Drug Development Tool (DDT) Tracking number: DDTBMQ000044

Submission: Drug Development Tool Biomarker Qualification Proposal, received by Center for Drug Evaluation and Research (CDER)

DDT name: *Plasmodium falciparum* 18S ribosomal (r)RNA/rDNA A type

Context of use:
The submitter proposed the following context of use:

> 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 controlled human malaria infection (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.

The context of use was modified during Biomarker Qualification Review Team discussions as follows:

> 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*.

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Executive Summary

The Submitter has provided analytical, nonclinical and clinical data to support the qualification of *Plasmodium* 18S rRNA/rDNA, measured by the TaqMan reverse transcription (RT)-polymerase chain reaction (PCR) assay, as a biomarker to replace thick blood smear (TBS) microscopy in controlled human malaria infection (CHMI) studies conducted in healthy volunteers from non-endemic sites. The subjects were challenged by either the bites of 5 infected mosquitoes or by direct intravenous inoculation of 3200 cryopreserved sporozoites of the NF54 strain of *P. falciparum*. The analytical performance of the assay as well as the nonclinical and clinical studies support the qualification of the biomarker. However, the context of use (COU) should be as a monitoring biomarker to inform initiation of rescue treatment with an anti-malarial drug following CHMI.

The Submitter proposed ≥250 estimated parasites/mL as a threshold for positive findings to initiate rescue treatment. However, the estimated parasite density was <250 estimated parasites/mL in a majority of the subjects; all subjects became TBS positive and developed signs and symptoms of malaria. Therefore, rescue treatment based on a threshold of ≥250 estimated parasites/mL, could delay treatment compared to any positive findings by the RT-PCR assay, although, this would still be earlier than time to TBS positive and development of signs and symptoms of malaria. It is recommended that timing for rescue treatment should be based on any positive findings by the RT-PCR assay as

- all subjects should be biomarker negative prior to challenge with *P. falciparum*,
- there is minimal chance of false-positive findings, and
- an improved sensitivity of the RT-PCR assay, compared to TBS, can facilitate initiating anti-malarial therapy before the onset of clinical signs and symptoms of malaria in the CHMI studies; this will also help reduce malaria-related symptoms.

Recommendations and Comments

The *P. falciparum* 18S ribosomal (r)RNA/rDNA A type should be qualified as a monitoring biomarker to inform initiation of rescue treatment with an anti-malarial drug following CHMI with *P. falciparum*.

Based on the information reviewed, changes recommended for the COU, previously discussed by the group (additions double underlined and deletions striked out) and recommendations for the detection of the biomarker by the 3rd generation RT-PCR assay performed at the University of Washington in future CHMI studies are as follows:

COU: A monitoring biomarker to inform initiation of rescue treatment with an anti-malarial drug following CHMI with *P. falciparum* sporozoites in healthy subjects from non-endemic areas enrolled in CHMI studies for vaccine and/or drug development ~~against *P. falciparum*~~.

Note: Monitoring for the *P. falciparum* 18S rRNA/rDNA biomarker should be initiated on Day 6 post-CHMI. Rescue treatment with an anti-malarial drug following CHMI should be based on any positive findings by the 18S rRNA/rDNA 3rd generation assay and the results expressed as number of copies/mL whole blood.

Additional comments:

Use of a calibration/standard curve: A standard curve was tested every 6 months or with each new reagent lot at three concentrations ($5.3x10^7$, $5.3x10^6$ and $5.3x10^5$ copies/mL). In future CHMI studies, appropriate dilutions for testing and establishing a standard curve should be included each time clinical specimens are tested. Also, concentrations <$5.3x10^5$ copies/mL should be tested. This will help in minimizing the variability.

Inclusion and Exclusion Criteria: Overall, the inclusion and exclusion criteria are appropriate. Due to a possibility of false-positive findings, either due to cross- reactivity or analytical interference, the following should be added to the exclusion criteria:

- Subjects from endemic areas or those who have recently travelled to regions where *Babesia* infection is known to be endemic.
- Subjects with leukocytosis.

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1. Introduction and Background

Conventional microscopy using blood smears is the established method for morphological identification of the *Plasmodium* parasite(s) and for measuring efficacy of drug or vaccine in treatment and/or prophylaxis clinical trials in subjects living in or travelling to endemic areas as well as controlled human malaria infection (CHMI) studies in healthy volunteers. Sensitivity of microscopic findings based on thick blood smears (TBS) is known to be low (~20 to 50 parasites/µL; range 3-100/µL).

Several investigators have reported an improvement in the sensitivity of parasite detection using different nucleic acid based tests such as polymerase chain reaction (PCR) for detecting parasite DNA and reverse transcription (RT)-PCR for detecting RNA. The RT allows a single strand of RNA to be translated into a complementary strand of DNA. Once that reaction occurs, the routine PCR method is then used to amplify the DNA. Any DNA in the extracted nucleic acids will also be amplified and detected in the RT-PCR assay.

An improved sensitivity of molecular tests compared to TBS can facilitate initiating anti-malarial therapy before the onset of clinical signs and symptoms of malaria; this will also help reduce incidence and severity of malaria-related symptoms. Molecular tests are less-operator dependent compared to TBS. However, none of the molecular assays have been cleared by the Center for Devices and Radiological Health (CDRH), FDA, to aid in the diagnosis of malaria.

The Submitter's proposed context of use for the CHMI studies is

> "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."

The testing was performed in the Submitter's laboratory at the University of Washington (UW). The TaqMan-based RT-PCR multiplex assay was used for measuring plasmodial (Pan) and *P. falciparum* specific 18S rRNA/rDNA. The detection of 18S rRNA/rDNA biomarker from *Plasmodium* species other than *P. falciparum*, in CHMI studies or any field trials (treatment or prophylaxis) conducted in subjects living in or travelling to endemic areas is beyond the scope of the proposed context of use (COU).

The Submitter has included studies supporting the analytical performance of the UW RT-PCR assay as well as results of nonclinical and clinical studies to qualify the *Plasmodium* 18S rRNA/rDNA as a biomarker to replace TBS microscopy in CHMI studies. The CHMI studies are performed in healthy volunteers living in non-endemic areas, who have not travelled to endemic areas, and are without any history of malaria infection or vaccination. The healthy subjects are likely to be negative for DNA or RNA of the *Plasmodium* parasites prior to challenge.

2. Analytical Validation

The studies supporting the analytical validation of the assay were reviewed by Dr Noel Gerald, CDRH and Dr Barry Rosenzweig, Office of Translational Sciences, CDER. Briefly, *P. falciparum*-specific and pan-*Plasmodium*,18S rRNA/rDNA was measured by RT-PCR and testing was performed at the UW. Multiplexed reagents and open version of the Abbott m2000 system were used. The sample consisted 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 (for details see review by Dr Rosenzweig). Run controls consisted 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 was spiked into lysed whole blood matrix as an absolute calibrator and standard curve plotted every 6 months or if new lot of reagents were used.

The assay performed at the UW has evolved over time; the clinical specimens were tested by either the 2nd or 3rd generation assays. The results of the 2nd and the 3rd generation assays were based on the *P. falciparum* and pan-*Plasmodium* channels, respectively. Some of the other differences between the 2nd generation and 3rd generation assays include differences in primers used, internal control, thermocycling program, the conversion factor used for converting number of copies/mL to estimated parasite equivalent/mL. Conversion factor for the 2nd generation assay was 3500 whereas for the 3rd generation assay was 7400. The differences in the conversion factor are due to the nucleic acid recovery and mastermix performance as these are platform and assay specific.

The UW RT-PCR assay is more sensitive than TBS. At an estimated parasite density of 20 parasites/mL, ≥95% of samples (20 per level) generated a positive cycle threshold (Ct) value by the UW assay. The Submitter calculated limit of detection (LoD) to be 20 estimated parasites/mL (1 estimated parasite/50 µL) of blood or $1.48x10^5$ rRNA copies/mL of blood. Since the 18S rRNA content of parasites may vary, the 'low positive' range was extended to any value ≥10 to <20 estimated parasites/mL. Experimental data provided in the submission suggests that the LoD could be between 60 and 100 estimated parasites/mL (for details see review by Dr Gerald). The linearity of the reportable range was demonstrated between the $1x10^2$ and $1x10^7$ parasites/mL.

Plasmodium parasites predominantly express 'A-type' 18S rRNA during asexual stages and 'S-type' 18S rRNA during sexual stages. However, the 18S rRNA was detected by the 3rd generation assay, in both the *P. falciparum* and pan-*Plasmodium* channels, in cultured sporozoite, erythrocyte (asexual) and gametocyte stages of *P. falciparum*. Other *Plasmodium* species known to infect humans as well as rodents can be detected in the pan-*Plasmodium* channel.

All malaria-negative samples tested (n=105) were negative. There was no cross-reactivity with any of the viruses (CMV, EBV, HIV-1, and HIV-2) or *Trypanosoma* species (*T. brucei rhodesiense*, *T. b. gambiense*, *T. b. brucei*, and *T. cruzi*) tested. However, *Babesia microti* tested positive in the pan-*Plasmodium* channel and negative in the *P. falciparum* channel by the RT-PCR assay. Although binding of *Babesia* to pan-*Plasmodium* specific primers was weaker compared to *Plasmodium* targets, a potential for false-positive results due to *Babesia* infection cannot be ruled out. *B. microti* infection is known to be endemic in upper North-Eastern and Central states of the USA. In Europe, *B. divergens* is the predominant species; a few cases of *B. microti* and *B. venatorum* infections also have been reported in Europe. Cross-reactivity with any of the bacterial or fungal species was not evaluated.

Analytical interference due to leukocytosis was reported. The Submitter states that the 3rd generation assay SOP contains a disclaimer about samples with very high leukocyte counts. However, no such disclaimer was found in the SOP.

The stability of the 18S rRNA/rDNA was optimal in whole blood samples when processed into lysis buffer within 48 hours of collection. If storage is required for more than 12 hours, storage at 4°C is recommended.

Comments:
The performance of the 3rd generation RT-PCR UW assay, supports the appropriateness of the detection of P. falciparum 18S rRNA/rDNA as a biomarker for administering rescue treatment in subjects enrolled in the CHMI studies. Although Plasmodium parasites predominantly express 'A-type' 18S rRNA during asexual stages and 'S-Type' 18S rRNA during sexual stages, the 18S rRNA was detectable by the 3rd generation UW assay in both the P. falciparum and pan-Plasmodium channels, in cultured sporozoite, erythrocyte and gametocyte stages of P. falciparum. Other Plasmodium species known to infect humans as well as rodents can be detected in the pan-Plasmodium channel.

There was a lot of variability in the assay. Also, there is a possibility of false positive findings due to cross-reactivity with B. microti and analytical interference due to high leukocyte count. Some considerations for the detection of 18S rRNA/rDNA as a biomarker, by the UW RT-PCR assay, for future CHMI studies are as follows:

Use of a calibration/standard curve:
A standard curve was included for testing every 6 months or if new lot of reagents were used. This could potentially lead to ~3-fold difference in quantitative results between calibrations (for details see review by Dr. Rosenzweig). Also, testing of the calibrator was limited to the middle range ($5.3x10^7$, $5.3x10^6$ and $5.3x10^5$ copies/mL of lysate). For future CHMI studies, a standard curve should be included each time clinical specimens are tested. Also, concentrations lower than $5.3x10^5$ copies/mL, should be included for testing.

Cross-reactivity (False positivity):
- *There is a potential for false-positive results due to cross-reactivity with Babesia. Therefore, subjects with a possibility of Babesia exposure (i.e., subjects from areas known to be endemic for Babesia or have travelled to endemic areas) should not be enrolled in the CHMI studies, especially when the assessment for development of infection is to be based on detection of rRNA/rDNA by the UW 3rd generation RT-PCR assay.*

- *Analytical interference due to leukocytosis was reported; the Submitter states that a disclaimer statement is included in the SOP. However, such a statement was not found in the SOP. Also, this should be part of the exclusion criteria.*

***Expression of results**:*
The results of the UW RT-PCR 3rd generation assay should be expressed as copies/mL and not estimated parasites/mL (for details see review by Dr Gerald).

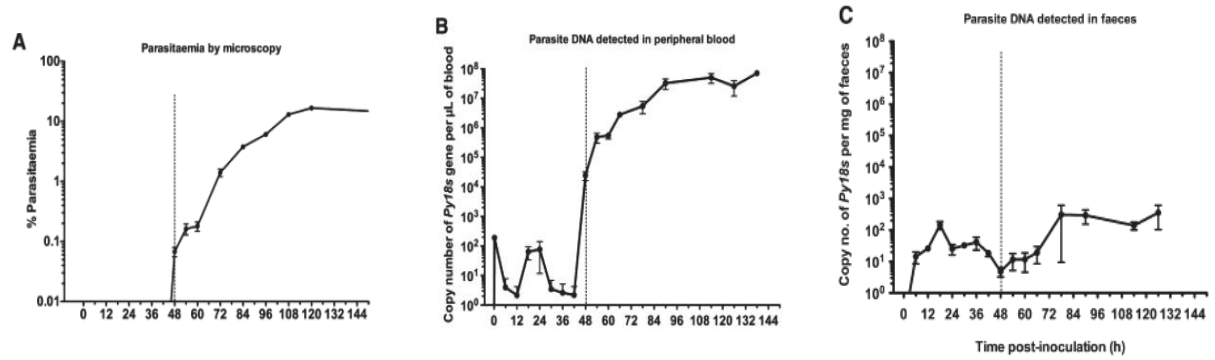
3. Nonclinical Studies

The Submitter has included 3 nonclinical studies to address the question whether or not the sporozoite derived biomarker persists in blood following CHMI and could cause false positive results in CHMI studies.

3.1. *Plasmodium yoelli* (7X1.1pp strain) infection with sporozoites in CBA mice

Abkallo *et al* (2014)[1] reported the presence of ~10^2 copies of rDNA/µL of blood (collected from the tail vein) by qPCR, within 10 minutes of challenge with 2.5x10^4 sporozoites, inoculated IV in the tail vein. Although, this was followed by a decrease in the number of copies by 96 hours, two peaks of rDNA were observed at 6 hours and 12-24 hours post-challenge during the pre-erythrocytic phase (developmental phase in liver) before emergence of the erythrocytic parasites in blood; ~10^2 copies/µL rDNA was detected (Figure 1). There was an increase in the detection of rDNA (≥10^4 copies/µL) between Days 2 and 6 post-challenge (Figure 1). Similar observations were made in fecal samples; rDNA was detectable in feces, throughout the parasite's developmental phase in the liver. The Submitter states that the presence of rDNA at 6, 12, and 24 hours is possibly due to the small total blood volume (~6.3 mL) in mice as well as the presence of residual sporozoites and/or DNA in the blood from tail vein, as the route of challenge was through the same site. However, this could not explain the presence of rDNA in feces during the pre-erythrocytic phase within 24 hours of challenge. rRNA was not measured.

Figure 1: *P. yoelii* parasites by TBS and 18S rDNA by qPCR in the tail vein blood and feces of CBA mice 0-144 h (0-6 days) post-challenge



10 µL of blood was sampled, by capillary action from the tail vein, from each mouse 10 min before inoculation, 10 min after inoculation, every 6 h for the following 60 h, every 12 h for the next 48 h, and then every 24 h for the next 5 days.

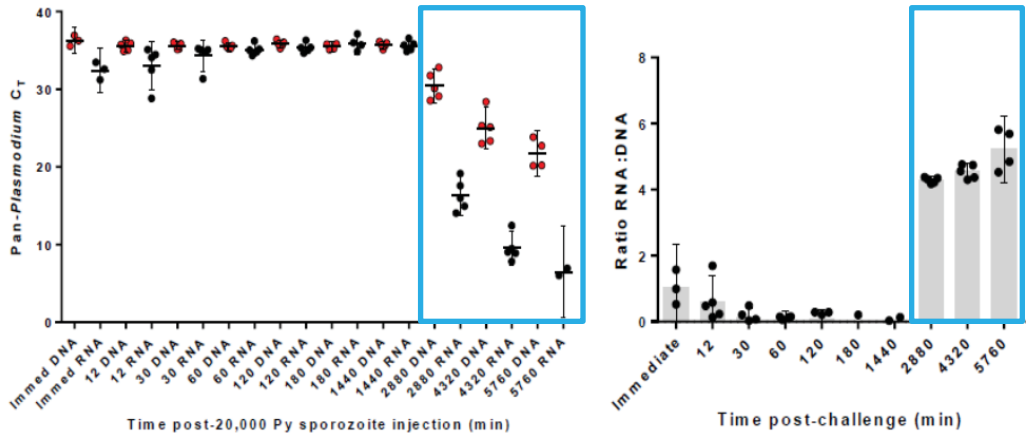
3.2. *Plasmodium yoelii* (17XNL) infection with sporozoites in BALB/c mice

The Submitter reported absence of rRNA in cardiac blood of mice at 24 hours post-challenge by IV (retro-orbital vein) route, with 2x10^4 sporozoites. Testing was done by the 3rd generation assay using pan-*Plasmodium* and mouse reagents.

In another experiment, mice were challenged with 10^4 sporozoites and blood collected for rRNA and rDNA testing in blood (~5-10 µL) from the tail vein by capillary wicking onto dried blood spot cards that were laser cut. The experimental design more closely mimicked the Abkallo study summarized above in Section 3.1. The results show that 18S rRNA/rDNA was detected by RT-PCR in blood immediately after challenge (e.g., 12 and 30 minutes post-challenge at >100 copies/µL blood). At 48 hours post-challenge, the 18S rRNA:rDNA ratio increased to >10,000, which is consistent with the start of erythrocyte stage infection (Figure 2). The Submitter hypothesized that biomarker detected at early time points reflects residual non-viable material, or may be simple contamination from the surrounding tail vein injection site itself. Please note that there is a possibility of cross-reactivity among the different stages of the *Plasmodium* parasites, as was reported in the *in vitro* studies.

[1] Abkallo HM, Liu W, Hokama S, Ferreira PE, Nakazawa S, Maeno Y, Quang NT, Kobayashi N, Kaneko O, Huffman MA, Kawai S, Marchand RP, Carter R, Hahn BH, and Culleton R. DNA from pre-erythrocytic stage malaria parasites is detectable by PCR in the faeces and blood of hosts. *Int J Parasitol* (2014) 44 (7): 467-473.

Figure 2: *P. yoelii* 18S rRNA vs. rDNA biomarker in BALB/c mice during 0-4 days post-challenge



Left: Whole blood was collected onto dried blood spots at the indicated minutes post-challenge, preserved in lysis buffer and extracted for total nucleic acids. qPCR or qRT-PCR was performed and the CTs are plotted. Red circles: qPCR (18S rDNA), Black circles: qRT-PCR (18S rRNA/rDNA). Right: Using the assay standard curve, the ratio of 18S rRNA:18S rDNA was calculated for each time point for each mouse. The shift from low ratio to a high ratio at 2880 minutes (24 hr) corresponds to onset of the erythrocyte stage.
The 18S rRNA:18S rDNA ratio immediately upon injection was set arbitrarily as 1.0. By 60 minutes, the 18S rRNA:18S rDNA biomarker ratio diminished to <1 and was maintained at this low 18S rRNA:18S rDNA ratio through 24 hr post-challenge
Represent 48, 72 and 96 hours

3.3. *Plasmodium falciparum* infection in non-human primates

Infection with *P. falciparum* sporozoites leads to establishment of liver stage parasites in rhesus macaques; however, blood-stage infection has not been reported. The Submitter reported absence of rRNA/rDNA in the blood of rhesus macaques, on Days 3 and 6 post-challenge with $6.5x10^6$ sporozoites of *P. falciparum*; detection of rRNA/rDNA was performed by the 2nd generation *P. falciparum* qRT-PCR assay. The rRNA/rDNA was detected in the liver on Days 3 (range 480 to $2.7x10^5$ copies/g of tissue; n=2) and 6 (range $1.7x10^7$ to $1.2x10^9$ copies/g of tissue; n=2). Spleen and lung, collected at the time of necropsy, on Days 3 and 6, were biomarker negative. Tissue samples from uninfected animals were negative. Overall, the study suggests that the biomarker is not detected in the blood at Days 3 and 6 post-challenge. Liver stage parasites tested positive by the assay.

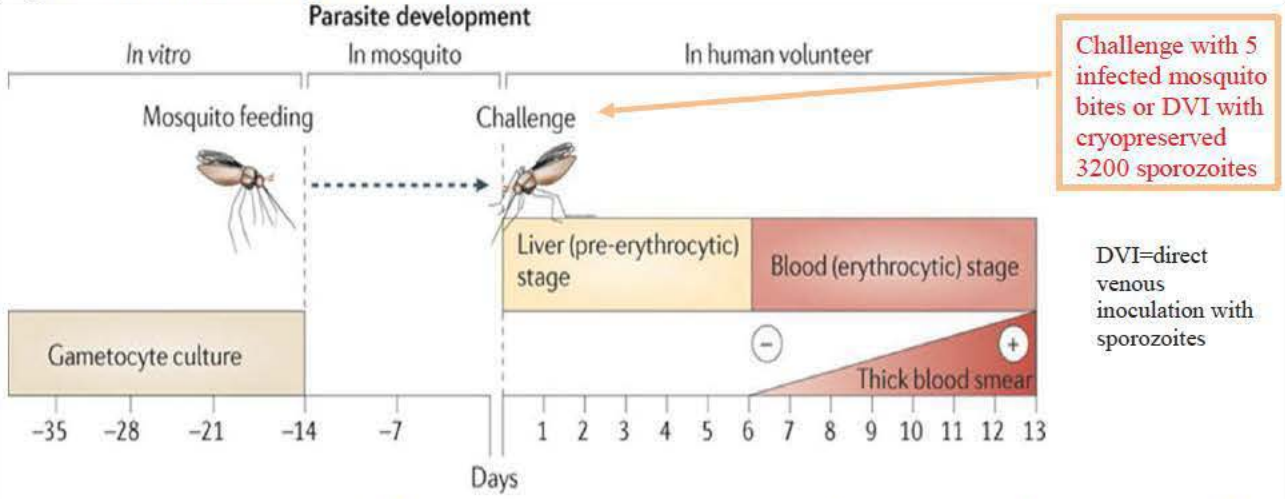
Comments:
In one published study in mice infected with P. yoelli, rDNA was reported in the blood and fecal samples collected at different time intervals after initiation of infection that includes pre-erythrocytic phase. The Submitter did similar experiments in mice and reported the presence of rRNA/rDNA by the 3rd generation assay in the tail vein. However, rRNA/rDNA was not detected in the cardiac blood. The Submitter hypothesized that the presence of rRNA/rDNA in blood is possibly due to the small blood volume (~6.3 mL) in mice and the presence of residual sporozoites and/or residual rRNA/rDNA in the blood from tail vein as the route of challenge was through the same site. However, this does not explain similar observations in fecal samples. In nonhuman primates with large blood volumes, rRNA/rDNA was not detected in peripheral blood until Day 6. Liver tested positive for rRNA/rDNA thereby suggesting that the UW assay detects all stages of the Plasmodium parasites. It is known that in humans erythrocytic stage parasites do not emerge until about Day 6 post-sporozoite inoculation. Therefore, use of the UW 3rd generation assay appears to be appropriate for the detection of the biomarker from Day 6 onwards, in the CHMI studies.

4. Clinical Studies

The protocols and results of four CHMI studies (MC-001, MC-003 ITV, PfSPZ-CVac PYR, and GAP3KO) were included. Testing for the biomarker was performed by the RT-PCR assay at the UW using stored whole blood samples. Additionally, the Submitter refers to 26 published studies to support the qualification of the biomarker. It is noted that the assays used in the published studies are different from the assay used by the Submitter. The details of the assays from these published studies were insufficient for review.

This review focuses on 3 studies (MC-001, MC-003 ITV, and PfSPZ-CVac PYR) where the UW assay was used and subjects were challenged with sporozoites of the N54 strain of *P. falciparum* by either infected mosquito bites (Studies MC-001 and MC-003 ITV) or direct IV inoculation (DVI; Study PfSPZ-CVac PYR) (Figure 3). The fourth CHMI study (MC-004 GAP3KO) tested administration of a genetically-attenuated sporozoites by mosquito bites and was not a wild-type CHMI; the study is briefly summarized to address the possible presence of sporozoite-derived biomarker in peripheral blood prior to patent parasitemia i.e., erythrocytic-stage infection.

Figure 3: The *Plasmodium* sporozoite CHMI model



Anopheles mosquitoes acquire *P. falciparum* gametocytes by membrane feeding on *in vitro* cultures and are reared under GMP-qualified conditions until salivary gland sporozoites develop. Eligible subjects receive the designated number of infected bites on Day 0 and remain asymptomatic during the pre-erythrocytic stage. In lieu of mosquito bites, direct venous injection of cryopreserved parasites is also possible on Day 0. On or after Day 6, parasites emerge from hepatocytes and initiate cyclical RBC infections that culminate in eventual detection by biomarker assay or TBS.

4.1. Studies MC-001, MC-003 ITV and PfSPZ-CVac PYR

Study design:

All the three studies were conducted in healthy volunteers and had an infectivity control group as outlined in Figure 3. In Studies MC-003 ITV and PfSPZ-CVac PYR, in addition to the infectivity control group, a group of subjects that were vaccinated/immunized [Infection-Treatment-Vaccination (ITV)] prior to challenge were included (see Table 1 and Appendix-1). All studies were conducted under an IND. Briefly, the ITV phase consisted of experimental infection with wild-type (non-attenuated) NF54 strain of *P. falciparum* sporozoites by the bites of 12-15 infected *Anopheles stephensi* mosquitoes (Study MC-003 ITV) or DVI with 51,200 sporozoites (Study PfSPZ-CVac PYR) in conjunction with causal prophylaxis with antimalarial drugs (chloroquine and/or primaquine in Study MC-003 ITV; chloroquine and/or pyrimethamine in the study PfSPZ-CVac PYR) or placebo. Primaquine treatment was timed to eliminate

parasites early in the liver stage of development, after hepatocyte invasion but prior to maturation and release into the bloodstream. Chloroquine and/or pyrimethamine treatment were administered to provide suppressive prophylaxis to prevent development of parasitemia and clinical malaria.

For details on inclusion and exclusion enrollment criteria, see Appendix-2.

Subjects were evaluated at different time intervals for parasites in the blood by TBS and RT-PCR as well as for clinical response (for details on schedule of events see Appendix-3). Trigger for rescue treatment post-CHMI, in studies MC-001 and MC-003 ITV, was based on TBS positive findings (at least 2 parasites per slide that was confirmed by a second reader). However, for Study PfSPZ-CVac PYR, a single positive peripheral TBS with two parasites or two positive real time NIH qPCR results on separate days post-CHMI were used for diagnosis and clinical decisions to initiate rescue treatment. The anti-malarial drugs administered for rescue treatment were chloroquine or Malarone as the N54 strain of *P. falciparum* is known to be sensitive to these drugs.

The endpoints measured in the clinical studies were:

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

- ***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.

- **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.

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. Evaluation of the performance of biomarker during the vaccination phase, i.e., prior to CHMI, in the two studies, is beyond the scope of this review.

Table 1: Clinical studies - Summary of study design

	MC-001 (n=6)	**MC-003 ITV (n=29)**	**PfSPZ-CVac PYR (n=21)**
Comparison of study design			
Open label (IND no.)	Yes (14224)	Partial double-blind - Subjects in the PQ Pilot Phase (Arms 1a and 1b) and subjects enrolled as infectivity controls (Arm 5) were unblinded throughout the study (14752)	Not specified (16650)
Study site	Seattle Biomed (now CIDR)	Seattle Biomed (now CIDR)	NIH
Route of infection	5 to 6 infected mosquito bites	5 infected mosquito bites	3,200 cryopreserved sporozoites, IV
Infectivity control group	Yes (n=6)	Yes (n=6)	Yes (n=5)
Vaccinated subjects	None	Yes* (n=23)	Yes** (n=16)
TBS and RT-PCR testing	Twice daily[1]	Twice daily[1]	Once daily
Out-patient (telephone) follow-up time	6-56 days (Up to 6 months)	Day 6 to 35 post-challenge (Up to 6 months)***	Days 6-21, 23, 25 and 29 post-CHMI***
Hoteling phase post-CHMI	Day 9-18	Day 8 to 21	None
Trigger for rescue treatment	TBS	TBS	TBS or 2 NIH PCR positive****
Number of subjects administered rescue treatment (CQ or Malarone) upon TBS^{+VE}	6	26	15
RT-PCR assay			
Type of assay used	3rd generation	2nd generation	3rd generation#
Conversion factor	7400	3500	7400

*Study MC-003 ITV: There were 5 groups in addition to infectivity control group; 5 subjects were not vaccinated (bitten by uninfected mosquitoes) but administered CQ (for details see Appendix-1).
**PfSPZ-CVac PYR: There were 3 groups in addition to infectivity control group (for details see Appendix-1).
***CHMI conducted on Day 147 i.e., 91 days after the final vaccination.
****A single positive peripheral TBS with two parasites OR two positive real time NIH qPCR results on separate days post-CHMI were used for diagnosis and clinical decisions to initiate curative treatment.
#Testing performed on separate aliquots of archived samples
DVI-Direct venous inoculation; CIDR- Center for Infectious Disease Research; TTP- Time to positivity
[1]Once daily results included in the submission
PQ=primaquine; CQ=chloroquine
For more details on Study design see Appendix-1

Results:

Infectivity control group:

The results show that all the subjects in the infectivity control group in Studies MC-001 and MC-003 ITV were TBS positive between Days 7 and 14 post-CHMI; all the subjects were biomarker positive between Days 7 and 10 post-CHMI and developed ≥1 signs/symptoms of malaria (Table 2 and Figure 4. However, only 2 of the 5 infectivity control subjects, in Study PfSPZ-CVac PYR, were TBS positive; 4 subjects were biomarker positive and developed signs and symptoms of malaria (Table 2). A low positive rate by TBS in Study PfSPZ-CVac is probably due to administration of rescue treatment based on two positive findings by the NIH real-time qPCR assay that detects *Plasmodium* 18S DNA.

Drug control group:
In Study MC-003-ITV, a group of drug control subjects (n=5) were exposed to **uninfected mosquito bites** and administered chloroquine and primaquine during the immunization phase prior to CHMI. All the 5 non-immunized/vaccinated subjects were TBS and RT-PCR positive; time to positivity was similar to that in the infectivity control group (Table 2).

Vaccinated group:
In vaccinated subjects, compared to the infectivity control group, there appears to be a trend towards a delay in TTP by both TBS and UW RT-PCR in both Studies MC-003 ITV and PfSPZ-CVac (Table 2). For subjects in Study PfSPZ-CVac, there appears to be a trend towards decrease in parasite density post-CHMI in vaccinated subjects compared to the infectivity control group; however, a similar trend was not apparent for Study MC-003 ITV. It is unclear if differences between the vaccinated subjects in the two studies are due to different endpoints used for administering rescue antimalarial therapy or other study design issues. As stated above, a single positive peripheral TBS with two parasites or two positive real time NIH qPCR results on separate days post-CHMI were used for diagnosis and clinical decisions to initiate rescue treatment in Study PfSPZ-CVac, whereas in Study MC-003 ITV, rescue treatment was based on TBS findings only.

TBS positive RT-PCR negative discordant results:
There was only one vaccinated subject in Study MC-003 (Patient ID (b) (6) in the primaquine/chloroquine group) that was TBS positive on Day 11 (parasite density 5000/mL) but remained PCR negative for the duration of the study; Grade 1 malaise and nausea were reported on Days 12 and 14, respectively. The reason(s) for PCR negative results in a TBS positive subject are not known.

For statistical analysis of study results please see the Statistics review by Dr Sue-Jane Wang.

Overall, the results of the three studies suggest that detection of 18S rRNA/rDNA is more sensitive than TBS in both infectivity control and vaccinated groups; the 18S rRNA/rDNA can be detected up to ~4 days prior to TBS detection.

The Submitter proposes to administer rescue treatment based on a threshold of ≥250 estimated parasites/mL by the RT-PCR assay. The results show that time to positive, based on a threshold of ≥250 estimated parasites/mL, would delay treatment compared to any positive findings by the RT-PCR assay, although, this would still be earlier than based on TBS (Table 3). However, it is recommended that timing for rescue treatment should be based on any positive findings by the RT-PCR assay as

- all subjects should be biomarker negative prior to challenge with *P. falciparum*,
- there is minimal chance of false-positive findings, and
- the aim is to use a more sensitive assay than TBS to minimize development of signs and symptoms of malaria in the subjects.

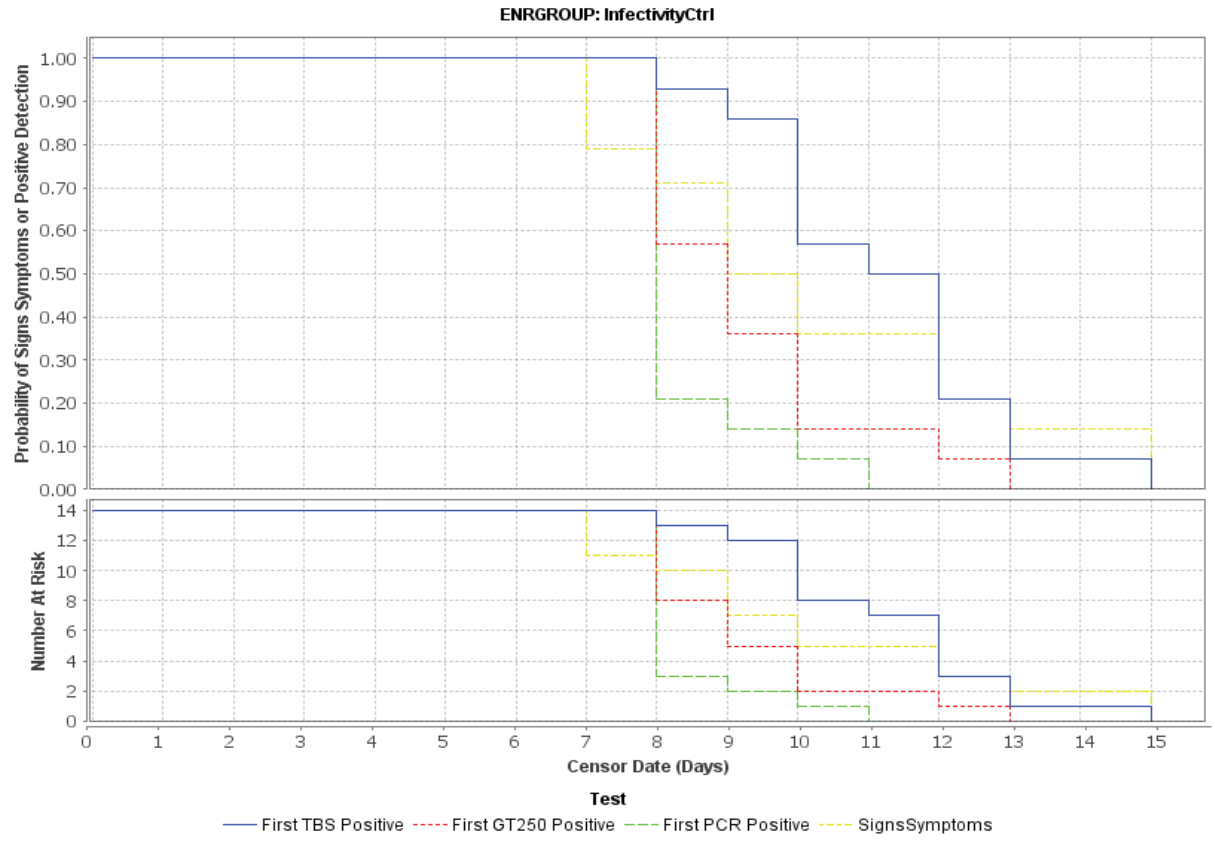
The Submitter states that the time needed to reach 250 estimated parasites/mL by biomarker measurement can be used in lieu of TBS time to positivity as evidence of partial protection. However, the number of vaccinated subjects or those treated with antimalarial drug in the two studies is very small for evaluation of partial protection.

Table 2: Clinical studies – Results

Parameter	MC-001 (n=6)		MC-003 ITV (n=29)				PfSPZ-CVac PYR (n=21)		
	Infectivity control (n=6)	Vaccinated (n=0)	Infectivity control (n=6)	Drug control (n=5)	Vaccinated CQ (n=3)	Vaccinated PQ/CQ (n=15)	Infectivity control (n=5)	Vaccinated CQ (n=5)	Vaccinated PQ/CQ (n=11)
RT-PCR Assay									
Type of assay used (Conversion factor for estimating parasites/mL)	3rd generation (7400)		2nd generation (3500)				3rd generation (7400)***		
Study Results									
TBS									
Number of subjects TBS positive	6	NA	6	5	3	12	2	0	4
TTP (Day): Mean (Range)	11.2 (9-14)	NA	8.7 (7-10)	9.8 (9-11)	14.3 (13-16)	10.6 (9-15)	12 (12)	NA	14.25 (12-18)
Parasites/mL Mean (Range)	19983 (2330-53700)	NA	9100 (1700-28500)	25100 (4000-72000)	31033 (14000-66400)	15492 (4000-82000)	45000 (8000-74000)	NA	17925 (6000-30000)
RT-PCR									
Number of subjects RT-PCR positive*	6	NA	6	5	3	11	5	1	9
TTP (Day): Mean (Range)*	7.7 (7-10)	NA	7 (7)	6.8 (6-7)	9.3 (8-10)	7 (7)	7-9 (7.6)	8 (8)	9.1 (7-15)
Ct (Range)	24.96-31.89	NA	23.33-29.91	23.42-33.55	27.62-29.8	24.72-32.73	27.00-30.46	30.18	26.75-28.7
Log_{10} Copies/mL (Range)	3.76-4.85	NA	4.06-5.98	3-5.95	4.09-4.73	3.24-5.57	3.27-4.33	3.25	3.81-4.41
Estimated parasites/mL (Range)	0 (3**)-379	NA	2766 (132-10904)	19-10265	142-612	19-4289	19-116	Not specified	35-139
Malaria related signs/symptoms									
Number of subjects with 1st any sign/symptom (Time to event in Days)	6 (9.2) [1]	NA	6 (7.8)[2]	5 (8.0)[2]	3 (11.0)[2]	14 (9.1)[2]	3 (10.7)[3]	1 (12)[3]	7 (12.3)[3]

*PCR positivity based on any Ct or copies/mL positive findings.
**Two patients were assigned a value of 3 estimated parasites/mL.
***Testing performed on separate aliquots of archived samples.
[1] All subjects experienced one or more systemic malaria-related signs/symptoms during the 28 days of follow-up post-challenge; the most frequent signs/symptoms were headache and myalgia (in 5/6 subjects) and fever, malaise, nausea and arthralgia (in 4/6 subjects). Most of these signs/symptoms were classified as mild (Grade 1) or moderate (Grade 2) in severity. At the time of positive TBS, one subject developed severe chills, headache, malaise and myalgia. In all subjects, symptoms peaked in severity on the day of diagnosis and decreased within 24 hours of CQ treatment. The symptoms were consistent with uncomplicated acute clinical malaria and all symptoms resolved within 48–72 hours of completion of treatment. No subject experienced a fever >38.0°C in this study. No serious adverse events occurred during the study.
[2] A majority (28/29) subjects experienced one or more systemic AE(s) during 28 days of follow-up post-challenge with symptoms in 26 subjects consistent with clinical malaria infection. The most frequent systemic AEs were malaise in 22/26 subjects who became TBS-positive, myalgia and headache in 20 such subjects and chills in 19 such subjects. Most AEs were classified as mild (Grade 1) or moderate (Grade 2) in severity. Symptoms in this study were consistent with uncomplicated acute clinical malaria and promptly resolved following rescue treatment.
[3] The mean time to symptoms in the PfSPZ-CVac PYR infectivity control arm was 10.7 days and two subjects experienced Grade 2 or higher symptoms starting on average on day 13.5, which was also similar to the experience with mosquito bite CHMI infectivity control subjects.
PQ=primaquine; CQ=chloroquine; TTP=Time to positive; NA=Not applicable; DVI-Direct venous inoculation; CIDR- Center for Infectious Disease Research

Figure 4: Time to parasitemia by TBS, different thresholds of positivity by PCR and symptoms in the Infectivity control group of subjects in the three Studies MC-001, MC-003 ITV, and PfSPZ-CVac PYR



GT250 = ≥250 estimated parasites/mL by RT-PCR
*Excluded 3 subjects in Study PfSPZ-CVac PYR that were TBS negative due to rescue treatment based on PCR^{+ve} findings
Figure created by Scott Garrett Runyan, Senior Analyst Software Developer, JReview Enhanced Support, CDER

Table 3: Summary of TBS and RT-PCR results combined for the 3 studies*

Parameter		Infectivity control*	Drug control**	Vaccinated*
TBS				
TTP	Range	7-14	9-11	9-18
	Mean	10.2	9.8	11.9
Number of positive subjects	n/N (%)	14/14 (100)	5/5 (100)	19/19 (100)
RT-PCR				
TTP (Any positive)	Range	7-10	6-7	7-15
	Mean	7.4	6.8	8.0
Number of positive subjects	n/N (%)	14/14 (100)	5/5 (100)	18/19 (94.7)
TTP (≥250 parasite equivalent/mL)	Range	7-12	7-9	7-17
	Mean	8.3	7.4	9.6
Number of positive subjects	n/N (%)	14/14 (100)	5/5 (100)	18/19 (94.7)
Malaria related signs/symptoms				
Time to 1st any symptom	Range	6-14	7-9	6-14
	Mean	9.2	8.0	10.2
Number of subjects with symptoms	n/N (%)	14/14 (100)	5/5	18/19 (94.7)

TTP=Time to positive
*3 subjects in the infectivity control group and 15 subjects in the vaccinated group, in Study PfSPZ-CVac PYR, were excluded as they received rescue treatment based on NIH PCR assay and were TBS negative at the time of rescue
**Nonimmunized subjects but administered chloroquine and primaquine during the immunization phase

4.2. MC-004 GAP3KO trial

Healthy adult volunteers (n=10) were infected with the triple-knockout genetically attenuated *P. falciparum* sporozoites (deleted for *p52*$^{-/-}$/*p36*$^{-/-}$/*sap1*$^{-/-}$) via the bites of ~150-200 infected *A. stephensi* mosquitoes. All subjects were TBS and biomarker negative; no signs or symptoms of malaria were reported throughout the 28-day study period.

Comments:
The Submitter used primers and probes for the RT-PCR assay, that are specific for A-Type 18S rRNA/rDNA gene of P. falciparum and Plasmodium species, which is mainly expressed in the liver/blood stages, however, it is not specific for those stages. Studies suggest that the primers and probes for the RT-PCR assay detects S-type gene also which is expressed in sporozoites and gametocyte stages as well; nonclinical studies showed that all stages of the parasite, i.e., sporozoites, liver stages, asexual forms and gametocytes in blood were detected in vitro and/or in infected animals. The Submitter was requested to perform testing for 18S rRNA/rDNA between Days 0 to 6 post-CHMI. However, no studies were performed.

Overall, the studies suggest that the *detection of 18S rRNA/rDNA by RT-PCR is more sensitive than TBS. The estimated parasite densities were <250/mL in most subjects and were positive early on i.e., prior to TBS positive or development of signs and symptoms. An improved sensitivity of the RT-PCR assay, compared to TBS, could facilitate initiating anti-malarial therapy before the onset of clinical signs and symptoms of malaria; this would also help reduce malaria-related symptoms.*

A decision for administering rescue therapy should be based on the presence of biomarker in whole blood; the positive findings should be based on any positive findings of the 3rd generation RT-PCR assay performed at the UW. If testing is performed in another laboratory or by a different assay, bridging studies should be performed.

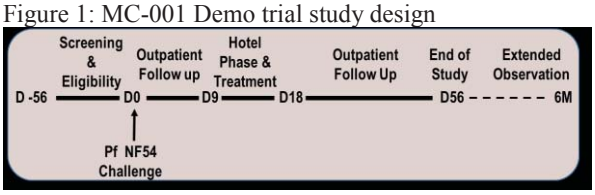
The schedule of events including blood sample collection time points for the RT-PCR assay are appropriate. However, due to cross-reactivity with B. microti, subjects with a possibility of Babesia exposure (i.e., subjects from endemic areas or those who have travelled to endemic areas) should be excluded. Also, to exclude the possibility of false positive findings due to high leukocyte count, subjects with leukocytosis should not be enrolled. Otherwise, the inclusion and exclusion criteria are appropriate.

Appendix-1: Clinical Study Designs

Study MC-001 - CHMI with 5 to 6 infected mosquito bites:

The study was a single-center, open-label Phase 1 trial to demonstrate the ability of the CIDR facility to conduct CHMI trials under an IND and to obtain immunological endpoints after a single challenge exposure with 5 infected mosquito bites (for details see Figure 1). One subject received 6 mosquito bites (protocol deviation).

Figure 1: MC-001 Demo trial study design

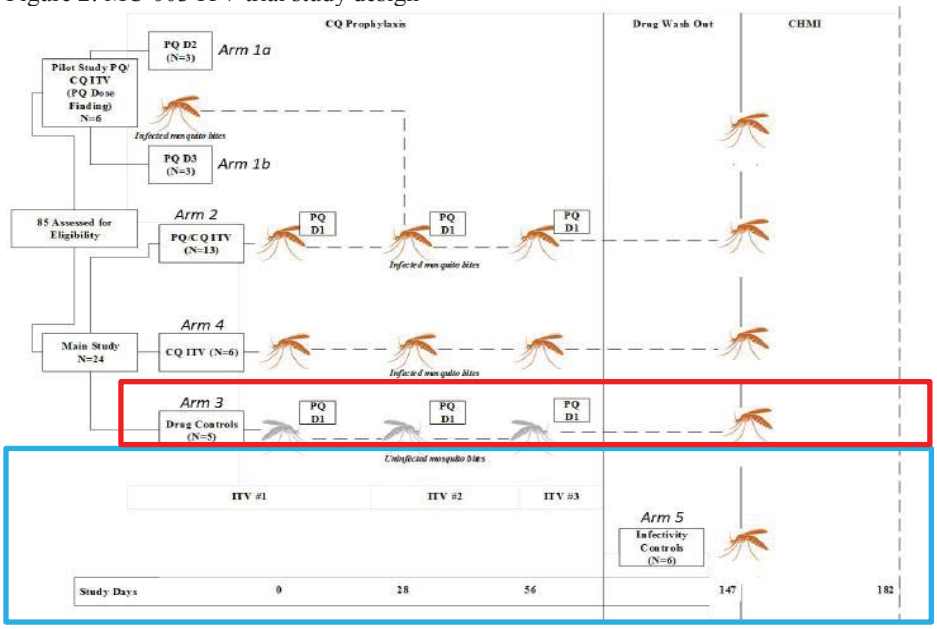


Study MC-003 ITV - CHMI with 5 infected mosquito bites:

This was a Phase 1, single center, randomized, partial double-blind, placebo-controlled trial of Infection-Treatment-Vaccination (ITV). Subjects were infected with wild-type NF54 *P. falciparum* sporozoites by mosquito bite in conjunction with antimalarial chemoprophylaxis with CQ plus PQ or placebo (Figure 2). There were two phases: ITV Phase and Challenge Phase. The ITV immunization phase consisted of:

- Experimental infection with wild-type (non-attenuated) NF54 strain *P. falciparum* sporozoites delivered by the bites of 12-15 infected *Anopheles stephensi* mosquitoes (ITV infection) administered in conjunction with
- Causal prophylaxis with PQ (45-mg single dose) or placebo, timed to eliminate parasites early in the liver stage of development, after hepatocyte invasion but prior to maturation and release into the bloodstream, and
- Continuous suppressive prophylaxis with the blood-stage antimalarial drug CQ (weekly dosing beginning eight days prior to first ITV infection [1g (600 mg base) loading dose then 500 mg (300 mg base) weekly) thereafter] and continuing for four weeks after the last ITV infection) to prevent development of patent parasitemia and clinical malaria.

Figure 2: MC-003 ITV trial study design



PQ=primaquine; CQ=chloroquine; ITV=Infection-Treatment-Vaccination

The protocol-defined study arms were vaccinated three times as follows:

- Arm 1a: Up to 3 pilot phase subjects (PQ/CQ) with PQ starting 2 days pre-ITV (n=2 to CHMI)
- Arm 1b: Up to 3 pilot phase subjects (PQ/CQ) with PQ starting 3 days pre-ITV (n=2 to CHMI)
- Arm 2: Up to 13 main phase subjects (PQ/CQ) with PQ starting 1 day pre-ITV (n=11 to CHMI)
- Arm 3: Up to 5 main phase 'drug control' subjects (CQ only) without ITV (n=4 to CHMI)
- Arm 4: Up to 6 main phase subjects (CQ only) with ITV (n=5 to CHMI)
- Arm 5: Up to 6 infectivity controls (n=6 to CHMI only)

The objective was to evaluate whether ITV can be limited to sporozoite/early liver stage parasites by addition of PQ to standard CQ prophylaxis (Arms 1-2) compared to CQ alone (Arm 4). During the immunization phase, CQ was provided to all ITV subjects and PQ was provided on Day 1 (Arm 2), 2 (Arm 1a) or 3 (Arm 1b) post-ITV to some subjects. During drug coverage, subjects underwent three rounds of ITV at monthly intervals via bites from 12-15 *P. falciparum*-infected *Anopheles stephensi* mosquitoes per ITV iteration. The drug control subjects were administered CQ and bitten by uninfected mosquitoes. After CQ clearance, all ITV subjects and six infectivity controls were challenged with bites from five infected mosquitoes. Thick blood smears (TBS) and second-generation biomarker qRT-PCR were used to monitor for infection during ITV and post-CHMI. Local and systemic adverse events (AEs) were monitored and detection of patent parasitemia by TBS served as the primary post-challenge endpoint.

The TBS and RT-PCR results during the immunization phase were not included. The Submitter states that during the immunization phase, no serious AEs occurred, although all subjects reported AEs at least once during the ITV phase, mostly during the first immunization. The overall results of the ITV phase of the study were as follows. All Main Phase subjects remained TBS-negative throughout the ITV phase with reductions in sub-patent estimated parasite densities measured by biomarker qRT-PCR with each subsequent ITV iteration.

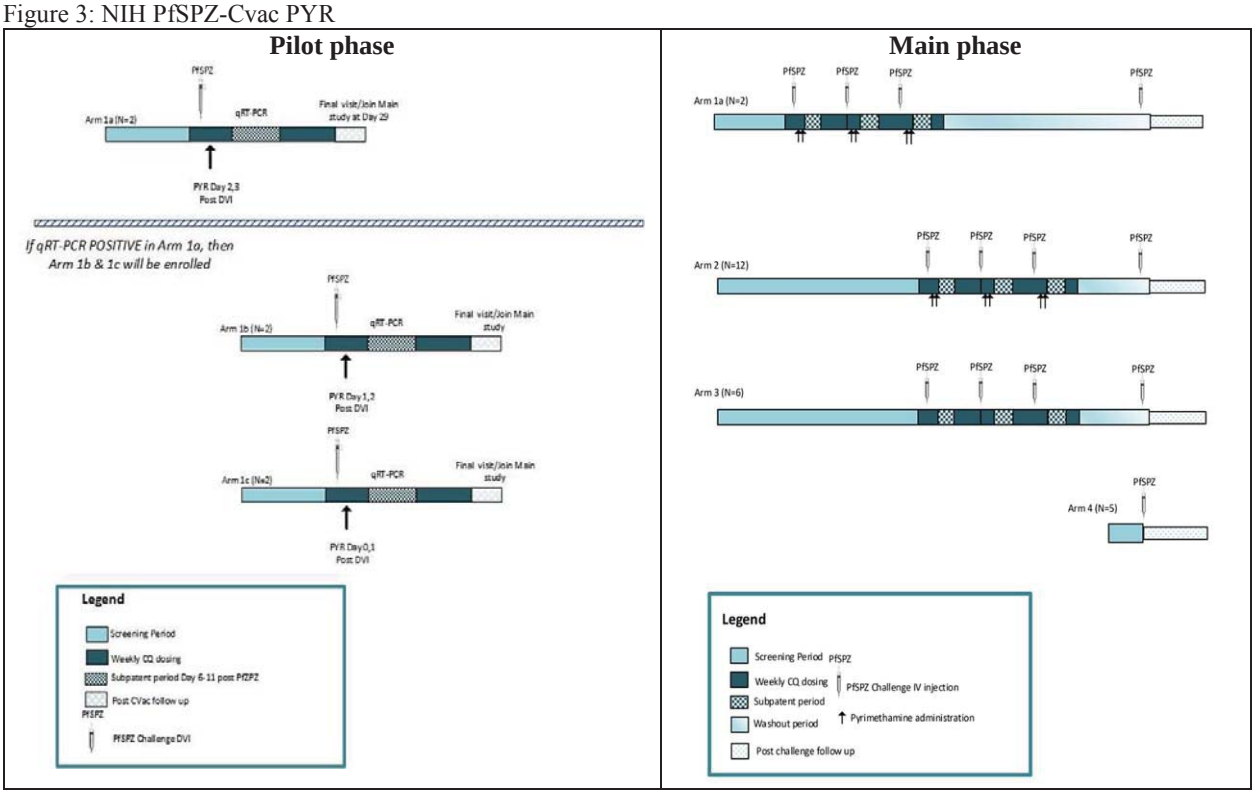
Study NIH LMIV PfSPZ-Cvac PYR trial – CHMI with 3200 *P. falciparum* DVI challenge:
To address DVI-based CHMI trials, the Submitter obtained samples and study data from the NIH trial that used the direct IV (DVI) challenge dose of 3200 *P. falciparum* sporozoite for CHMI and relied on a primary TBS endpoint. This was a Phase 1 single center trial of ITV consisting of wild-type, aseptic, purified, 51,200 cryopreserved *P. falciparum* sporozoites [Sanaria® *P. falciparum* sporozoite (PfSPZ) Challenge] administered by DVI in conjunction with antimalarial chemoprophylaxis with CQ and pyrimethamine (PYR) to induce stage-specific sterile protection. The pilot and main phase study designs are shown in Figure 3.

A pilot group (Arm 1a) was first tested to determine optimal timing of PYR dosing relative to challenge to maximize antigenic exposure to developing liver-stage parasites. Since biomarker was not detected in either pilot phase subject (using the protocol-defined real time qPCR assay performed at NIH), pilot Arms 1b and 1c were never initiated and the study proceeded to the main phase with PYR dosing 2 and 3 days post-DVI. The protocol-defined subjects in the different study arms were vaccinated three times as follows:

- Arm 1a: Up to 2 pilot phase subjects (CQ/PYR) with PYR 2 and 3 days post-ITV (n=2 to CHMI)
- Arm 2: Up to 12 main phase subjects (CQ/PYR) with PYR 2 and 3 days post-ITV (n=9 to CHMI)
- Arm 3: Up to 6 main phase subjects (CQ only) (n=5 to CHMI)
- Arm 4: Up to 5 infectivity controls (n=5 to CHMI only)

The Submitter combined the subjects in Arms 1a and 2 to a single group.

Figure 3: NIH PfSPZ-Cvac PYR



After screening, enrollment and vaccinations, 21 malaria-naïve healthy adult subjects underwent CHMI with 3200 PfSPZ by DVI including subjects in Arm 1a (2 subjects), Arm 2 (9 subjects), Arm 3 (5 subjects) and Arm 4 (5 subjects). Subjects underwent CHMI on Study Day 147 (91 days after the final vaccination).

Subjects were followed with daily outpatient on Days 6-21, 23, 25 and 29 post-CHMI. Blood samples for TBS and PCR were collected once daily post-CHMI and were the main laboratory safety endpoints post-CHMI that were used for decision making during the trial. For same-day PCR, the NIH Clinical Center's Malaria Genus Species 4-plex biomarker PCR was used. Two positive real time NIH qPCR results on separate days *OR* a single positive peripheral TBS post-CHMI were used for diagnosis and clinical decisions to initiate curative treatment. A positive TBS was defined as detection of at least two unambiguous parasites per slide that were identified and confirmed by a second microscopist. The NIH PCR assay had a LoD of ~500 parasites/mL of whole blood. Upon reaching the treatment definition, subjects were treated with the approved curative oral dose of Malarone® (1 g atovaquone/400 mg proguanil hydrochloride daily) for three consecutive days. In-person follow-up ended 29 days post-CHMI with telephone follow-up until six months post-CHMI.

Testing by the 3rd generation UW assay was performed retrospectively.

Appendix-2: Inclusion and Exclusion Criteria

Volunteers participating in CHMI trials should meet the following criteria. Some variations in these criteria are permitted from trial to trial depending on the nature of the drug or vaccine product under investigation.

1. **General CHMI inclusion criteria**

- Aged 18-50 years old. In specific situations, if it is determined that it is appropriate for individuals >50 years old to be included, additional screening should be included as necessary to protect the safety of volunteers.
- Volunteer must understand and sign written informed consent form.
- Volunteer should be free of clinically significant health problems as established by medical history, clinical examination and general laboratory evaluation.
- Volunteer should be available for the duration of the study, reachable by mobile phone or pager and should provide information on two emergency contacts to assist with making contact.
- Volunteer should reside nearby the trial center during days 0-42 following CHMI.
- Female volunteers should take adequate contraceptive precautions or be of non-childbearing potential. (Note that in some studies, women of childbearing potential may be excluded.)
- Volunteer must agree not to travel to a malaria endemic region during the in-person visit portion of the trial.
- Volunteer should be in good health, as determined by vital signs (heart rate, blood pressure, oral temperature), medical history, screening 12-lead ECG and laboratory tests*, and a physical examination.
    - *Typical screening laboratory tests include hemoglobin, white blood cell count, platelet count, glucose (random), serum alanine aminotransferase (ALT), serum aspartate aminotransferase (AST), serum creatinine, HIV/hepatitis screening, urine protein and urine blood.

2. **General CHMI exclusion criteria**

- History of malaria infection or vaccination, continuous residence in malaria-endemic area for ≥5 years, travel to malaria-endemic area in previous 6 months, or prior participation in malaria research study.
- Use of malaria chemoprophylaxis or chemotherapy within 90 days prior to study.
- Use of systemic antibiotics with known antimalarial activity within 30 days prior to the study (such as trimethoprim-sulfamethoxazole, doxycycline, tetracycline, clindamycin, erythromycin, fluoroquinolones and azithromycin)
- Plans to travel to malaria endemic areas during the study period
- A history of a chronic systemic immune modulating disorder, such as lupus, rheumatoid arthritis, vasculitis, scleroderma, cancer and diabetes mellitus. In addition, all significant contraindications to the antimalarial to be used should be exclusion criteria. For example, psoriasis and porphyria are contraindications to chloroquine use.
- Symptoms, physical signs and laboratory values suggestive of systemic disorders, including renal, hepatic, cardiovascular, pulmonary, neurological, skin, immunodeficiency, psychiatric (including suicide risk within 3 years), active neoplastic disease or other conditions, which could interfere with the interpretation of the study results or compromise the health of the volunteer.
- Any confirmed or suspected immunosuppressive of immunodeficient condition including HIV and asplenia.
- A history of allergic disease or reactions likely to be exacerbated by mosquito bites, malaria infection or antimalarial drugs.

- The confirmed or suspected presence of Hepatitis B, C or HIV.
- The confirmed or suspected presence of hemoglobin S, hemoglobin C, hemoglobin E, thalassemia or Glucose-6-phosphate dehydrogenase deficiency.
- Pregnant or lactating volunteers (or planning to start during study period).
- The chronic use (defined as more than 14 days) of immune modulating drugs within 6 months prior to malaria challenge or the use of immunoglobulins or blood products within 3 months prior to malaria challenge. Inhaled and topical steroids are generally allowed.
- Suspected or known alcohol or illicit drug abuse that, in the opinion of the investigator, may interfere with the subject's ability to comply with the protocol. Some protocols include partial or complete restrictions on alcohol, tobacco and/or other recreational drug use and some protocols delineate a specific timeframe to which the abuse applies.
- Volunteers unable to be closely followed, for social, geographical or psychological reasons
- The participation in another clinical trial which requires the use of an investigational or non-registered drug, vaccine or medical device within 30 days before the malaria challenge or any time during the study period for that participant
- Plans to undergo surgery (elective or otherwise) between enrollment and the end of the study.
- Vital sign abnormalities including systolic blood pressure >140 mm Hg or diastolic blood pressure >90 mm Hg; resting heart rate <40 or >100 beats per minute or oral temperature ≥ 38°C (100.4°F).
- Body mass index (BMI) ≥ 35 (exact cutoff varies by study)
- Any Grade 1 or higher clinically significant laboratory abnormality on screening
    - Screening laboratory tests include hemoglobin, white blood cell count, platelet count, glucose (random), serum alanine aminotransferase (ALT), serum aspartate aminotransferase (AST), serum creatinine, HIV/hepatitis screening, urine protein and urine blood. Some studies also assess for sickle trait. Any clinically significant Grade 1 or higher value for any screening test is exclusionary. Testing can be repeated in persons with non-clinically significant abnormalities to ascertain eligibility.
- Algorithmic evidence of moderate to high coronary risk, using locally appropriate coronary risk evaluation scoring systems. At our center, such risk consists of moderate risk or higher categories for fatal or non-fatal cardiovascular event within 5 years (>10%) determined by non-invasive criteria for cardiac risk according to the National Health and Nutrition Examination Survey (NHANES I) and/or an abnormal ECG.
    - Risk factors include sex, age (years), systolic blood pressure (mm Hg), smoking status (current vs. past or never), body mass index (BMI; kg/mm2), reported diabetes status (yes/no), current treatment for raised blood pressure (yes/no).
    - Abnormal screening ECG findings include evidence of pathologic Q waves and significant ST-T wave changes; left ventricular hypertrophy; any non-sinus rhythm excluding isolated premature atrial or ventricular contractions; right or left bundle branch block; QT/QTc interval >450 ms; or advanced (secondary or tertiary) A-V heart block.
- Acute febrile illness (oral temperature ≥ 38°C [100.4°F]) or other acute illness on or up to 3 days before product administration (drug or vaccine) and/or CHMI.
- Any condition that would, in the opinion of the site investigator, place the subject at an unacceptable risk of injury or render the subject unable to meet the requirements of the protocol or compromise the interpretation of data or the scientific integrity of the protocol.

Appendix-3: Example for the Study Schedule During the Challenge Phase of a CHMI study

Study day relative to CHMI	Once on -7 to -1	0	1-2	6-18	21	28	90	180
	Pre-CHMI assessment	CHMI	Reactogenicity Monitoring	Daily Outpatient Monitoring			Phone assessments	
Assessment of eligibility	X	X						
Review of medical history	X	X	X	X	X	X	X	X
Concomitant meds	X	X	X	X	X	X	X	X
Targeted physical exam	X	[X]	[X]	[X]	[X]	[X]		
Vital signs & oral temp.	X	X	X	X	X	X		
Contraceptive/menstrual history	X	X	X	X	X	X		
Pregnancy test		X						
Blood for clinical safety labs	X	X		X*				
Dipstick urinalysis	X							
Drug/vaccine response assays								
Serum		X		Per protocol				
PBMC		X		Per protocol				
Identification of AEs/SAEs	X	X	X	X	X	X	X	X
Review memory aid data		X	X	X				
P. falciparum challenge		X						
18S rRNA/rDNA biomarker and/or TBS	X			X	X	X		
Drug treatment (e.g., Malarone®)				Per protocol				

[X] required at this visit only if clinically indicated

*On days 12 and 18 (Safety labs include CBC and chemistry panel (creatinine, ALT, AST, alkaline phosphatase, total bilirubin).

†Blood for thick blood smear preparation may be obtained on the day of and prior to administration of treatment but at the proposed treatment threshold the TBS will routinely be negative and therefore prospective addition of TBS does not add a safety in treated subjects known to be biomarker positive. TBS should be maintained as a backup methodology in the event of a laboratory problem that precluded biomarker testing.

Data in this BQ submission is based on once-daily (morning) blood draws; twice daily sampling did not enhance infection detection (data not shown). This is approach in agreement with experiences at other centers [1].