OFFICE OF CLINICAL PHARMACOLOGY (OCP) AND OFFICE OF BIOTECHNOLOGY PRODUCTS (OBP) FULL BIOMARKER QUALIFICATION (BQ) PACKAGE REVIEW

Application	Qualification of *Plasmodium falciparum* 18S rRNA or rDNA, A type, as a monitoring biomarker to inform initiation of rescue treatment with an anti-malarial drug following controlled human malaria infection (CHMI) with *P. falciparum* sporozoites in healthy subjects from non-endemic areas enrolled in clinical studies for vaccine and drug development against *P. falciparum*.
Submission Date	March 10, 2017
Requestor	Sean C. Murphy, M.D., Ph.D.
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EXECUTIVE SUMMARY

Dr. Murphy submitted a biomarker qualification (BQ) package in support of *Plasmodium falciparum (P. falciparum)* 18s rRNA or rDNA as a biomarker to inform initiation of rescue treatment with an anti-malarial drug following controlled human malaria infection (CHMI) studies. To this aim, *P. falciparum* 18s rRNA/rDNA was measured in 3 studies using quantitative RealTime (RT)-PCR. Overall, it is the opinion of the reviewers that the analytical portion of requestor's package supports that P. falciparum 18s rRNA or rDNA can serve as a monitoring biomarker for CHMI studies as described in the FDA's proposed context of use (COU) below.

1 BACKGROUND

Controlled human malaria infection (CHMI) is a clinical research tool used to infect malaria naïve adults to anti-malarial drugs and vaccines. Human subjects are infected via direct venous inoculation, or through controlled malaria-infected mosquito bites. The current "gold-standard" for malaria infection diagnosis is the thick blood smear, a thick smear of whole blood, usually obtained from a finger prick, is spread across a glass slide followed by microscopic analysis to test for the presence of parasites. However, the desire for a more rapid, less subjective, and less time-consuming method of diagnosis has led to the development of nucleic acid tests. Polymerase chain reaction (PCR) assays are being explored as a more sensitive nucleic acid test, for earlier and more accurate detection for the presence of malarial nucleic acids in human whole blood samples.

A biomarker qualification (BQ) package was submitted to the FDA seeking to qualify the use of Plasmodium 18s rRNA/rDNA as a biomarker to replace thick blood smear (TBS) microscopy in CHMI studies conducted in non-endemic sites. The requestor proposed the following COU:

Detection of the *P. falciparum* 18S rRNA/rDNA is a safety (and efficacy) endpoint for initiating treatment before clinical malaria symptoms appear in subjects who have undergone P. falciparum sporozoite CHMI in non-endemic regions. The biomarker can be tested for at ≥6 days post-CHMI in human whole blood. The P. falciparum 18S rRNA/rDNA biomarker must have been measured with one or more specific nucleic acid amplification-based methods. This biomarker is intended to replace the use of thick blood smear (TBS) microscopy for this endpoint.

During the review, the FDA revised the biomarker and COU to the following:

Biomarker: *Plasmodium falciparum* 18s rRNA or rDNA, A type

COU: A monitoring biomarker to inform initiation of rescue treatment with an anti-malarial drug following controlled human malaria infection (CHMI) with *P. falciparum* sporozoites in healthy subjects from non-endemic areas enrolled in clinical studies for vaccine and drug development against *P. falciparum*.

2 SUBMISSION CONTENTS

The BQ package includes both analytical and clinical validation data to support the qualification request. This review only covers the analytical component of the submission.

Biomarker Detection Methodology: The assay is a lab-developed quantitative RT-PCR assay that detects *Plasmodium* A-type 18S rRNA and rDNA. This assay detects the *P. falciparum* and pan-*Plasmodium* 18S rRNA biomarker coupled with detection of human TATA-Binding Protein (TBP) mRNA as an endogenous internal control. TBP is a commonly used endogenous mRNA control marker that is of low-moderate abundance and serves as a suitable extraction and amplification control to ensure the ability of the assay to detect low amounts of *Plasmodium* template. The TBP mRNA target is more susceptible to degradation than the *Plasmodium* 18S rRNA template, which ensures that samples with intact TBP mRNA will also have intact *Plasmodium* 18S rRNA if present. The pan-*Plasmodium* channel is the primary channel with all quantification based on this channel. The assay reports the *P. falciparum* channel as positive, low positive, or negative.

The assayed sample consists of 50 µL of EDTA-anticoagulated whole blood stabilized in 2 mL of bioMérieux NucliSENS lysis buffer. This material can be freshly extracted or frozen and extracted later. Extraction is performed on the Abbott m2000sp followed by qRT-PCR on the Abbott m2000rt using Bioline's SensiFast One-Step RT-PCR kit and primers and hydrolysis (TaqMan) probes sourced from LCG Biosearch Technologies. The Abbott m2000 platform employs barcoded sample management from sample collection through automated extraction, amplification and reporting. Run controls consist of *P. falciparum* parasitized human erythrocytes in EDTA-anticoagulated whole human blood. Armored RNA (Asuragen) encoding the full-length *P. falciparum* 18S A-type rRNA is spiked into lysed whole blood matrix as an absolute calibrator.

The current version of the assay corresponds to its 3rd generation. All trials supporting this BQ submission were conducted with the validated 3rd generation assay except for study MC-003, which used the validated 2nd generation assay. A comparative study conducted by the

requestor showed that the test performance characteristics were comparable between the 2nd and 3rd generation assays. The latter, however, is improved to detect *P. falciparum* even in the presence of mixed species infection. From here after, this review will focus on the 3rd generation assay.

The University of Washington (UW) third generation Pan-*Plasmodium* primers and probe consist of:

Forward: PanDDT1043F19: 5'- AAAGTTAAGGGAGTGAAGA -3'

Reverse: PanDDT1197R22: 5'- AAGACTTTGATTTCTCATAAGG -3'

Probe: 5'-[CAL Fluor Orange 560]-ACCGTCGTAATCTTAACCATAAACTA[T(Black Hole Quencher-1)]GCCGACTAG-3'[Spacer C3]

The UW *P. falciparum* specific primers and probe consist of:

Forward: PfDDT1451F21: 5'- GCGAGTACACTATATTCTTAT -3'

Reverse: PfDDT1562R21: 5'- ATTATTAGTAGAACAGGGAAA -3'

Probe: 5'-[6-FAM]-ATTTATTCAGTAATCAAATTAGGAT-3'[Black Hole Quencher 1]

TBP primers and probe consist of:

TBP Forward Primer: 5'- GATAAGAGAGCCACGAACCAC -3'

TBP Reverse Primer: 5'- CAAGAACTTAGCTGGAAAACCC -3'

5'-[Quasar 670]- CACAGGAGCCAAGAGTGAAGAACAGT-3'[Black Hole Quencher-2 (BHQ-2)]

BHQ-2 chemistry where deoxycytosine (dC) and deoxyuridine (dU) are "propyne" moieties.

A custom lot of Armored RNA (Pf-Armored RNA) was purchased from Asuragen, cat# 49594. This material encodes the *P. falciparum* 18S RNA and provides absolute quantification in the 3rd generation assay. Absolute quantification is achieved using a standard curve of the Armored RNA (Pf-Armored RNA) diluted in malaria-negative whole blood.

Pf 18S (WAS-1) Target Sequence

5'AACCTGGTTGATCTTGCCAGTAGTCATATGCTTGTCTCAAAGATTAAGCCATGCAAGTGAAAGTATATATATATTTTA
TATGTAGAAACTGCGAACGGCTCATTAAAACAGTTATAGTCTACTTGACATTTTTATTATAAGGATAACTACGGAAAAG
CTGTAGCTAATACTTGCTTTATTATCCTTTGATTTTTATCTTTGGATAAGTATTTGTTAGGCCTTATAAGAAAAAAGTTAT
TAACTTAAGGAATTATAACAAAGAAGTAACACGTAATAAATTTATTTTATTTAGTGTGTATCAATCGAGTTTCTGACCT
ATCAGCTTTTGATGTTAGGGTATTGGCCTAACATGGCTATGACGGGTAACGGGGAATTAGAGTTCGATTCCGGAGAGGG
AGCCTGAGAAATAGCTACCACATCTAAGGAAGGCAGCAGGCGCGTAAATTACCCAATTCTAAAGAAGAGAGGTAGTG
ACAAGAAATAACAATGCAAGGCCAATTTTTGGTTTTGTAATTGGAATGGTGGGAATTTAAAACCTTCCCAGAGTAACAA
TTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCGTATATTAAAATTGTTGCAGTTAAAACG
CTCGTAGTTGAATTTCAAAGAATCGATATTTTATTGTAACTATTCTAGGGGAACTATTTTAGCTTTTCGCTTTAATACGC
TTCCTCTATTATTATGTTCTTTAAATAACAAAGATTCTTTTTAAAATCCCCACTTTTGCTTTTGCTTTTTTTTGGGGATTTTG
TTACTTTGAGTAAATTAGAGTGTTCAAAGCAAACAGTTAAAGCATTTACTGTGTTTGAATACTATAGCATGGAATAACA
AAATTGAACAAGCTAAAATTTTTTGTTCTTTTTTCTTATTTTGGCTTAGTTACGATTAATAGGAGTAGCTTGGGGACATT
CGTATTCAGATGTCAGAGGTGAAATTCTTAGATTTTCTGGAGACGAACAACTGCGAAAGCATTTGTCTAAAATACTTCC
ATTAATCAAGAACGAAAGTTAAGGGAGTGAAGACGATCAGATACCGTCGTAATCTTAACCATAAACTATGCCGACTAG
GTGTTGGATGAAAGTGTTAAAAATAAAAGTCATCTTTCGAGGTGACTTTTAGATTGCTTCCTTCAGTACCTTATGAGAA
ATCAAAGTCTTTGGGTTCTGGGGCGAGTATTCGCGCAAGCGAGAAAGTTAAAAGAATTGACGGAAGGGCACCACCAGG

CGTGGAGCTTGCGGCTTAATTTGACTCAACACGGGGAAACTCACTAGTTTAAGACAAGAGTAGGATTGACAGATTAAT
AGCTCTTTCTTGATTTCTTGGATGGTGATGCATGGCCGTTTTTAGTTCGTGAATATGATTTGTCTGGTTAATTCCGATAAC
GAACGAGATCTTAACCTGCTAATTAGCGGCGAGTACACTATATTCTTATTTGAAATTGAACATAGGTAACTATACATTT
ATTCAGTAATCAAATTAGGATATTTTTATTAAAATATCCTTTTCCCTGTTCTACTAATAATTTGTTTTTTACTCTATTTCTC
TCTTCTTTTAAGAATGTACTTGCTTGATTGAAAAGCTTCTTAGAGGAACATTGTGTGTCTAACACAAGGAAGTTTAAGG
CAACAACAGGTCTGTGATGTCCTTAGATGAACTAGGCTGCACGCGTGCTACACTGATATATATAACGAGTTTTTAAAAA
TATGCTTATATTTGTATCTTTGATGCTTATATTTTGCATACTTTTCCTCCGCCGAAAGGCGTAGGTAATCTTTATCAATAT
ATATCGTGATGGGGATAGATTATTGCAATTATTAATCTTGAACGAGGAATGCCTAGTAAGCATGATTCATCAGATTGTG
CTGACTACGTCCCTGCCCTTTGTACACACCGCCCGTCGCTCCTACCGATTGAAAGATATGATGAATTGTTTGGACAAGA
AAAATTGAATTATATTCTTTTTTTTCTGGAAAAACCGTAAATCCTATCTTTTAAAGGAAGGAGAAGTCGTAACAAGGTT
TCCGTAGGTGAACCTGCGGAAGGATCATTA-3’

The biomarker assay provides raw data in 18S rRNA copies/mL of whole blood. Ring-stage *P. falciparum* parasites have a stable concentration of 18S rRNA that allows conversion from copies/mL to estimated parasites/mL. To derive a conversion factor relating 18S rRNA copies/mL to estimated parasites/mL, archival EQA samples were assayed and copy numbers were generated using the Pf-Armored RNA standard curves. The assay estimated that there were $7.4x10^3$ 18S RNA copies per parasite (3.87 log10 18S rRNA copies per parasite; median 3.87 log10; 95% CI 3.79 – 3.94 log10; n=22 EQA-validated samples). Thus, to calculate the estimated parasite density /mL of whole blood in this assay, the 18S rRNA copy number/mL of lysate (equivalent to 50% of the nucleic acids from 50 µL of whole blood) is divided by 7400 (18S rRNA-to-parasite conversion) and multiplied by 40 (conversion to 1 mL of whole blood). The 18S rRNA-to-parasite conversion factor is platform and assay specific.

3 RESULTS

3.1 Is the requestor’s choice of a nucleic acid detection platform and study design reasonable and acceptable?

Yes, the requestor’s platform choice is acceptable. However, some concerns exist regarding the methodology and protocols for standard curve analysis and instrument calibration.

The requestor used the Abbott M2000 RealTime system which consists of two instruments: 1) the m2000sp, a fully automatic specimen nucleic acid extraction instrument for 96-well PCR plate preparation, and 2) an m2000rt, a fully automatic nucleic acid amplification and detection instrument.

The requestor’s submission states that the malarial RT-PCR assay approach is based on the Clinical & Laboratory Standards Institute guidelines (CLSI guidelines) and three FDA-approved Abbott m2000-based RT-PCR assays (HIV-1, HBV, and HCV) which use the same platform. To assess the proposed *Plasmodium* 18S rRNA RT-PCR assay, a comparison to these three assays was evaluated. These three FDA-approved Abbott m2000-based assays, which utilize stored calibration curves, have been rigorously validated in formal external quality assurance (EQA) programs and each assay is standardized against World Health Organization (WHO) standards. Each Abbott RealTime assay contains PCR specific reagents, a control kit consisting of both negative and positive PCR controls, and a calibration kit consisting of two calibrators. The Abbott m2000 System software processes sample preparation and amplification/detection protocols based on pre-determined, assay-specific parameters that are contained in individual assay application specification files that are installed on the SCC workstation. The three FDA approved tests mentioned above contain these assay-specific parameters and are run on the Abbott instrument in a close-mode application.

In contrast to the three FDA-approved Abbott m2000-based RT-PCR assays for HIV-1, HBV, and HCV, the requestor's *Plasmodium* 18S rRNA RT-PCR assay was designed as an open-mode protocol for performing laboratory-defined (user-validated) applications, and therefore, may contain some technical deficiencies due to the lack of a comparative formal EQA program and lack of calibrators which have been standardized against WHO standards.

The requestor's platform choice and approach are appropriate and have been properly validated by formal EQA validation using alternative FDA-approved RealTime assays. However, the requestor's *Plasmodium* 18S rRNA RT-PCR assay does not use a WHO reference material product as one is not currently available. Lack of a WHO standard means any quantifiable results derived from PCR results obtained in the requestor's studies may only be relevant to their intra-laboratory datasets based solely on their in-house reference materials using their platform and their calibrators. Copy numbers of the 18S RNA PCR target are only relative to studies using this same characterized reference material. Therefore, a more rigorous EQA validation of the performance of the Armored RNA material used as a reference standard in this BQ submission may be required for absolute quantification of true target copy numbers relative to parasite concentration. As stated by Murphy et al.[1], "a formalized funded program is needed to ensure assay validation and provide malaria NAT EQA in line with that commonly used for assays detecting HIV and other infectious pathogens." It is reasonable that a future WHO standard would be comparable to the approach in this BQ submission. However, more data would be needed to compare any future WHO standard reference material to the armored RNA control used in the current submission.

3.2 Are the proper quality control metrics for the proposed quantitative RT-PCR assay used for Plasmodium A-type detection captured and described in the requestor's submission?

Yes. In general, proper evaluation of qPCR assays require minimal technical documentation to support scientific claims and conclusions derived from PCR generated datasets. "Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE)" was modeled after other genomic initiatives coordinated under the Minimum Information for Biological and Biomedical Investigations (MBBI; http://www.mibbi.org). The MIQE guidelines describe the metrics and analytical methods required for minimal evaluation of real-time PCR experiments. A checklist for these parameters is provided for authors, reviewers, and editors in the MIQE publication.

Based on the MIQE Guidelines, no gross deficiencies were observed in the supporting evidence for technical evaluation of the 18S rRNA *Plasmodium* biomarker in this review. Most of the checklist items were fully met, although there are a few minor concerns over quantification that would benefit from further review. These concerns are described in more detail in the following sections 3.2.A (calibrators & dynamic range) and 3.2.B (LOD).

3.2.A Are the requestor's calibrators and dynamic range acceptable for standard curve analysis?

Yes. Although the requestor's dynamic range and number of calibrators are both insufficient to cover the intended range of the assay, this concern could be mitigated with a reasonable change to the method standard operating procedure (SOP) to decrease the variance of the data.

Each of the three FDA-approved assays mentioned in section 3.1 utilize two calibrators as specified in each of the approved assays and supply the slope and y-intercept validity criteria in their supporting documentation. The use of only two calibrators, as stated in the approved assays,

was qualified because primary calibrators are assigned lot specific target concentrations based on results of direct testing against the WHO standard, and product calibrators are assigned concentrations based on comparison to primary calibrators as stated in each of the package inserts. In contrast, three-point calibration curves were routinely performed in the requestor's *Plasmodium* 18S rRNA RT-PCR assay, using a custom-made reference material lacking WHO standardization spanning a narrow 2-log range of 5.3E7 to 5.3E5 nominal rRNA log10 copies/mL lysate. This range lacks coverage near the lower end of the concentration range, which could affect interpretation of copy number sensitivity near the LOD of the assay.

Since, the current assay for *Plasmodium* 18S rRNA has not been officially validated via a formal EQA program, and calibrators have not been standardized against WHO standards to assess the dynamic range of the assay, a wider dynamic range with more than three points is recommended to assess proper evaluation of concentrations at the lower end of the concentration range. To support this recommendation, FDA recommends that quantitative assays have between two and ten calibrators, as specified by the assay, to yield a log-linear calibration curve (cycle number vs. log of calibrator concentration), that quantitative calibration curves satisfy slope and y-intercept validity criteria specified for each assay, and that assays be recalibrated for each lot of assay reagents and as directed by assay reagent labeling. Based on these requirements, the calibration curves and dynamic range of the standard curves were insufficient for proper evaluation. For proper evaluation, accurate calculation of PCR efficiency depends on the range of template being used for the efficiency calculation. To properly evaluate PCR efficiency for future use of the *P. falciparum* 18S RNA biomarker, a minimum of 3-5 logs of template concentration are necessary, with a minimum of three replicates per concentration[2,3].

Concerns about the calibration curves in this submission were addressed by supporting evidence for comparison of 2-log, 3-log, and 4-log ranges. Of note, the sponsor's narrative regarding the log span covered by the calibration curves can be misleading. Calibration curves referenced as "3-, 4- and 5-log calibration curves" show 3-, 4-, and 5-log10 dilutions (points) of the standard, and correspond to only 2-, 3- and 4-log span or range, respectively. The reviewer will refer to the log range of the curve from hereafter in this report. Of the six calibrations described in the submission, two of the calibrations cover a 2-log range, one of the calibrations covers a 3-log range, and 3 of the calibrations cover a 4-log range (however, two of the 4-log range calibrations contain only one replicate per concentration). The Excel dataset submitted by the requestor contained minor worksheet errors that were corrected by the reviewer. The reviewer calculated standard deviations (SDs) differ slightly from those provided in the submission, but are not significantly different. Based on the reviewer-calculated data, the slopes (efficiencies), y-intercepts, and R^2 values appear to be within acceptable criteria limits, however, it is difficult to verify this analysis because of the requestor's spreadsheet calculation errors. Compared to the requestor submitted data which was not corrected for Excel errors (Figure 1A), a stronger trend upwards in the log parasite/mL concentrations is seen at each Ct interrogation (30, 26, 21) with increasingly wider calibration range, 2-log range towards 4-log range, based on the reviewer's analysis of the corrected dataset (Figure 1B). This indicates a potential bias or shift in the calibration data across different log ranges.

Figure 1A. Reviewer analysis of log parasite/mL concentrations at Ct 30, 26, and 21 (using requestor's uncorrected data).

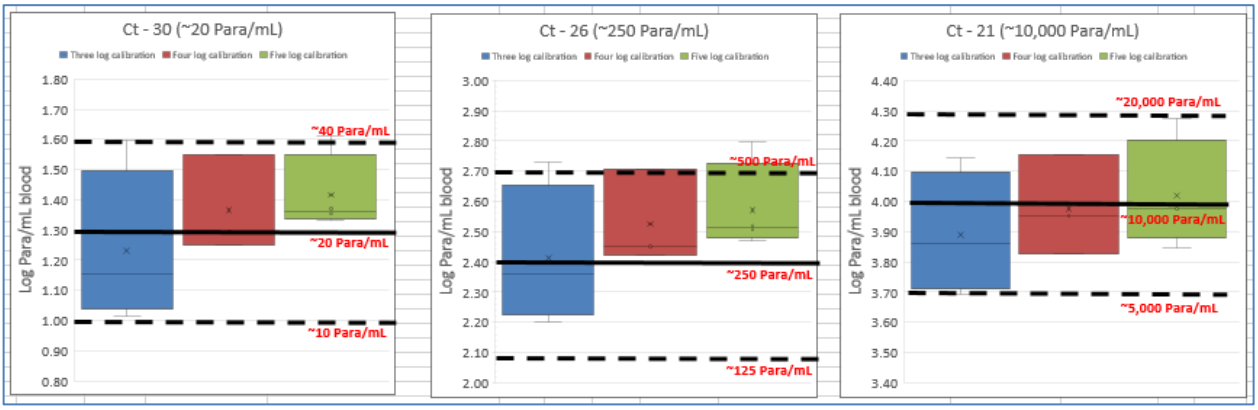
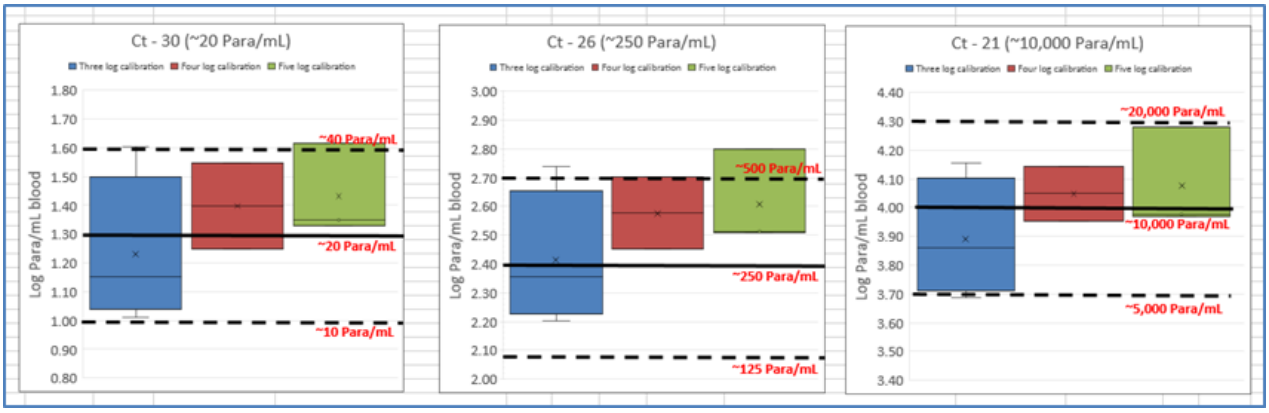


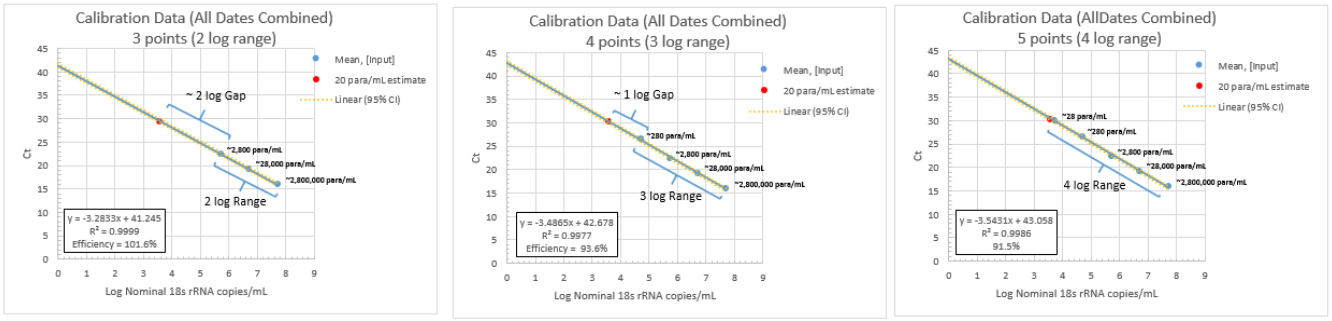
Figure 1B. Reviewer analysis of log parasite/mL concentrations at Ct 30, 26, and 21 (using requestor's corrected data).



Each box represents the requestor's measured parasite/mL concentration (~20, ~250, or ~10,000) output for each of the three evaluated Cts (30, 26, or 21). The solid black line represents the nominal input parasite/mL concentration. The dashed black lines depict a single doubling in each direction from the nominal input parasite/mL concentration at each Ct. Source: Reviewer generated plots based on *P. falciparum* 18S rRNA/rDNA BQ Package, Appendix 54 (uncorrected and reviewer corrected).

The reviewer also generated three Excel charts (Figure 2) using Ct data combined from the six separate calibrations dates to emphasize the differences between the three evaluated concentration ranges covering 2 logs, 3 logs, or 4 logs of input concentrations. The 3 point curves, spanning 2 log ranges, leave approximately a 2-log gap between the lowest evaluated concentration point and the LOD. Although each evaluation (2 log range, 3 log range, or 4 log range) was within acceptable limits of 90-110% efficiency, a decrease in efficiency and an increase in the y-intercept was observed as the dynamic range was increased to cover concentrations at the lower end of the assay, particularly as it approaches the proposed LOD (20 parasites/mL or 185 copies 18S rRNA/ mL). As such, extrapolation towards the lower end of the curve could potentially skew data for successive runs.

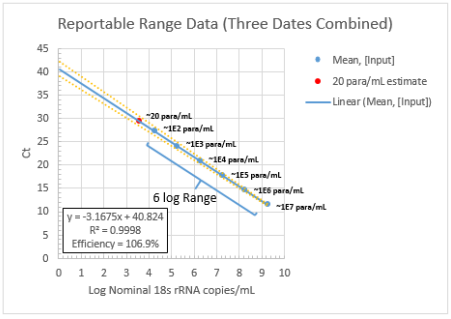
Figure 2. Reviewer analysis of Calibration Data (using requestor's data).



Source: Reviewer generated plots based on *P. falciparum* 18S rRNA/rDNA BQ Package, Appendices 14 and 54 (reviewer corrected).

To support the reviewer's recommendation for a wider standard curve and calibration range, the reviewer performed an analysis of the reportable range data supplied by the requestor (Figure 3). Reportable ranged data was generated by diluting 1E7 parasite/mL sample into whole blood to create a titration curve over the evaluated concentrations covering a six log range. Although, this data was derived from parasite/blood extractions and not derived from an Armored RNA titration, the data was combined from three separate dates, with each concentration point repeated in triplicate. A more accurate evaluation of the variance across the entire concentration range was determined from this titration, as indicated by the 95%confidence interval (95% CI). Metrics for slope, y-intercept, and efficiency were all within acceptable criteria limits, however, the fold change near the LOD of 20 parasite/ml (185 copies 18S rRNA/mL) was 4.3, slightly higher than the proposed cutoff criteria of 3.5 described in the BQ submission. In contrast, the requestor's calibration data had insufficient replicates to evaluate concentration points at the lower end of the concentration range, making 95% CI potentially misleading. In addition, only one of the six evaluated dates, 7/1/16, contained a set of triplicate samplings across the entire 4 log range.

Figure 3. Reviewer analysis of Reportable Range Data (using requestor's data).



Source: Reviewer generated plots based on *P. falciparum* 18S rRNA/rDNA BQ Package, Appendix 22.

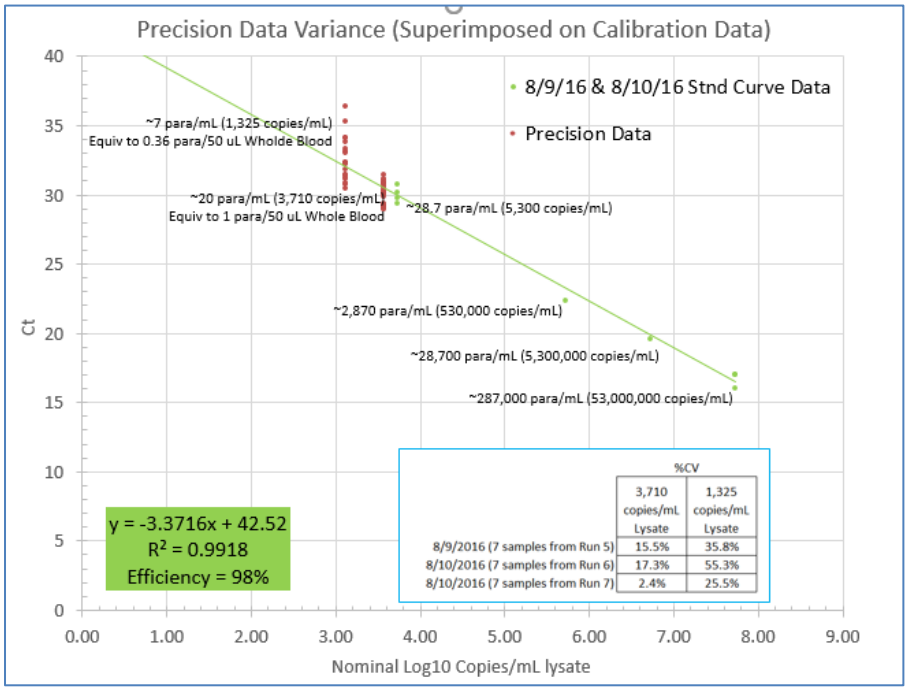
In summary, changes are recommended to the method SOP to decrease the variance of the data through the addition of one extra point on the calibration curve to four concentration points spanning a three-log range as proposed by the requestor. However, the addition of two extra points on the calibration curve, for a total of five concentrations points spanning a four-log range, would be even more suitable. Utilizing a four-log range with closer proximity to the proposed LOD of 20 parasites/mL (equivalent to approximately 5.3E3 copies 18s rRNA/mL), with a minimum replicate number of three per concentration point, may be necessary for proper evaluation of the calibration

curves until full cross validation of this reference material can be tested in a more formalized and documented manner.

3.2B Is the requestor's proposed LOD of 20 parasites per mL of lysate acceptable?

Yes. Although the calibration data was weak due to insufficient replicates (as noted earlier in this review), it is the opinion of the reviewers that the data supports a LOD of 20 parasites/mL in this assay. To evaluate the requestor's LOD of 20 parasites per mL of whole blood lysate, the reviewer analyzed a combination of precision data and calibration data collected by the requestor. These data were provided from sample runs on two dates, 8/9/16 & 8/10/16. Figure 4 shows the variance around the two concentrations evaluated by the requestor to support this LOD. The 7 parasite/mL concentration point, equivalent to 0.36 parasites per mL of lysate, is too variable and unacceptable for use in LOQ determination. This same determination was concluded by the requestor's analysis for LOD.

Figure 4. Reviewer analysis of Precision Data superimposed on Calibration Data (using requestor's Pf-Armored RNA data).



The reviewer's calculated coefficient of variation (%CV) and 95% CI for the LOD of 20 parasites per mL of lysate, equivalent to one parasite in 50 µL of whole blood lysate (approximately 3,710 copies of 18S rRNA based on the requestor's conversion factor), can be seen in Figure 4.

4 SUMMARY AND CONCLUSIONS

It is the opinion of the reviewers that the requestor's platform choice and approach are appropriate and have been properly validated by formal EQA validation using alternative FDA-approved RealTime assays. Based on the MIQE Guidelines, no gross deficiencies were observed in the supporting evidence for technical evaluation of the 18S rRNA *P. falciparum* biomarker in this review. Most of the checklist items were fully met, although there are a few minor concerns over quantification that would benefit from further review. These concerns include: calibration methodology, dynamic range of the standard curve, lack of a WHO recognized standard, and LOD analysis. The requestor's approach to instrument calibration was suboptimal, due to a narrow dynamic range of the titrated Armored RNA reference material, which couldn't be calibrated against a known standard. In addition to the narrow dynamic range, the number of replicates used to support the calibration curves was also suboptimal. LOD analysis to support a concentration of 20 parasites per mL was justified using the requestor's data, assuming support of the requestor's conversion factor, but only for their specific instrument, assay, and lot specific reference material for use within their intra-lab setting. Copy number claims and extrapolated Ct measurements cannot be generalized for other platforms or assays, as noted by the requestor.

5 RECOMMENDATIONS

Based on the data provided, the analytical aspects of this BQ package support qualification of *Plasmodium* 18s rRNA/rDNA as a monitoring biomarker to replace thick blood smear (TBS) microscopy in controlled human malaria infection (CHMI) studies conducted in non-endemic sites.

Minor technical assay concerns were highlighted in this review regarding the methods for generating calibration curves and standard curves for the quantitative PCR (qPCR) assay, particularly with the dynamic range and number of replicates at each interrogated input concentration. Due to the lack of a WHO recognized standard, the proposed approach of using an intra-lab reference material of Armored RNA is acceptable. However, a wider dynamic range to cover a minimum of 4-5 logs and triplicate interrogation at each concentration point is recommended for future studies and calibration runs until a standardized calibrator is available.

REFERENCES

1) Murphy SC, Hermsen CC, Douglas AD, Edwards NJ, Petersen I, Fahle GA, Adams M, Berry AA, Billman ZP, Gilbert SC, Laurens MB, Leroy O, Lyke KE, Plowe CV, Seilie AM, Strauss KA, Teelen K, Hill AV, Sauerwein RW. External quality assurance of malaria nucleic acid testing for clinical trials and eradication surveillance. PLoS One. 2014 May 16;9(5):e97398. PMID: 24838112.

2) Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL, et al. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem. 2009 Apr; 55(4):611-22. Epub 2009 Feb 26. PMID: 19246619.

3) Applied Biosystems APPLICATION NOTE Real-Time PCR: Understanding C_T. 05/2008 Publication 136AP01-01.