QUALIFICATION DECISION AND EXECUTIVE SUMMARY

The FDA is issuing this Qualification Decision and Executive Summary to Dr. Sean Murphy of the Department of Laboratory Medicine at the University of Washington [herein referenced as "Submitter"], in response to your biomarker Full Qualification Package submitted to the Center for Drug Evaluation and Research (CDER) Biomarker Qualification Program (BQP). We have completed our review of your Full Qualification Package[1] submission, and have concluded to **Qualify** this biomarker panel for the Context of Use (COU) as described below. In addition, this document includes a summary of the discipline-specific reviews and recommendations by the members of the Biomarker Qualification Review Team (BQRT):

- Reviews from Division of Anti-Infective Products/CDER, Division of Vaccines and Related Products Applications/CBER and Office of Biostatistics/CDER as well as a consolidated review
- Consolidated Review from Office of Clinical Pharmacology/CDER and Office of Biotechnology Products/CDER
- Analytics Review from Division of Microbiology Devices/CDRH

This biomarker qualification represents the current thinking of the Food and Drug Administration (FDA or Agency) on this topic. This biomarker can be used by drug developers for the qualified COU in submissions of investigational new drug applications (INDs), new drug applications (NDAs), and biologics license applications (BLAs) without the need to resubmit the biomarker information or rereview by the relevant CDER disciplines.

Biomarker Description

Plasmodium falciparum 18S rRNA/rDNA (copies/mL) measured in blood samples by a nucleic acid amplification test assay

[1] Since the Full Qualification Package was submitted and under review prior to the enactment of the 21st Century Cures legislative 507 process, this qualification determination was made following the legacy process.

Context of Use (COU)

"A monitoring biomarker, that when positive, informs initiation of treatment with an anti-malarial drug ≥6 days following controlled human malaria infection (CHMI) with *P. falciparum* sporozoites in healthy subjects (18-50 years old) from non-endemic areas enrolled in clinical studies for vaccine and/or drug development."

The following section lists considerations when using the biomarker for this COU:

General Considerations

- This biomarker is qualified for use in monitoring subjects in CHMI studies to inform initiation of treatment with anti-malarial drug.
- Studies utilizing this biomarker should apply inclusion/exclusion criteria sufficient to protect subject safety. Moreover, due to the potential for false-positive findings (see "Analytical Validation" below), which may result from cross-reactivity, analytical interference, or other factors, the following additional exclusion criteria should be added:
    - Subjects from endemic areas or those who have recently travelled to regions where *Babesia* infection is known to be endemic.
    - Subjects with leukocytosis.
- All subjects should be biomarker negative prior to challenge with *P. falciparum*.
- The biomarker is not cleared or approved for diagnosis of malaria. Patients who test negative should not be assumed to be malaria-free, and study protocols should take appropriate precautions to ensure that false negative findings do not lead study subjects to forgo necessary treatment.
- Informed consent forms should indicate that CHMI subjects will be treated with an appropriate anti-malarial drug, immediately upon reaching the protocol-defined treatment threshold of biomarker positive and/or developing signs and symptoms of malaria. However, if the protocol defined threshold is not achieved, anti-malarial therapy will be administered at the conclusion of the trial.

Background

In CHMI studies, healthy volunteers from non-endemic regions are infected with *P. falciparum*, usually via mosquito bites or intravenous inoculation of sporozoites, before or after administration of novel drugs or vaccines intended for treatment of infection or prophylaxis. To ensure the safety of CHMI study volunteers, those who manifest symptoms and/or Thick Blood Smear (TBS)-positivity are typically treated. Subjects who do not show signs of illness or any TBS positivity are treated upon conclusion of the study.

Microscopic detection of parasites by TBS is the current standard for diagnosing *Plasmodium* infection in CHMI trials. Volunteers that have contracted *Plasmodium* through CHMI generally become TBS-positive 10-12 days after infection if no vaccine or therapy has been administered (e.g., control arm of a CHMI study). While there have been no reports of patient deaths in a CHMI trial, CHMI subjects may suffer from malaria symptoms and run the risk of potentially serious (grades 3-5) adverse events. Biomarkers that can identify infection earlier will improve healthy volunteer safety by allowing earlier initiation of anti-malarial treatment that may reduce the likelihood of clinical symptoms of infection and grades 3-5 adverse events.

Sources of Data and Major Findings

The Submitter has provided analytical, nonclinical and clinical data to support the qualification of *P. falciparum* 18S rRNA/rDNA measured by a TaqMan reverse transcription (RT)-polymerase chain reaction (PCR) assay as a biomarker to complement TBS microscopy in CHMI studies. To support the proposed context of use, the submission included post-hoc analyses of data from three CHMI studies. These studies were all conducted in healthy malaria-naïve adults from non-endemic sites, with infection achieved either by subjecting participants to the bites of 5 infected mosquitoes or by direct intravenous inoculation of 3200 cryopreserved sporozoites of *P. falciparum* NF54 strain. The submission also references 26 clinical studies in 28 published papers that in aggregate support the proposed context of use.

Analytical Considerations

Assay Description

The 3rd generation assay submitted here is used to detect the biomarker in blood samples. The assay is a single-step real time quantitative reverse transcription polymerase chain reaction (real time qRT-PCR) that uses multiplexed reagents and an open version of the Abbott m2000 system to target a *P. falciparum*-specific region of the asexual-stage 18S rRNA/rDNA as well as a pan-*Plasmodium* genus-specific region of the asexual 18S rRNA/rDNA. A multiplexed control primer/probe set also targets the human endogenous TATA-Box binding protein (TBP) mRNA as a low copy number endogenous internal control. Samples consist of 50 µL of EDTA-anticoagulated whole blood stabilized in 2 mL of bioMérieux NucliSENS lysis buffer. A standard curve generated from *P. falciparum* 18S RNA-encoded Armored RNA diluted in malaria-negative whole blood is used to calculate RNA copies/mL.

The 2nd generation assay, which was used in one of the clinical studies presented here (MC-003), differs in design in several ways. Notably, it does not contain pan-*Plasmodium* primers and can only detect *P. falciparum.* For both the 2nd and 3rd generation assays, an estimation of parasites/mL of blood can be calculated from biomarkers/mL of blood using the conversion factors 3500 and 7400 respectively. The accuracy of estimated parasites/mL of blood has not been established. Clinical decisions should be based on any biomarker positive findings.

Assay Threshold

This biomarker is qualified for use with any positive cycle threshold (Ct) value indicating a positive test result. However, if any specific threshold value in the context of a particular study under an IND involving CHMI needs to be established, the information supporting the proposed thresholds would then be reviewed by FDA as part of an IND application for the specific therapeutic product being studied. Using detection of a positive Ct to determine when to initiate anti-malarial treatment requires that enrolled subjects are biomarker negative at the beginning of the study.

A clinically relevant assay threshold based on parasite density cannot be reliably estimated from quantitative biomarker measurements (i.e., biomarker copies/mL). It is expected that a single parasite will contain multiple copies of rRNA/rDNA, and that rRNA/rDNA from the dead/lysed parasites will contribute to overall biomarker levels. There is also greater experimental uncertainty in measuring low biomarker levels. Use of the biomarker assay for detection rather than quantitation is recommended. The expression of results as biomarker copies/mL rather than estimated parasites/mL, will allow future CHMI studies to test the validity of protocol-defined thresholds.

Assay Validation

The description and validation of the 3rd generation assay used to detect *P. falciparum* 18S rRNA/rDNA followed the "Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE)" guidelines (www.mbbi.org), with no gross deficiencies observed. Data supporting the analytic performance of the 3rd generation assay was generated from parasite-containing whole-blood samples (10 parasites/mL – $4x10^7$ parasites/mL). The following performance characteristics were determined:

a. Standard curve. A standard curve was calculated using *P. falciparum* (Pf)-Armored RNA standards at three concentrations. Standard (calibration) curves were within limits but had decreases in efficiency and increases in the y intercept as the curve approached the Limit of Detection (LoD). The differences in LoD and precision between the calibrators and whole parasite samples suggests that Pf-Armored RNA may perform differently in the assay than clinical samples. However, because calibration was performed with an insufficient number of calibrators and lack of coverage near the LoD, including additional calibrators at low concentrations is recommended.

b. Analytical sensitivity. An LoD study was conducted using *Plasmodium*-infected whole blood (10 – 250 parasites/mL) from previously-quantified clinical samples. An LoD of 20 estimated parasites/mL ($1.48x10^5$ biomarker copies/mL), with a 'low positive' range of any value between 10-20 estimated parasites/mL ($7.4x10^4$ - $1.48x10^5$ biomarker copies/mL) to account for variations in 18S rRNA content in parasites, was based on a 95% detection rate at 50 and 20 estimated parasites/mL. However, in a separate study

using External Quality Assessment (EQA) samples[2], the pan-*Plasmodium* channel only detected 81% of samples at 60 nominal parasites/mL. While there is insufficient data from the EQA study to calculate an LoD, the LoD could be more credibly set at 100 nominal parasites/mL because 100% of the samples were detected at this higher concentration in the EQA study. This does not affect qualification, however, as the clinical significance of this difference is not known.

c. Correlation. Correlation between nominal and observed estimated parasites/mL were determined by testing parasite-containing whole blood samples (high, medium, and low levels) in duplicate over 20 runs with different operators. $R^2 = 0.99$ for both *P. falciparum* and pan-*Plasmodium* primers. No false positives or false negatives were detected (n = 106), and low, medium, and high parasitemia samples were correctly categorized. Using a Bland-Altman plot, average bias = 0.03 $\log_{10}$ estimated parasites/mL for *P. falciparum* primers and 0.11 $\log_{10}$ estimated parasites/mL for pan-*Plasmodium* primers.

d. Precision. Intra-assay (within-run or repeatability) and inter-assay (between-run or reproducibility) precision was determined using samples covering a range of parasite concentrations. For both *P. falciparum* and pan-*Plasmodium*, the %CV at all concentrations met acceptance criteria for %CV of <10% and < 15% for intra-assay and inter-assay precision, respectively.

e. Reference interval. The reference interval of the healthy, uninfected population is "not detected".

f. Analytical specificity.
   i. Inclusivity. When spiking pre-extracted DNA from different *Plasmodium* species (*P. falciparum, P. vivax, P. malariae, P. knowlesi,* and *P. ovale)* into extracted malaria-negative eluates, *P. falciparum* primers detect only *P. falciparum* and pan-*Plasmodium* primers detect all species. Additionally, clinical specimens containing intact *P. vivax* RNA and samples containing two *Plasmodium* rodent strains tested negative with *P. falciparum* primers and positive with pan-*Plasmodium* primers.
   ii. Exclusivity. *Babesia* is a potential source of false positives, as pan-*Plasmodium* but not *P. falciparum* primers yield signal from *B. microti* approximately 10 cycle higher Ct values later compared to *B. microti*-specific primers. Positive results may also result from *P. falciparum* sexual stage gametoyctes and sporozoites, as 18S RNA from both was detected with both pan-*Plasmodium* and Pf primers.

[2] Murphy SC et al. (2014) *External quality assurance of malaria nucleic acid testing for clinical trials and eradication surveillance.* PLoS One **9**(5):e97398.

iii. Analytical interferences. Very high leukocyte counts may interfere with the assay. At <$25x10^9$ white blood cells (WBC)/L, the pan-*Plasmodium* channel was 0.12 log_{10} estimated parasites/mL less than controls; at >$25x10^9$ WBC/L, this channel was 0.34 log_{10} estimated parasites/mL less than controls. No interference was observed due to hemolysis, lipemia, bilirubinemia, and heparinized plasma.

g. Reportable range. The reportable range is from the LoD (see "Analytical Sensitivity" above) to 1 x 10^7 estimated parasites/mL. Higher parasite concentrations should be diluted before testing.

h. Analyte stability. Aliquots of an infected clinical whole blood sample were stored at 4°C or room temperature (24°C) for 24 – 96 hours. Values from both channels showed statistically significant decreases at 96 hours at 4°C when compared to samples processed immediately, while those from the PF channel also showed statistically significant decreases at 48 and 72 hours at 24°C. All individual aliquots showed decreases within acceptance criteria (< 0.55 log_{10} copies/mL decrease from baseline) up to 48 hours at 4°C and 24 hours at 24°C. Samples lysed before storage were within acceptance criteria up to 96 hours at either temperature. These studies indicate that samples should be processed into lysis buffer within 48 hours of collection if stored at 4°C and 24 hours after collection if stored at room temperature. Lysed samples can be stored at 4°C or room temperature for up to 96 hours. Whole blood samples may be frozen, but should not undergo repeated freeze-thaws.

i. Carryover. No carryover was detected after interspersing high target (n = 207) and negative samples (n = 105) in the same run, as no negative samples were misidentified as positive.

j. Comparison of 2nd and 3rd Generation Assays: A comparison of 2nd and 3rd generation assays was conducted. EQA samples were run repeatedly on both 2nd and 3rd generation assays. The 3rd generation assay yielded similar but higher qualitative point estimates in samples at lower nominal parasite concentrations.

Table 7. Comparison of second- and third-generation assay detection of low parasite densities

Nominal Para/mL	Gen. 3 assay			Gen. 2 assay	
	# of replicates	*P. falciparum* # detected (%)	Pan *Plasmodium* # detected (%)	# of replicates	*P. falciparum* # detected (%)
600	18	18 (100%)	18 (100%)	20	20 (100%)
60	21	17 (81%)	17 (81%)	15	11 (73%)
6	21	3 (14%)	3 (14%)	15	1 (6%)

Plasmodium falciparum 18S rRNA/rDNA was evaluated using both the 2nd and 3rd generation assays, but the studies were not intended to provide a complete comparison of these two

assays. Agreement between assays should be calculated based on biomarker copy/mL particularly given questions about the validity of the conversion factors.

Clinical Summary

Literature

The submitter analyzed 26 clinical studies in 28 published papers, most from established CHMI sites, to support the qualification of the biomarker. In total, these studies enrolled 761 subjects, of which 686 became TBS-positive; 395 naïve subjects at non-endemic sites from 22 studies had CHMI induced by mosquito bite and 144 naïve subjects by Pf sporozoite (SPZ) parasite injection. The analysis indicated that biomarker time-to-positivity (TTP) preceded TBS TTP by 0.9 – 7.1 days, depending on the study. For previously vaccinated subjects at non-endemic sites, 11 studies examined a total of 315 subjects. 288 subjects were challenged by mosquito bites, and 27 by PfSPZ. Both the biomarker and TBS TTP were delayed, but the biomarker was still detected 1.2 – 3.1 days earlier, depending on the study.

The Submitter noted that the assays used in the published studies are different from the assays used by the Submitter. The details provided on the assays that were used in the published studies were insufficient for review.

Submitted studies

Three clinical studies were submitted to support qualification of the biomarkers.

1. MC-001 clinical study: a single-center, open-label Phase 1 trial to demonstrate mosquito bite CHMI under an IND and obtain immunological endpoints after a single exposure by mosquito bites or sporozoite inoculation. This study enrolled 6 subjects in the infectivity control group. Biomarker and TBS were monitored, and subjects treated with an anti-malarial drug upon becoming TBS positive. The biomarker was detected using the 3rd generation RT-PCR assay for sample testing.

2. MC-003 ITV clinical study: a single center, randomized, partially double-blinded, placebo-controlled Phase 1 trial of Infection-Treatment-Vaccination (ITV) using chloroquine (CQ) with or without primaquine (PQ) under an IND. There were six arms, with 6 subjects enrolled in the infectivity control arms and 23 subjects in the vaccinated arms. The biomarker was detected using the 2nd generation RT-PCR assay. 26 subjects became TBS-positive, and 25 of these became biomarker positive. There is no explanation for the appearance of a TBS-positive, biomarker-negative findings in one subject.

3. NIH LMIV PfSPZ-Cvac PYR clinical study: a single center Phase 1 study of ITV consisting of wild-type, aseptic, purified, cryopreserved 3200 *P. falciparum* sporozoites (Sanaria® PfSPZ Challenge) administered by DVI in conjunction with CQ and pyrimethamine (PYR) under an IND. This study had 4 arms, and enrolled 5 subjects in the infectivity control group and 16 subjects in the vaccinated group. The biomarker was detected using the 3rd generation RT-PCR assay for sample testing. 15 subjects were PCR-positive; 6 of these were also TBS-positive, while the remaining 9 did not become TBS positive (these 9 were treated based on a study-performed PCR positivity alone – it is noted that the PCR assay used was different from the 2nd or 3rd generation RT-PCR assay used for the biomarker detection).

Endpoints

The endpoints measured in the clinical studies were:

1. TBS: positive/negative; time to positivity (TTP); estimated parasite density.

2. *Plasmodium* 18S rRNA/rDNA biomarker: positive/negative; TTP; estimated parasite density at first positive and at TBS positive. The biomarker positivity was based on positive results in the pan-*Plasmodium* channel, by the 3rd generation assay, in Studies MC-001 and PfSPZ-CVac PYR. In Study MC-003 ITV, biomarker positivity was based on positive results in the *P. falciparum* channel by the 2nd generation assay.

3. Malaria-related symptoms: Time to any or Grade 2 symptom; number of Grade 3 symptoms. Malaria-related clinical symptoms included fever, chills, headache, nausea, vomiting, generalized malaise, myalgia, arthralgia, diarrhea, abdominal, lower back pain, arthralgia, and/or chest pain.

Study Results and Conclusions

Comparisons of TTP based on TBS and biomarker findings were made against each other as well as against time to any malaria-related symptom in infectivity control and vaccinated subjects. Results are summarized in Tables 1 and 2.

Table 1. Clinical study data comparing TBS, biomarker by RT-PCR, and appearance of malaria-related symptoms.

		TBS		RT-PCR*		Malaria-related symptoms	
	N	**# positive**	**TTP# Mean (95% CI)**	**# positive**	**TTP Mean (95% CI)**	**# positive**	**Time to event Mean (95% CI)**
MC-001 DEMO							
Infectivity control	6	6	11.2 days (9.5, 12.8)	6	7.7 days (6.4, 8.9)	6	9.7 days (6.4, 13.0)
MC-003 ITV							
Infectivity controls	6	6	8.7 days (7.6, 9.8)	6	7.0 days (7.0, 7.0)	6	8.0 days (6.7, 9.3)
Vaccinated subjects*	23	20	11.0 days (10.0, 11.9)	19	7.3 days (6.8, 7.8)	22	9.5 days (8.6, 10.5)
PfSPZ-CVAC PYR							
TBS-positive subjects only	6	6	13.5 days (11.0, 16.0)	6	9.2 days (6.1, 12.2)	4	12.8 days (10.4, 15.1)
TBS- and/or PCR-positive subjects	15	0	12.0 days*** (10.8, 13.2)	15	8.6 days (7.6, 9.6)	10	11.3 days (9.6, 13.0)
Infectivity control	5	2	12.0 days (12.0, 12.0)	5	7.6 days (6.8, 8.4)	4	10.3 days (7.3, 13.2)
All studies combined							
TBS-positive subjects	38	38	11.03 days (9.71, 15.85)	37	7.62 days (6.07, 9.17)	38	9.66 days (7.27, 12.05)

TTP = time to positivity.

* RT-PCR positivity based on any Ct or copies/mL positive finding.

**combination of all trial arms except infectivity controls.

***protocol-defined diagnosis (TBS+ and/or study-performed RT-PCR^{+}).

PQ = primaquine; CQ = chloroquine.

Table 2. Comparison of times to TBS- and biomarker by RT-PCR-positivity.

	Mean difference between TBS and RT-PCR (95% CI)	Mean difference between 1st malaria symptoms and RT-PCR (95% CI)	Mean difference between 1st malaria symptoms and TBS (95% CI)
MC-001 DEMO			
Infectivity control	3.5 days (2.6, 4.4)	2.0 days (-0.8, 4.8)	1.5 days (-0.7, 3.7)
MC-003 ITV			
Infectivity control	1.7 days (0.6, 2.8)	1.0 days (-0.3, 2.3)	0.7 days (-0.8, 2.1)
Vaccinated group	3.6 days (2.9, 4.4)	2.1 days (1.2, 3.0)	1.4 days (0.4, 2.4)
NIH LMIV PfSPZ-Cvac PYR			
TBS-positive subjects only	4.3 days (3.1, 5.6)	4.8 days (3.2, 6.3)	0.0 days* (-2.3, 2.3)
TBS- and/or PCR positive subjects	3.4 days** (2.7, 4.1)		
Infectivity control			

*4/6 TBS-positive subjects developed malaria-related symptoms.

**difference between protocol-defined diagnosis (TBS+ and/or PCR^+) and PCR^+ only.

Qualification is supported by the findings that the biomarker is consistently identified at about 7 days post-CHMI and appears approximately 2 – 4 days before TBS-positivity and appearance of malaria symptoms. Analysis of time to positivity of the three clinical studies described indicate that use of the biomarker will accelerate detection of malaria positivity by approximately two days, eliminate Grade 3 symptoms, and reduce the number of Grade 3 adverse events.

Considerations

There is sufficient evidence supporting the analytical performance of the 3rd generation biomarker assay as a qualitative test for the purposes of qualifying the biomarker for the COU. However, several considerations should be noted, and guide future assay development.

- *Plasmodium* 18S rRNA/rDNA is not a diagnostic biomarker. The assay used to detect *Plasmodium* 18S rRNA/rDNA in the full qualification package is not a diagnostic test for presence of malaria.

- Due to the potential for false-positive findings, which may result from cross-reactivity, analytical interference, or other factors, the following additional exclusion criteria should be added:
    - Subjects from endemic areas or those who have recently travelled to regions where *Babesia* infection is known to be endemic.
    - Subjects with leukocytosis.

- This biomarker is not qualified for other CHMI studies such as those in subjects challenged with erythrocytic stage of the *P. falciparum* parasites or non-CHMI studies (e.g., field studies).

- Biomarker measurement in blood is not a substitute for TBS for studies other than the context of use discussed above i.e., for identifying infection to inform initiation of anti-malarial drug in CHMI studies (infection induced by sporozoites).

- Biomarker measurements should be reported in biomarker copies/mL of blood.

- In the absence of a WHO standard, calibration curves should cover a minimum of 3-5 logs with a minimum of 3 duplications per concentration, but preferably 5, and one sample close to the LoD. Additionally, a standard curve should be included each time clinical specimens are tested. Development of a WHO standard for *Plasmodium* 18S rRNA/rDNA is encouraged.

- Because the clinically relevant levels or changes in levels of biomarker that define the medical decision point in CHMI are unknown, it is not possible to designate a maximum allowable error for the test.

- Any Ct value should be considered a positive assay result indicating *P. falciparum* infection.

- False positives may lead to the treatment of subjects who have the biomarker but would not have otherwise developed malaria symptoms. False negative might lead to subjects not receiving necessary treatment. Because TBS and/or the appearance of malaria symptoms are accepted endpoints in CHMI, risks due to failure to detect the biomarker when present will be mitigated by observation of either. For this reason, biomarker negative subjects should be monitored throughout the course of a CHMI trial for TBS-positivity and emergence of malaria-like symptoms.

- It is noted that the sample size in each individual study was small and in some cases not powered for establishing statistical significance. It is understood that sample size is generally small in exploratory studies in early phase drug or vaccine development. More

data collection on the use of the biomarker is encouraged to accumulate data evidence as a tool for drug or vaccine development.

- While the COU here applies to the qualitative use of the biomarker, future uses may include quantitation of parasite levels. To inform the development of the biomarker for quantitation, we note that it is difficult to establish how accurately biomarker quantitation estimates blood parasite densities below the LoD of TBS, as technical and biological limitations will become an increasing concern as parasite concentrations decrease.