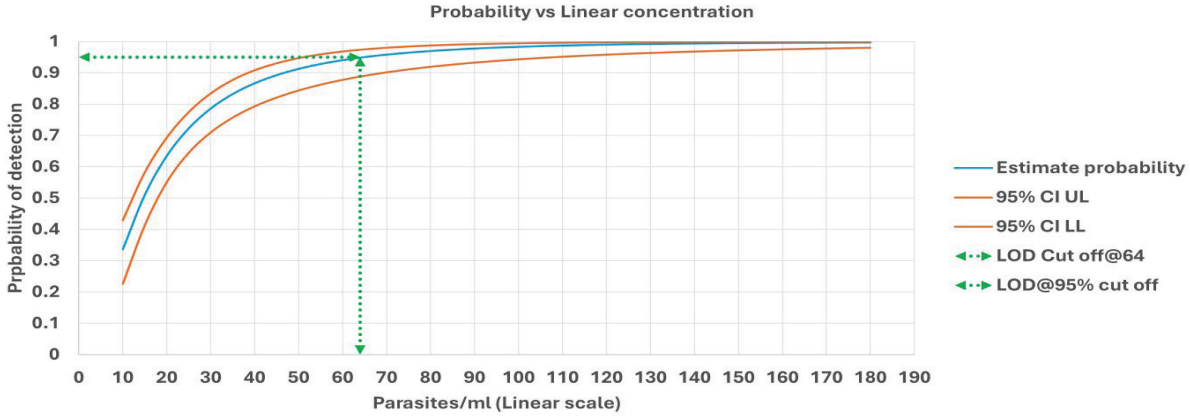

Figure 14: WB probability prediction model with 95% confidence intervals and LoD cutoff at 64 parasites/mL.

3.4.3 Sensitivity of *P. falciparum* Armored RNA controls

Because the parasite-derived LoD samples are limited by Poisson statistics and because we know that these assays can reliably detect the 18S rRNA derived from a single parasite or less in a given lysis buffer volume, we also performed LoD studies using Armored RNAs in the blood matrices. The Armored RNA calibrators at levels E and F contain RNA copies of less than a single parasite per sample. CAL-E control with 2,650 copies of *P. falciparum* Armored RNA per 50 µL blood

volume and CAL-F control with 663 copies of *P. falciparum* Armored RNA per 50 µL blood volume showed a 100% detection rate in all the twenty replicates that were tested. The tested average value for CAL E was 5.54 (SD: 0.05) log_{10} copies/mL and for CAL F was 5.13 (SD: 0.16) log_{10} copies/mL, which showed high reproducibility and strong detection capability at the lowest density range **(Table 8).** This approach was not performed for DBS.

Table 8: Detection of *P. falciparum* 18S rRNA Armored RNA controls CAL E and CAL F in whole blood samples.

***P. falciparum* Armored RNA sample ID**	**Nominal copies18S rRNA per 50µl blood processed**	**Nominal Log10 copies/mL blood**	**Nominal parasites / mL**	**Number of replicates tested**	**Average (SD) Log10 copies/mL blood**	**Number detected (%) by *P. falciparum* qRT-PCR**
CAL E	2,650	5.33	28	20	5.54 (0.05)	20 (100%)
CAL F	663	4.72	7	20	5.13 (0.16)	20 (100%)

3.5 Precision

The intend of the study was to determine within-run (repeatability) and within-lab (reproducibility) precision of the DBS cobas® *P. falciparum* 18S rRNA qRT-PCR assay. Precision reflects the assay's ability to produce consistent results across repeated measurements under the same or varied conditions. The percent geometric coefficient of variation (%GCV) for within run and within-lab precision were calculated as recommended by Clinical and Laboratory Standard Institute [28]. Precision was evaluated across three parasite concentrations high (HI: 400,000 parasites/mL), mid (MID: 2,000 parasites/mL), and low (LO: 100 parasites/mL).

Precision studies evaluated repeatability at a given concentration over repeated runs. **Table 9** illustrates the higher density samples demonstrated acceptable precision with HI and MID samples at within-run %GCV of 31.45% and 45.75% and within-lab % GCV of 34.31% and 52.51%, respectively. These results were within the set acceptable limit of ≤100%GCV, supporting the assay's reliable precision at moderate to high parasite densities. However, at the lower parasite density, the assay showed more variability (within-run %GCV 237.69; within-lab %GCV 280.97), which exceeded the set acceptable limit. We think that this imprecision is due to somewhat mixed culture stages in the validation sample set. The parent culture sample contained a significant number of rings, trophozoites, schizonts and gametocytes that were seen in the Giemsa-stained smear microscopy.

Table 9: Precision analysis of DBS samples across HI, MID, and LO parasite densities.

Description of DBS samples and testing schedule				**Summary of precision-*P. falciparum* qRT-PCR**				
Sample name	**Estimated parasites/ mL WB**	**Testing**	**Nominal log10 parasites/ mL WB**	**Average means log10 parasites/ mL WB**	**Within-run SD log10 parasites/ mL WB**	**Within-lab SD log10 parasites/ mL WB**	**Within-run %GCV**	**Within-lab %GCV**
HI	400,000	Duplicate x 10 days	5.6	4.83	0.12	0.13	31.45	34.31
MID	2,000	Duplicate x 10 days	3.3	3.33	0.16	0.18	45.75	52.51
LO	100	Duplicate x 10 days	2.0	1.9	0.53	0.58	237.69	280.97
Acceptable criteria threshold							≤100%	

3.6 Accuracy

To gauge the accuracy of the modified cobas® *P. falciparum* 18S rRNA qRT-PCR assay, bias evaluations were performed using Bland-Altman analysis. Bias here is a quantitative difference between the measured value and the true (expected or nominal) value of an analyte. It reflects the degree to which test results deviate consistently in one direction and is expressed as the mean difference between paired measurements.

Bias assessment of nominal vs liquid samples with liquid calibrators

We aimed to evaluate the accuracy of the results produced by the modified cobas® *P. falciparum* 18S rRNA qRT-PCR assay when liquid samples were calibrated with whole blood liquid calibrators. We compared the results obtained against the nominal expected values derived by microscopy count of the parent *P. falciparum* culture sample.

Figure 15, illustrate a bias of +0.70 $\log_{10}$ parasites/mL obtained after the assessment of agreement between measured concentrations of *P. falciparum* in liquid whole blood samples and nominal parasite concentrations determined by microscopy. This indicated that liquid samples overestimated *P. falciparum* parasite concentrations relatively to the nominal values. This was due to biological characteristic variability of the parent culture material that had mixed stages of ring forms and non-ring forms. The parent culture sample material was serially diluted to generate linearity validation samples of densities ranging from 1×10^8, 1×10^7, 1×10^6, 1×10^5, 1×10^4, 1×10^3, to 1×10^2 parasites/mL which were used as the nominal expected value baselines.

WB liquid Calibrators on Liquid samples		*P. falciparum* qRT-PCR	Acceptable criteria	Performance
Bias		0.70	Within +/- 0.55 log_{10} parasites/mL	Unacceptable
95% limits of agreement	From	0.51		
	To	0.89		

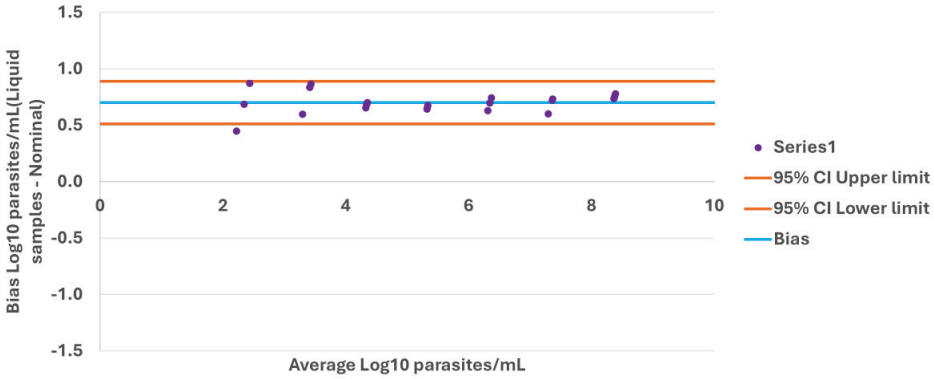

Figure 15: Bias assessment of liquid whole blood samples quantified using whole blood liquid calibrators against nominal microscopy-based concentrations.

Notably, the parent culture sample concentration was established based on microscopy counts during initial characterization prior to serial dilution. The count results showed a mixed-stage population consisting of trophozoites, schizonts, and gametocytes, rather than being enriched with ring-stage parasites. Because only ring-stage parasites are typically counted in standardized quantification for clinical malaria diagnostics, the inclusion of more mature parasite forms may have led to inflated parasite counts relative to what would be expected from nucleic acid–based detection of ring-stage–equivalent targets. This discrepancy led to a bias that exceeded the acceptable threshold limit of ±0.55 log_{10} parasites/mL when comparing *P. falciparum* quantification in liquid samples to nominal values derived from microscopy count.

To further investigate whether the modified cobas® *P. falciparum* 18S rRNA qRT-PCR assay was operating at optimal RNA recovery efficiency, we used known input quantities of spiked *P. falciparum* Armored RNA in liquid whole blood that generated the standard curve. Six target

concentrations were prepared ranging from 2.65×10^7, 2.65×10^6, 2.65×10^5, 2.65×10^4, 2.65×10^3, and 6.63×10^2 copies/mL **(Table 2)**. Two replicates of each concentration were tested.

A Bland–Altman analysis was used to assess the agreement between the observed measurements and the nominal values of the listed concentrations. The results in **Figure 16** showed the mean bias was +0.13 $\log_{10}$ parasites/mL, with 95% limits of agreement ranging from −0.27 to +0.52 $\log_{10}$ parasites/mL. All the observed values fell within the acceptable threshold of ±0.55 $\log_{10}$ parasites/mL, which indicated acceptable assay performance in achieving consistent optimal recovery of spiked RNA across the quantification ranges.

P. falciparum Armored RNA spiked in liquid whole blood (Observed-Nominal values)		*P. falciparum* qRT-PCR	Acceptable criteria	Performance
Bias		0.13	Within +/- 0.55 $\log_{10}$ parasites/mL	Acceptable
95% limits of agreement	From	-0.27		
	To	0.52		

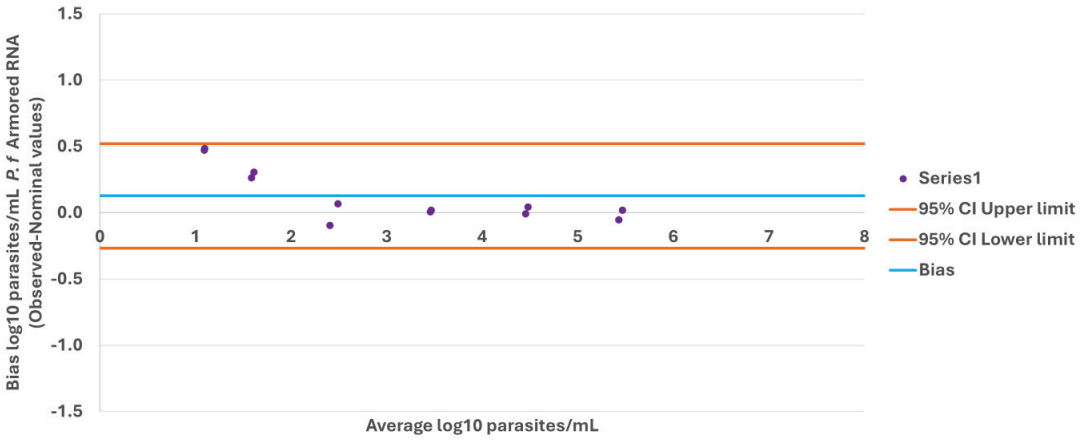

Figure 16: Bias assessment of qRT-PCR quantification of *P. falciparum* Armored RNA in liquid whole blood samples compared to known input concentrations.

3.6.1 Bias assessment for liquid samples vs DBS samples with Matrix Matched calibrators

To evaluate assay bias introduced by the choice of calibration material, we compared cobas® qRT-PCR quantification results for DBS samples using either whole blood (liquid) calibrators or DBS-based calibrators.

Next, we considered whether the calibrator matrix mismatch was responsible for the observed bias of 0.79 log_{10} parasites/mL for DBS results underestimation **(Figure 11)**. Here we recalibrated the DBS samples using DBS-based calibrators. The use of matrix matched DBS calibrator approach resulted in a mean bias of -0.07 log_{10} parasites/mL that corrected the underestimation of DBS results, with 95% limits of agreement ranging from -0.33 to +0.20 log_{10} parasites/mL **(Figure 17).** The reduced bias showed no significant quantification difference between liquid and DBS samples.

DBS Calibrators on DBS samples		*P. falciparum* qRT-PCR	Acceptable criteria	Performance
Bias		-0.07	Within +/- 0.55 log_{10} parasites/mL	Accepted
95% limits of agreement	From	-0.33		
	To	0.20		

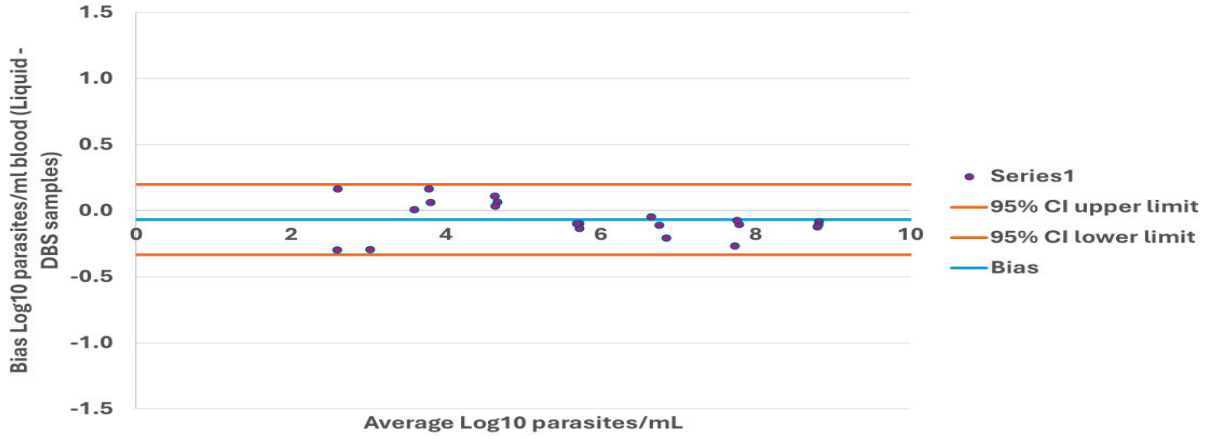

Figure 17: Bland-Altman plot showing bias analysis for liquid vs DBS samples with Matrix matched calibrators.

3.7 Carryover

The aim of this experiment was to determine whether *P. falciparum* 18S rRNA copies from a previously highly positive concentrated sample contaminated the subsequent negative sample during cobas® assay processing test run. To answer this question, we used an approach of interspersed distribution **(Figure 18)** of high *P. falciparum* DBS samples with a concentration of $4x10^5$ parasites/mL and malaria negative DBS samples within a run day. For this study, twelve DBS samples of *P. falciparum* with high parasites density for the precision dilution series and twelve negative DBS samples were used.

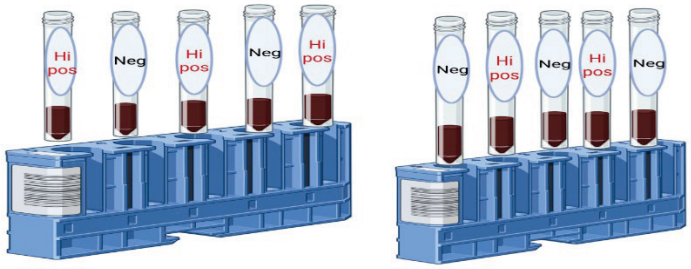

Figure 18: Interspersed sample layout for carryover evaluation during cobas® process testing.

Evidence for any cross-contamination effects was determined by calculating the percentage of positive assay results among the negative samples in the study. As shown in **Table 10,** none of the negative DBS samples demonstrated any detectable copies of *P. falciparum* 18S rRNA following the cobas® processing of a high-concentration positive sample. All twelve negative replicates consistently yielded a result of 0 parasites/mL**.** Accordingly, all calculated carryovers were 0%, which confirmed the absence of detectable cross-contamination during the cobas® process testing. This suggested that the steps on the Roche cobas® do not introduce any cross-contamination during automated qRT-PCR analysis.

Table 10: Carryover assessment of interspersed DBS samples tested using the cobas® *Plasmodium* 18S rRNA qRT-PCR assay.

Carryover analysis			
Interspersed sample pair	**DBS High Result_H (parasites/mL)**	**DBS Negative Result_N (parasites/mL)**	**Carryover (%)**
H9↔N9	591,928	0	0
H10↔N10	705,388	0	0
H11↔N11	302,606	0	0
H12↔N12	596,458	0	0
H13↔N13	591,928	0	0
H14↔N14	663,648	0	0
H15↔N15	248,191	0	0
H16↔N16	392,158	0	0
H17↔N17	404,302	0	0
H18↔N18	240,736	0	0
H19↔N19	605,623	0	0
H20↔N20	302,606	0	0
N_Result (Measured result of negative sample after High one) =0 N_Baseline (Expected true value of Negative sample) =0 H_Result (Value of the preceding high sample) Carryover (%) = [N_Result -N_Baseline)/H_Result] *100			

3.8 DBS stability

This study sought to examine the preservation of *P. falciparum* 18S rRNA in DBS samples under different storage conditions. The DBS samples under investigation were stored for 1 and 6 weeks

at 22-25ºC and -80ºC. A total of forty DBS samples were tested across 2 parasite concentrations, LOW (100 parasites/mL) and MID (2000 parasites/mL). The performance of these DBS samples was assessed and compared to whole blood liquid controls stored at -80 °C. Forty DBS samples and twenty whole blood liquid samples from the 2 parasite densities LO (100 parasites/mL) and MID (2000 parasites/mL) were tested after the elapse of the storage time points. The DBS positivity rate results were assessed and compared with whole blood liquid controls results.

Table 11 shows, the Low-density DBS samples stored at room temperature (22–25 °C) and -80°C consistently maintained a 100% positivity hit rate across all the two timepoints (weeks 1 and 6). All the MID parasite density DBS samples exhibited a 100% positivity rate across both timepoints and temperature storage conditions as seen in **Table 12**. The control liquid samples for both parasite densities showed a 100% positive detection rate of *P. falciparum* 18S rRNA. This indicated the reliability of DBS sample matrix stored at 22-25°C or -80°C conditions for the stable detection of *P. falciparum* RNA over time, supporting their use in decentralized sample collection.

Table 11: Stability of *P. falciparum* 18S rRNA detection in low-density (100 parasites/mL) DBS samples over 6 weeks.

DBS LOW (100 parasites/mL)				
Storage temperature (°C)	**Tested duration (weeks)**	**Number of replicates tested**	**Positivity rate (%)**	**Average means (Average SD) log10 copies/mL blood**
22-25	1	5	100	5.93 (0.16)
22-25	6	5	100	5.52 (0.26)
-80	1	5	100	5.67 (0.13)
-80	6	5	100	5.48 (0.17)
Control Whole Blood (100 parasites/mL)				
-80	1	5	100	6.29 (0.21)
-80	6	5	100	6.20 (0.16)

Table 12: Stability of *P. falciparum* 18S rRNA detection in mid-density (2000 parasites/mL) DBS samples over 6 weeks.

DBS MID (2000 parasites/mL)				
Storage temperature (°C)	**Tested duration (weeks)**	**Number of replicates tested**	**Positivity rate (%)**	**Average means (Average SD) log10 copies/mL blood**
22-25	1	5	100	7.19 (0.03)
22-25	6	5	100	7.06 (0.03)
-80	1	5	100	7.08 (0.09)
-80	6	5	100	6.73 (0.13)
Control Whole Blood (2000 parasites/mL)				
-80	1	5	100	7.82 (0.04)
-80	6	5	100	7.80 (0.07)

3.9 Further optimization opportunities

3.9.1 Improving DBS RNA elution

In method validation of the cobas® *P. falciparum* 18S rRNA qRT-PCR assay, the efficiency of RNA extraction from DBS is a key determinant for accuracy. The cobas® assay utilizes the specimen pre-extraction reagent (SPER) buffer, which has a guanidinium concentration of 2.68 M (28% w/w) [29], which was the reagent undergoing validation.

The reference m2000Gen3.5 *P. falciparum* 18S rRNA qRT-PCR assay uses the NucliSENS buffer, which has a guanidinium concentration of 5 M [30]. The Bland-Altman plot, **Figure 11** illustrated that DBS samples which were calibrated with liquid calibrators showed a bias of 0.79 $\log_{10}$ parasites/mL, underestimating parasite concentration.

The bias was reduced to -0.07 $\log_{10}$ parasites/mL when we changed to matrix-matched DBS specific calibrators **(Figure 17).** But matching calibrators only compensated for the

underestimated parasite concentration, but it did not solve the root cause: poor RNA recovery from DBS using the Roche SPER buffer.

The SPER buffer used by the cobas® assay contains about 2.68 M guanidinium thiocyanate. In contrast, the bioMérieux NucliSENS lysis buffer, which is used in *Plasmodium* 18S rRNA lab-developed tests (LDTs) assay, contain 5 M guanidinium concentration which is much stronger. Guanidinium thiocyanate is a strong chaotropic agent essential for effective cell lysis, protein denaturation, and RNase inactivation [31].

The lower RNA yield from the cobas® DBS protocol reflects this difference in lysis strength. The suboptimal guanidinium concentration in SPER may result in incomplete lysis of dried matrix-bound cells and less efficient release and stabilization of RNA in DBS samples. Improving DBS parasite quantification may require the evaluation of SPER buffer content supplemented with a higher guanidinium concentration that will help boost RNA recovery and reduce bias.

4 Discussion

This method validation study evaluated the performance of a modified cobas® *P. falciparum* 18S rRNA qRT-PCR assay for detecting and quantifying *P. falciparum* RNA in non-standard sample formats specifically 50 µL liquid whole blood and dried blood spots (DBS). The aims were (1) to validate assay compatibility with small-volume blood and DBS samples and (2) to establish a robust standard curve for accurate estimation of absolute quantification of the detected *P. falciparum* 18S rRNA in the tested samples by the cobas® 6800 system.

4.1 Feasibility of low blood-volume and DBS sampling

The pilot feasibility study confirmed that 50 µL of liquid whole blood samples were compatible with the cobas® 6800 system. The whole blood samples spiked with *P. falciparum* Armored RNA demonstrated 100% positive detection across all seven tested parasite densities. These results supported the use of liquid whole blood small volume in malaria diagnostic studies where venous blood collection is limited. However, the sensitivity for DBS samples prepared using *P. falciparum*-positive whole blood decreased at lower parasite densities, with the detection below 95% at the least parasite densities. These findings were consistent with prior studies that indicated RNA degradation, inefficient elution, and matrix effects had an impact on the performance of DBS samples at lower parasite densities [32].

4.2 Standard curve-based quantification

The generation and validation of a standard curve was essential to enable absolute quantification of *P. falciparum* 18S rRNA in tested samples using the cobas® 6800 system, which delivers qualitative result outputs. The generated 4-point and 5-point standard curves using *P. falciparum* Armored RNA liquid whole blood calibrators, demonstrated excellent strong correlation ($r^2 \geq$

0.998) and acceptable amplification efficiency (slopes ranged between -3.30 and -3.05), which met all the set performance acceptable limits. The 4-point standard curve with density ranging from 2.65 x 10^7 to 2.65 x 10^4 copies/mL was selected due to high precision and improved reproducibility across the eleven independent runs performed. The chosen 4-point curve was used in the accurate calculation and estimation of absolute quantification of *P. falciparum* 18S rRNA copies in the validation study samples.

4.3 Linearity and bias for liquid and DBS samples

Linearity which refers to the ability of an assay to produce results that are directly proportional to the concentration of the analyte within a given range. It is a key performance characteristic for quantitative assays, that ensure changes in signal correspond predictably to changes in target concentration. We subjected the cobas® *P. falciparum* 18S rRNA assay to linearity validation study. The assay demonstrated strong linear correlation for whole blood liquid samples, with an r^2 of 0.997, and DBS samples, with an r^2 of 0.991. These results confirmed a good proportional signal response over parasite densities which ranged from 1×10^8 to 1×10^2 parasites/mL.

Bias is the systematic difference between the measured value and the true value of an analyte. It reflects the degree to which the results deviate constantly in one direction and expressed as the mean difference between paired values. The examination of bias for our cobas® assay indicated a notable matrix effect when we used liquid calibrators on DBS samples. This showed a mean bias of +0.79 $\log_{10}$ parasites/mL that exceeded the ±0.55 $\log_{10}$ parasites/mL acceptable threshold limit. This indicated that DBS samples underestimated the parasite concentration relative to liquid samples.

We switched the approach, used DBS calibrators on DBS samples and liquid calibrators on liquid samples. The bias was –0.07 $\log_{10}$ parasites/mL and was not significant. The reduced bias indicated no significant quantification difference between liquid and DBS samples measurement. The cobas® assay with SPER buffer for elution requires matrix matched calibrators. This outcome indicated that same matrix-matched calibrators determined the quantification accuracy and validated the effectiveness of matched-matrix calibration approach in cobas® DBS samples workflow processing.

4.4 Analytical sensitivity (LoD)

The Limit of Detection (LoD) was defined as the lowest concentration of the *P. falciparum* 18S rRNA that was detected in ≥95% of the replicate samples tested using the cobas® 6800 system. Twenty replicate liquid blood and DBS samples with *P. falciparum* nominal concentrations ranging from 2.5×10^2, 1×10^2, 5×10^1, 2×10^1, to 1×10^1 parasites per mL were tested using cobas® *Plasmodium* 18S rRNA qRT-PCR assay. The analytical sensitivity studies demonstrated a LoD of 90 parasites/mL for DBS samples and 64 parasites/mL for whole blood liquid samples. Both sample format types achieved ≥95% detection at 100 parasites/mL density threshold, which indicated that the 50 µL blood sample volume achieved a stable detection capability at ≥100 parasite/mL density limit. The DBS samples performance had increased variability at lower densities, which confirmed its limitation for ultra-low parasitemia detection. Nonetheless, the LoD of ≥100 parasites/mL is adequate for diagnostic and surveillance context studies, especially in malaria endemic regions.

4.5 Precision

We aimed to determine the within-run (repeatability) and within-lab (reproducibility) precision of the DBS cobas® *P. falciparum* 18S rRNA qRT-PCR assay. Precision reflected the assay's ability to produce consistent results across repeated measurements under the same or varied conditions. Precision was evaluated across three parasite concentrations high (HI: 400,000 parasites/mL), mid (MID: 2,000 parasites/mL), and low (LO: 100 parasites/mL).

The cobas® assay demonstrated acceptable within-run and within-lab precision at HI and MID parasite densities, with %GCV ≤100% acceptable threshold limit. However, at LO parasites density the DBS samples yielded poor precision (%GCV > 200%) which exceeded the ≤100% acceptable limit and indicated high variability. This imprecision was due to somewhat mixed culture stages in the validation sample set. The parent culture sample contained a significant number of rings, trophozoites, schizonts and gametocytes that were seen in the Giemsa-stained smear microscopy.

4.6 Carryover contamination and assay workflow integrity

The aim of this experiment was to determine whether *P. falciparum* 18S rRNA copies from a previously highly positive concentrated sample contaminated the subsequent negative sample in an assay run. We were able to answer this question when we used the approach of interspersed distribution of high *P. falciparum* DBS samples with a concentration of $4x10^5$ parasites/mL and malaria negative DBS samples then tested using the modified cobas® *P. falciparum* 18S rRNA qRT-PCR assay. No evidence of carryover contamination was observed in any of the interspersed malaria negative DBS sample runs. With no false positive results among the tested negative

samples, this demonstrated the reliability of the cobas® automated processing system for high throughput.

4.7 DBS RNA stability under different storage conditions

The DBS sample matrix spotted with 50µL of blood was the subject of the stability assessment. Our aim was to examine the preservation of *P. falciparum* 18S rRNA in DBS samples stored for 1 and 6 weeks at 22-25°C, and -80°C. We tested forty DBS samples and twenty whole blood liquid samples from the 2 parasite densities, LO (100 parasites/mL) and MID (2000 parasites/mL), after the elapse of each storage time points. The assessment of the DBS samples stored at -80°C and the DBS samples stored at room temperature(22-25°C) showed that all the samples stored at 22-25°C and -80°C room retained the *P. falciparum* 18S rRNA integrity and the detection rate was 100% over 6 weeks for both LO and MID parasite densities. This indicated that reliable qualitative detection of *P. falciparum* RNA for DBS samples stored at 22-25°C or -80°C conditions. Supporting their use in decentralized sample collection and delayed testing.

4.8 Improving DBS RNA elution

In this study, the efficiency of *P. falciparum* RNA extraction from the DBS samples is a critical determinant feature of accuracy for the cobas® *P. falciparum* 18S rRNA qRT-PCR. The cobas® assay utilizes the specimen pre-extraction reagent (SPER) buffer, which has a guanidinium concentration of 2.68 M. The m2000Gen3.5 *P. falciparum* 18S rRNA qRT-PCR assay uses the NucliSENS buffer, which has a guanidinium concentration of 5 M. The difference in the RNA yield and quantification exhibited by the DBS samples which utilized the SPER buffer during processing reflects the disparity in lysis strength.

This difference instituted a systematic bias as shown in **Figure 11**. The DBS samples that were calibrated using liquid whole blood calibrators, showed consistent underestimation of *P. falciparum* quantification represented by a bias of 0.79 $\log_{10}$ parasites/mL. The under reported DBS results necessitated for change of approach, we switched to match the matrix using DBS calibrators to calibrate DBS samples which substantially reduced the bias to –0.07 $\log_{10}$ parasites/mL.

However, while matrix matching effectively corrected the DBS samples underestimation error, it did not tackle the root cause of inefficient RNA recovery due to suboptimal lysis conditions in the cobas® DBS protocol.

The lower guanidinium concentration in SPER buffer is the likely cause that led to incomplete lysis and suboptimal RNA release from the DBS samples. To resolve the weak strength of the SPER buffer, increasing the guanidinium thiocyanate concentration or supplementing it with additional chaotropic agents may improve RNA extraction efficiency for DBS samples. Future studies need to explore buffer modifications in parallel with matrix-matched calibrator development to minimize quantification bias and improve clinical utility of DBS sampling in molecular malaria diagnostics.

5 Conclusions

This method validation study aimed to demonstrate the successful adaptation of the modified FDA approved Roche cobas®6800 assay for the detection and quantification of *P. falciparum* 18S rRNA in low-volume (50 µL) whole blood and DBS samples. We evaluated standard curve, linearity, analytical sensitivity (LoD), precision, accuracy, carryover and dried blood spot stability. We found that the assay validation parameters demonstrated successful adaptation of the modified FDA approved Roche cobas® 6800 platform for the detection and quantification of *P. falciparum* 18S rRNA in 50 µL whole blood and DBS samples. Analytical sensitivity studies confirmed the assay's LoD at 64 parasites/mL for liquid whole blood and 90 parasites/mL for DBS samples, with both formats achieving ≥95% detection at the 100 parasites/mL threshold.

The DBS samples detection rates for moderate and high parasite densities were within the acceptable limit, while detection at the lowest tested densities fell below 95% which revealed limitations in sensitivity. Precision studies reinforced this, which indicated high reproducibility at high and mid parasite densities but significant imprecision at the low density (100 parasites/mL) for DBS samples. The variability was attributed to mixed-stage parasite composition in the cultured validation samples and likely compounded by inefficient RNA recovery from DBS samples.

The study showed a systematic quantification bias of +0.79 $\log_{10}$ parasites/mL for DBS samples when calibrated with liquid-based standards, that resulted in a consistent underestimation of parasite load. This was resolved by employing matrix-matched DBS calibrators, which effectively mitigated the quantification bias to –0.07 $\log_{10}$ parasites/mL, thereby validating the importance of calibrator/sample matrix alignment for quantitative accuracy.

There was no evidence of carryover contamination observed when interspersed high-density *P. falciparum* samples and malaria negative DBS samples were tested. In addition, DBS stability studies revealed that *P. falciparum* RNA was stable for at least six weeks under both room temperature (22-25°C) and –80°C storage, emphasizing the practicality of DBS samples for decentralized collection and delayed analysis without compromising RNA integrity.

A key insight from this study was the role of lysis buffer chemistry in RNA extraction efficiency from DBS samples. We identified suboptimal RNA recovery from DBS, likely due to the lower guanidinium concentration in the cobas® SPER buffer. Further optimization of the DBS sample workflow is required for to improve lysis by increasing the guanidinium concentration from that in the cobas® SPER buffer – this is expected to boost the RNA recovery from DBS samples.

Collectively, these findings support the modified FDA approved Roche cobas® 6800 malaria assay for 50 µL liquid blood samples but improvements are needed to achieve optimal results for 50 µL DBS samples.

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