Letter of Intent - Biomarker Qualification

Submission title: ***Plasmodium 18S rRNA/rDNA biomarker for prophylactic efficacy studies in malaria-endemic regions***

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1. **Drug Development Need Statement**
    - **a.** The malaria pipeline contains an increasing number of drug and vaccine candidates. Although thick blood smears (TBS) long served as a gold standard for detection of *Plasmodium* infection in human blood, more sensitive molecular tests have been developed. The University of Washington (UW) (Point of contact: Sean C. Murphy, MD/PhD) worked with FDA from 2014-2018 to qualify the *Plasmodium* 18S rRNA/rDNA biomarker to replace blood smears in controlled human malaria infection (CHMI) studies conducted *in non-endemic sites* (DDTBMQ000044). This biomarker achieves earlier and more analytically sensitive detection of *Plasmodium falciparum* infections than TBS and is now qualified for use in initiating treatment of participants post-challenge. The Qualification Package for the now-qualified non-endemic COU provides extensive history and rationale for the *Plasmodium* 18S rRNA/rDNA biomarker, and we refer FDA reviewers to that DDTBMQ000044 submission for historical and technical context. In addition to the clinical validation information, the analytical validation of the reverse transcription polymerase chain reaction (RT-PCR) used for biomarker detection were also extensively reviewed by the FDA DDT Biomarker Qualification review team for the qualified DDTBMQ000044 COU.
    - **b.** This new LOI proposes to extend the COU as **an indicator of infection in efficacy studies of prophylactic interventions at endemic sites**. Such prophylactic or preventive interventions could include drugs, vaccines, or other therapeutics like monoclonal antibodies. This submission will utilize many of the analyses performed for the non-endemic site CHMI COU except using samples from field efficacy studies in endemic settings. These analyses may identify unique aspects of the biomarker COU for endemic field efficacy studies. Some likely differences are noted below. Please also note that we also submitted an endemic site CHMI COU LOI (DDT000100) in February 2020.
    - **c.** As seen in non-endemic sites, use of the proposed biomarker in endemic field prophylactic efficacy studies will likely improve TBS endpoints by providing more sensitive, more specific, and more quantitative data to inform study endpoints. The data will be analyzed to determine how the biomarker identifies blood-stage infections in comparison to TBS and whether biomarker endpoints would change participant safety and/or the understanding of the clinical trial endpoint(s). The biomarker may detect subpatent infections that do not achieve patent parasite densities. Such infections may be due to underlying anti-erythrocyte stage immune responses known to occur in persons in endemic regions who have been repeatedly exposed to *Plasmodium* parasites. Biomarker testing will add a layer of information to such studies, and adverse events will be able to be evaluated against the biomarker concentration. This information could help us better understand differences between vaccine/drug treatment and control groups.
    - **d.** Another important difference between non-endemic and endemic COUs is that there is a greater risk of pre-existing *Plasmodium* infection at endemic sites. At such site, a variety of drug clearance approaches using approved medicines have been taken during the time leading up to the prophylactic efficacy endpoint phase of vaccine/drug trials. During the vaccination phase for instance, some sites treat participants with one or more anti-malarial drugs to eliminate existing parasites whereas other studies have not included clearance treatments. Studies have been done in areas of varying endemicity, making the

need for clearance treatments somewhat site-specific. As a study approaches the beginning of the efficacy endpoint phase, drug treatment stops being an option so that residual concentrations of approved drugs to not complicate the measurement of the efficacy endpoint. On or just prior to the start of the efficacy phase, a blood sample is always obtained and can be used to determine if the person was infected or not *prior to* this efficacy phase. In our work, we will systematically compare "pre-efficacy phase" samples to true efficacy phase samples. Unlike CHMI studies, these participants are not domiciled and therefore encounter *Plasmodium*-infected mosquitoes in the community.

- **i.** For persons who are biomarker-negative just prior to the efficacy endpoint phase and then become biomarker-positive thereafter, it is reasonable to conclude that biomarker reflects a naturally-acquired infection during the efficacy endpoint window.
- **ii.** For persons who are biomarker-positive just prior to the efficacy endpoint phase, this would indicate a pre-existing infection.
    1. If this positive was a non-*P. falciparum* biomarker positive, such an infection could be discerned from *P. falciparum* infections since the former result would be positive for pan-*Plasmodium* targets in the biomarker assay, but not the *P. falciparum*-specific targets.
    2. If a pre-existing positive indicated a *P. falciparum* infection, additional tools like Sanger sequencing and next-generation sequencing could possibly be used to determine whether the pre-existing infection was the same or different from the latter infection.

e. The frequency of diagnostic testing in field efficacy studies is much less than in CHMI studies because the participants are not deliberately infected and because their naturally-acquired infections may occur at any time during the longer efficacy evaluation window (if at all). In addition, less sensitive tests like TBS or RDTs are often used as the on-site assays to guide immediate patient care, while more sensitive biomarker tests are often done on batches of samples at a later time. In some more recent trials, biomarker testing is being done at the endemic site as the primary test.

f. Unlike CHMI studies, where 100% of infectivity control participants are expected to become biomarker positive, in field studies the prevalence in the placebo control group is usually no higher than the community prevalence. As such, larger numbers of participants are enrolled, and these participants are tested at fewer scheduled visits or at on-demand unscheduled visits for malaria-compatible symptoms. Because biomarker testing is more analytically sensitive, it is possible that a substantially higher prevalence of infection will be detected in studies reliant on biomarker testing. Particularly for interventions that achieve high rates of sterile blood-stage protection, this increased analytical sensitivity is likely to improve the ability to discern efficacy differences between groups. On the other hand, a increase in biomarker-defined infections could also narrow the difference between incompletely effective interventions and the placebo control groups. Because biomarker tests can be quantitative, such differences can be evaluated across a continuum, whereas TBS and RDTs usually provide a qualitative-only measure.

2. Biomarker Information and Interpretation

a. ***Biomarker name***: *Plasmodium* 18S rRNA/rDNA

b. ***Analytical methods***: RNA or total nucleic acid extraction of liquid whole blood samples or dried blood spots (DBS) followed by quantitative reverse transcription PCR (qRT-PCR). For this effort, we will utilize an assay refined from that in DDTBMQ000044. It may also be possible to fulfill the COU when using appropriately-designed 18S rDNA PCR-only assays (as is performed by several other CHMI centers).

   i. Pan-*Plasmodium* 18S rRNA (with Armored RNA calibrator in whole blood)

   ii. *P. falciparum*-specific 18S rRNA (with Armored RNA calibrator in whole blood)

   iii. Host mRNA or rRNA control target

c. ***Measurement units and limit(s) of detection***:

   i. Copies/mL of whole blood (LOD 5.3×10^4 copies/mL whole blood)

   ii. Estimated parasites/mL of blood (*P. falciparum* only)

d. ***Biomarker interpretation and utility***:

   i. In this COU, the biomarker is intended to serve as an indicator of infection that could replace TBS. Briefly, the biomarker assay provides copies/mL of the qRT-PCR target sequences (for pan-*Plasmodium* and *P. falciparum* targets). These values are reported to the study team. In addition, we determined a conversion factor for *P. falciparum* ($7.4x10^3$ copies/ring stage *P. falciparum* parasite) to provide an 'estimated' parasites/mL value. This estimated parasite density was found to agree with DNA-based measures of parasite density (where the target number is genomically fixed). This conversion factor was extensively discussed with FDA in the previous DDTBMQ000044 submission. Thus, we report a copies/mL value and an estimated parasites/mL value. It is our strong recommendation to include parasite density estimates to help align trial results with historical trials and studies tested by TBS endpoints.

   ii. TBS is the gold standard diagnostic test for clinical malaria in endemic regions. In some settings outside of the U.S., RDTs are alternatively used. Both are likely to be used for some time to guide clinical decision making to protect the safety of subjects in endemic site trials. We do not intend to stipulate a specific treatment threshold for biomarker-detected infections. This is consistent with the practice in the now-qualified non-endemic CHMI COU DDTBMQ000044 and the proposed endemic CHMI COU LOI DDT000100. In practice, if the biomarker was used to judge efficacy while TBS, RDTs, and symptoms were used to monitor participant safety, then criteria for interpretation of the biomarker-based infection detection would be listed in the trial protocol. Alternatively, if the biomarker was used at the endemic site in real time to monitor safety, the site would need to indicate a biomarker-based treatment threshold that would trigger treatment (akin to the situation in CHMI studies). Such a threshold would be indicated in the clinical trial protocol as well and allows for trial-specific tailoring of the exact biomarker cutoff for treatment. This field study COU is intended to qualify the biomarker as a true indicator of infection.

iii. Reportable range: $1.48x10^5$ - $7.4x10^{10}$ copies/mL (equivalent to $2x10^1$ to 1×10^7 estimated *P. falciparum* parasites/mL (note that the approximate LOD of TBS is $5x10^3$ to $2x10^4$ parasites/mL).

3. Context of Use Statement

a. A monitoring and surrogate endpoint biomarker that indicates the status of infection in *Plasmodium falciparum* field prophylactic efficacy trials of drugs, vaccines, or other therapeutics in enrolled subjects in endemic areas. When used as a monitoring biomarker, this biomarker may be used to inform the initiation of treatment with an approved anti-malarial drug. The presence of the biomarker may also be used as a surrogate endpoint to make efficacy determinations in *Plasmodium falciparum* prophylactic efficacy trial of drugs, vaccines, or other therapeutics in endemic areas. The concentration of biomarker required for monitoring and/or surrogate endpoint determinations will be made on a trial-specific basis and defined in each clinical trial protocol.

4. Analytical Considerations

a. ***General description of biomarker being measured and methods***: The biomarker assay is a standard qRT-PCR for 100-350 bp long regions within the 2.1 kb 18S rRNA sequences of *Plasmodium* parasites. The amount of 18S rRNA biomarker is an indicator of total parasite burden. The assay is substantially equivalent to the assay presented in DDTBMQ000044 with slight refinements to further enhance performance. Analytical and clinical validation of the DBS verison of the assay will also be provided to FDA.

b. ***Brief description of sample source***: Whole blood collected in EDTA anticoagulant or DBS. Typical volume is 50 µL of blood for both sample types.

c. Description of pre-analytical factors and quality assurance/quality control (QA/QC) plans to preserve specimen integrity:

i. ***Liquid whole blood samples***: Preferably aliquoted into bioMerieux NucliSENS lysis buffer (guanidinium-based lysis buffer) and frozen at ≤-70°C. Alternatively can be frozen as-is as whole blood at ≤-70°C. The biomarker has been shown to be stable under these conditions and this data was reported in DDTBMQ000044.

ii. ***DBS***: Preferably stored in desiccated, gas-impermeable bags. Storage at room temperature is suitable for short term storage. Storage at ≤-70°C is preferable for longer-term storage. Analytical validation data for DBS will be provided to FDA. DBS are intended to be cut with contamination-free methods such as laser cutting.

iii. ***Control samples*** (run controls): cultured, ring-synchronized *P. falciparum* parasites added to whole blood and then prepared as above as liquid samples and/or DBS. Run control performance is monitored by Levey-Jennings plots following each run (high, low, negative) as in DDTBMQ000044.

iv. ***Calibration***: achieved by an Assuragen Armored RNA calibrator that encodes the full-length *P. falciparum* 18S rRNA and provides traceable quantification as in DDTBMQ000044.

d. Analytical Validation Plan

i. The original biomarker detection assay was tested for liquid samples for accuracy, precision, analytical sensitivity, sample stability and analytical specificity (interferences), reportable range, and carryover using whole blood samples and was provided to FDA for liquid venous blood samples under DDTBMQ000044. The assay has since been refined slightly to further increase analytical specificity (see changes in non-public attachment). Data showing substantial equivalence between the revised assay and that reviewed under DDTBMQ000044 will be submitted for this endemic CHMI COU. DBS validation will cover the same topics to ensure reliable testing for the DBS sample type.

5. Clinical Considerations

a. Precedent for biomarker use in endemic site prophylactic efficacy studies

i. There is excellent precedent for qualification of this biomarker for endemic prophylactic efficacy (non-CHMI) studies of preventive drugs and vaccines. To date, clinicaltrials.gov lists the following trials in malaria-endemic sites that have used TBS and *Plasmodium* 18S rRNA/rDNA biomarker-based endpoints (**Table 1**). A subset of these endemic site trials will be included in the Biomarker Qualification for the proposed COU.

Table 1. Vaccine trials in endemic regions with TBS and molecular biomarker endpoints

Clinicaltrials.gov (& affiliated IDs)	**Study title**	**Location; Endpoints; Status**
NCT02687373 (KEMRI/SERU/CGHR/017/3129)	Safety, Tolerability and Efficacy of PfSPZ Vaccine in Healthy Children and Infants 5 Months - 9 Years Living in Kenya	Kenya; TBS & DBS pPCR; Completed
NCT02663700 (15-0001)	Safety and Immunogenicity of Sanaria's Irradiated Sporozoite Vaccine (PfSPZ Vaccine) in Malaria-Experienced Adults in Burkina Faso	Burkina Faso; TBS & qPCR; Completed
NCT03521973 (LaSPZV1)	Safety, Tolerability and Protective Efficacy of PfSPZ Vaccine in Gabonese Children	Gabon; TBS & qPCR; Recruiting
RTS,S vaccines	Numerous RTS,S studies	Many sites; RDT/TBS & symptoms; completed & ongoing

ii. In addition to studies with both TBS and biomarker endpoints, several trials have been done using biomarker-based endpoints alone **(Table 2)**.

Table 2. Other notable vaccine trials in endemic regions with molecular biomarker endpoints

Clinicaltrials.gov	Study title	Location; Endpoints; Notes; Status
NCT00121823	Malaria Infection Diagnosed by Polymerase Chain Reaction (PCR) as a Means of Evaluating Pre-erythrocytic Candidate Malaria Vaccines	Gambia; qPCR only; Parasite multiplication rates for subpatent and patent infections were lower in Gambian adults than in malaria-naïve adults challenged with *P. falciparum* in the UK (PMID: 21459819); Completed.
NCT01658696	Efficacy of Candidate Malaria Vaccines in Senegalese Adults	Senegal; qPCR only; Similar rates of biomarker positivity for vaccinated and control groups (PMID: 27978537); Completed.
NCT01666925	Efficacy of Malaria Vaccines in Kenyan Adults	Kenya; qPCR only; Monitored thrice weekly for 8 weeks and demonstrated 67% change versus controls (PMID: 25947165); Completed.

a. How will the biomarker measurement inform drug and vaccine development?

iii. As proposed herein, the biomarker is intended to tell investigators when an erythrocyte-stage *P. falciparum* infection has developed during the efficacy stage of a drug or vaccine trial. For the proposed COU, this information is used to determine whether or not the experimental drug or vaccine was effective at preventing erythrocyte-stage *P. falciparum* infection. If the trial is conducted with real-time testing of the samples, this data may also serve as a safety endpoint to initiate treatment.

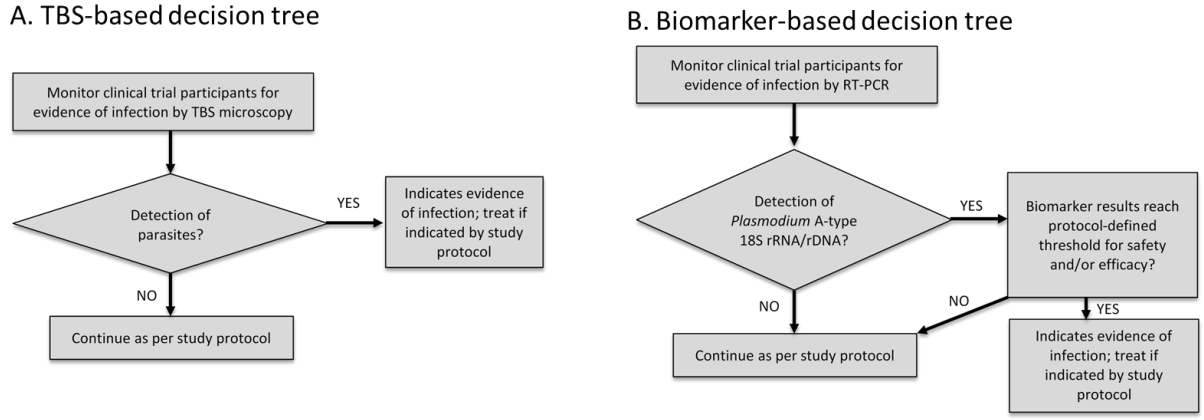
iv. With respect to drug/vaccine development, biomarker positivity in a participant that received an investigational product intended to prevent erythrocyte stage infection could indicate that the product did not achieve complete pre-erythrocytic protection. However, since the vaccine and drug products may vary, exactly how the biomarker will be used to inform prophylactic efficacy endpoints would be contained in each clinical trial protocol.

v. These clinical considerations are similar to those addressed in DDTBMQ000044 and in our endemic site CHMI COU submission DDT000100.

b. ***Decision tree***

i. The decision tree for this endemic site COU is shown in **Figure 1**.

Figure 1. Decision trees for TBS microscopy (A) and 18S rRNA/rDNA biomarker detection for endemic site field efficacy studies (B). The threshold listed in B would be defined in each clinical trial protocol. Biomarker positivity is an indicator of true *Plasmodium* infection for this COU and action taken in response to that threshold may vary from study-to-study.



c. ***Patient population***: Field efficacy studies first enroll healthy adult volunteers and gradually extend inclusion to children, pregnant women, and other at-risk populations. Inclusion/exclusion criteria are more relaxed than in CHMI studies submitted under DDTBMQ000044.

d. ***Setting***: Field prophylactic efficacy studies in malaria-endemic sites (examples in Table 1).

e. ***Clinical validation***: The study design will be similar to that used for the non-endemic qualification DDTBMQ000044 with adjustments to take into account the change from CHMI study designs to non-CHMI field efficacy study designs. Key aspects are briefly summarized below.

i. ***Format of data***: Clinical validation data will be provided in the same format as in DDTBMQ000044 (Qualification Package with Appendicies including Subject-level Data in .sas format) to support the biological and clinical relevance of the biomarker for the proposed endemic field efficacy COU.

ii. ***Reference values***: As in the non-endeimc CHMI COU, the ideal reference interval of the healthy, normal population is "not detected". Given the risk of malaria infection in the community, baseline/pre-efficacy phase and efficacy phase samples can be compared and contrasted to determine if pre-existing infection was present in enrolled participants. Since the biomarker assay is more sensitive than TBS, it is likely that we will find some persons with low density *Plasmodium* infections prior to the start of the efficacy phase. Thus, this endemic site qualification study will determine if low density biomarker positivity is a common or rare occurrence in such studies. Some studies employ anti-malaria drug clearance prior to the start of the efficacy phase and this will be taken into account as well.

iii. ***Proposed clinical validation:***

1. **Literature review for endemic site studies:** Existing data from the peer-reviewed literature on endemic site field studies will be provided to support the proposed COU.
2. **Data from prospective testing of endemic site prophylactic efficacy studies**
    - **a.** Using the analytically-validated venous blood and DBS protocols, we will obtain archival samples from a subset of the trials listed in Table 1 and potentially from comparable trials that are not yet reported in clinicaltrials.gov. These samples will be tested by the UW *Plasmodium* 18S rRNA qRT-PCR in Seattle and/or by another laboratory running the same assay at endemic sites and participating in external quality assurance with the UW laboratory. The RT-PCR data will be compared against TBS results that served as the infection detection endpoint in these studies. Data will be evaluated on a cohort-by-cohort basis, on a trial-by-trial basis, and on a composite basis across all trials. We will assess concordance between biomarker and TBS as well as overall concordance (e.g., Does a biomarker-positive person eventually go on to become TBS-positive as well?). This approach is similar to that taken in DDTBMQ000044.
    - **b.** We will determine how biomarker qualitative and quantitative positivity relate to the presence of Grade 1, 2 or 3 symptoms (if any) and to TBS positivity (if any). We will determine which of these differences are statistically significant using t-tests. We will also determine the mean copy number and mean estimated parasite density (±95%CI) for the first biomarker positive sample and at the time of TBS positivity. We will evaluate whether different biomarker-based treatment thresholds are likely to reduce symptoms. These comparisons are consistent with the approaches taken in DDTBMQ000044.
    - **c.** We will also determine whether biomarker data recapitulates conclusions about group differences originally made on the basis of TBS and/or clinical symptoms.
    - **d.** We will also compare to available biomarker data from outside laboratories (where available) to perform discrepant analysis in situations that are qRT-PCR biomarker-positive/TBS-negative. This analysis will follow FDA guidance on this topic and is consistent with the discrepant analysis performed in DDTBMQ000044.
    - **e.** Special additional analyses may be undertaken to address issues more likely in endemic settings such as evaluations of biomarker

positivity prior to the efficacy phase or the presence of non-*P. falciparum* parasites.

iv. ***Benefits and Risks of applying the biomarker in drug development or a clinical trial****:* With the recognition that low-density subpatent infections occur in endemic regions and that these infections may impact and be impacted by anti-malarial immunity, the abiltity to more sensitivity and quantitatively detect *Plasmodium* infections means that we will obtain a much richer dataset with which to evaluate malaria drug and vaccine candidates. For instance, we may discover that drug or vaccine candidates that prevented TBS-positive infections did not prevent subpatent infections. While a major goal of malaria vaccine and drug development is to advance products that eliminate malarial disease, another goal is to advance products that eliminate malaria infections altogether. More sensitive and quantitative diagnostics are key to this process. Since malarial symptoms generally increase with rising parasite densities, the quantitative nature of these tests can also be leveraged to understand the thresholds between infection and disease. Even trials that use clinical endpoints (disease) could benefit from this sort of biomarker-based insight.

v. ***Current knowledge gaps, limitations and assumptions for the proposed COU***: The biggest knowledge gap pertaining to this proposed endemic field study COU is that we do not know how asymptomatic infection varies across the malaria-endemic world. This knowledge gap also applies to the endemic CHMI COU submitted as DDT000100. We presume that asymptomatic infections modify immune responses and may periodically or continuously boost the immune system, mitigating severe disease signs and symptoms. However, we do not know how asymptomatic infections are geographically dispersed because, until recently, relatively insensitive detection methods were used for field prevalence surveys. As such, less is know about asymptomatic subpatent infections. By testing a subset of samples in this project from studies in Table 1, we will be able to rigorously evaluate the biomarker's potential to fulfill the proposed endemic field efficacy study COU. By doing so, we will also add to our collective understanding of patent and subpatent malaria in endemic regions.

6. Supporting Information

a. The supporting evidence for a link between biomarker positivity and true TBS-defined malaria infection are explained in detail in DDTBMQ000044 and in the attached paper by Seilie, Chang et al (Attachment 1).

b. The Qualification Letter for DDTBMQ000044 is included (Attachment 2).

c. The specific COU for endemic site prophylactic efficacy studies will be addressed by performing biomarker testing and then data analyses from the endemic field studies as described above. These studies will ascertain whether the general findings from CHMI studies apply to field-acquired infections at endemic sites as well. At least a subset of the included studies from endemic sites will contain a TBS gold standard comparator.

7. Previous Qualification Interactions and Other Approvals

a. Our non-endemic site CHMI COU was qualified in October 2018 under DDTBMQ000044 (Attachment 2).
b. The LOI for our endemic site CHMI COU was submitted to FDA in February 2020.
c. During our interactions with FDA, we discussed our plans to expand the qualified COU to include endemic site COUs. This LOI concerns the endemic field efficacy study COU.
d. This COU is part of a process of COU expansion for the *Plasmodium* 18S rRNA/rDNA biomarker for a wider array of drug and vaccine trials globally.

8. List of Attachments

a. Attachment 1: Seilie, Chang et al. 2019. Beyond Blood Smears. *Amer J Trop Med Hyg*. (this paper describes the findings from DDTBMQ000044)
b. Attachment 2: Non-endemic site CHMI COU Biomarker Qualification Letter