Step I: White Paper Application

White Paper Application

Project Title:

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1. Executive Summary *(Please limit to 500 words.)*

inoculates sporozoites into the human host. The sporozoites travel through blood vessels and infect liver cells to multiply into thousands of blood stage infective merozoites. In the case of *P. falciparum* this takes a minimum of 5.5 days. The replication in hepatocytes ensures the release of high numbers of merozoites into the blood stream. Thus liver stages of *Plasmodium* have enormous potential for anti-malarial intervention, either by drugs or by vaccine, before any clinical symptoms.

B. Existing Genome Data

PlasmoDB version 9.0 was released on May 2012, and constitutes a complete rebuild of the database, where all genomes, data and analysis were redone and improved. This is one of the main resources for *Plasmodium* and it contains genome information from 17 datasets that correspond to 9 species and strains of the genus *Plasmodium*. Data includes strand-specific, amplification-free transcriptome sequence (**FRT-Seq**) from *P. falciparum* 3D7 parasites (pooled red blood cell stage). It has been shown that the expression of both coding and non-coding RNA is different in different stages of blood-stage Plasmodium parasites. No RNAseq data from sporozoite stages is present. We anticipate to uncover many different both coding and non-coding RNAs that are unique to sporozoites and liver stages of *Plasmodium*. In addition, The Broad Institute, in collaboration with Dyann Wirth, Sarah Volkman and Daniel Hartl from Harvard University, is sequencing two clones of *P. falciparum* used in studies of malarial genetics, HB3 and Dd2, to better understand polymorphism in *P. falciparum*. Other strains and clones described as part of that project are: D10, 7G8, D6, FCC-2, RO-33, SL, K1, Senegal_V34.04, VS-1, IGH-CR14, NF54, and NF135/5.C10. In some instances, nucleus, mitochondrion, and apicoplast were sequenced. Strains correspond to different geographical regions. *Plasmodium vivax* was sequenced at JCVI (TIGR) as part of the MSC, under Jane Carlton direction and Lis Caler project management. Since Sanaria recently succeeded in making aseptic sporozoites, deep sequencing of both coding and non-coding RNA from *P. vivax* sporozoites will be considered during Phase II of this proposal.

C. **Value to the community:**

Successful deep sequencing of the proposed stages of *Plasmodium* will help the scientific community to better understand the biological basis of liver stage infection and help to develop malarial interventions against this stage.

D. **Clinical and scientific valu**

clear association with parasite development in liver, which will ultimately enable discovery of new therapies or interventions.

Genomic sequences of axenic versus salivary gland stage sporozoites will help us understand the development of liver stage parasites. Successful completion of this project will yield means to extend analogous studies to other stages in the *Plasmodium* life cycle and possibly the host-parasite interactions.

3. Rationale for Strain Selection

4. *Provide the rationale behind the selection of strains and the number of strains proposed in the study. The focus of the program is on potential agents of bioterrorism or organisms responsible for emerging or re-emerging infectious diseases. Non-select agents or non-pathogenic organisms will be considered when they can provide insight into these scientific areas.*

and commercialize a pre-erythrocytic stage vaccine composed of attenuated PfSPZ. Sanaria has succeeded in manufacturing sufficient quantities of an attenuated whole sporozoite vaccine that meets regulatory standards, including purity, sterility, safety, and potency using the NF54 strain of *P. falciparum.* The NF54 strain has been one of the most favored by researchers around the word due to its ease of culturing in laboratory conditions, and the large number of sporozoites per mosquito it yields. The genomic sequence

comparative analysis with the sequence of the 3D7 clone from NF54 is now underway (e.g. 3D7 is a clone of NF54).

A. Sporozoites and 72 Hour Liver Stage Parasites:

Sanaria has unique expertise to produce, extract and purify aseptic PfSPZ based on the proprietary technology developed for S porozoite based human vaccines. These sporozoites are not only free of any mosquito materials but also free of any microorganisms. The manufacturing and release of the sporozoites are described in Epstein et al 2011[4]. Sanaria will use similar methodology to produce 300 million total PfSPZ in three different manufacturing campaigns, 100 million each in three biological replicates, and use 40 million sporozoites for RNA extraction and 60 million sporozoites for axenic transformation into 72 hour liver stage parasites. PfSPZ manufactured will be tested for their ability to invade human hepatocytes using the 3-day hepatocyte potency assay [4]. For axenic development, 60 million sporozoites will be incubated in complete hepatocyte culture medium (10% FBS in 1X HBSS with penicillin and streptomycin) for three days with daily media change. 72 hours later *in-vitro* axenically transformed liver stage parasites

sequencing of the 3D7 clone of NF54 of blood stage parasites has been reported recently [5]. RNA sequencing of NF54 asexual blood stage parasites will serve as an internal control for RNA extraction, library preparation as well as sequencing. We will compare the sequences that we get from NF54 to that of 3D7.

Table 1. Summary of strain and stages proposed for deep RNA sequencing

Strain	Number and Stage	Purpose of RNA deep sequencing	Time-frame of availability and collaboration
NF54	4×10^7 aseptic, purified salivary gland sporozoites.	**1)** To compare the gene expression profiles of sporozoites and axenically cultured liver stage parasites **2)** To identify novel coding and non-coding RNAs in sporozoites.	3-4 months for material generation 6 months for sequencing, analysis and annotation
NF54	6×10^7 aseptic, purified salivary gland sporozoites axenically transformed to yield 4×10^7 liver stage parasites.	**3)** To compare the gene expression profiles of sporozoites and axenically cultured liver stage parasites **4)** To identify novel coding and non-coding RNAs in liver stages of Pf.	3-4 months for material generation 6 months for sequencing, analysis and annotation
NF54	4×10^7 asexual blood stage parasite as a control	**5)** This will serve as a comparator for deep sequencing as well as sequence analysis	Will be provided to JCVI at the same time as the sporozoites

4a. Approach to Data Production: **Data Generation**

C. State the data and resources planned to be generated. (e.g draft genome sequences, finished sequence data, SNPs, DNA/protein arrays generation, clone generation etc.)

RNA isolation and proposed libraries from sporozoites and 72 hour axenic liver stage parasites:

Based on preliminary experiments for each set (PfSPZ and 72 hour liver stage parasites), we anticipate requiring $4x10^7$ PfSPZ for the PfSPZ sequencing, and $6x10^7$ PfSPZ to produce the 72-hour liver stage parasites. Thus, $3x10^8$ aseptic, purified PfSPZ in total will be required. Sanaria will produce and prepare the material and JCVI will produce the appropriate libraries, conduct the sequencing, analyze and report the results. Infections /experiments will be performed in triplicates. From these datasets we will perform polyA mRNA enrichment and we will utilize the remai
ncRNA libraries from pooled material.

We will prepare directional (strand specific) RNA-Seq libraries to enhance the value of our samples for transcriptome annotation, profiling and potential gene discovery[6]. Increasing evidence suggests that noncoding RNAs (ncRNA) perform important regulatory functions. Currently, there is very limited or no high throughput small RNA data on any given Plasmodium strain, and no data at all on non-erythrocyte stages such as sporozoites we are proposing[5]. Therefore, our protocols for RNA processing and library preparation will be performed following the kits indicated below.

Protocols for ncRNA and mRNA extraction. For total RNA isolation, including miRNA,

-Seq Library Preparation Kit. This kit also generates the

sequencing library so the final product is ready for sequencing. For mRNA, we will use an adaptation of a protocol for RNAseq from Illumina TruSeq RNA Sample Preparation Kits v2.
Bioinformatic analyses to identify differentially expressed genes and pathways will be performed at JCVI and will be compared with existing transcriptomes (data from PlasmoDB) and our internal control sample.

Quality control of RNA sample: In order to ensure the RNA quality, we will make sure that the RNA is not degraded and it is free of DNA (RNA will be treated with DNAse).

RNA Concentration: We will produce RNA samples with a concentration of 100 ng/µl or above and if necessary, we will dilute the RNA solution in 1X TE to achieve appropriate concentration. RNA will be submitted for sequencing as pellets in 70% ethanol.

Documentation: Samples submitted for deep sequencing shall have the following accompanying documentation -

- Estimation of RNA concentration.
- Estimation of total RNA.

In case a different RNA preparation method is used, additional relevant information will also be documented.

sporozoites is a fundamental step towards understanding the effects of irradiation in the vaccine targets. This information will be unique, and a stepping stone for future research.

5. Community Support and Collaborator Roles:

E. *the proposed sequencing or genotyping data for this organism or group of organisms. Please provide specific examples.*

F. *List all project collaborators and their roles in the project*

G. *List availability of other funding sources for the project.*

There are several laboratories in the world working on the liver stages of *Plasmodium*, and quite a few of them are interested in knowing the RNA sequencing results of sporozoites and liver stages by deep sequencing.

Names of few investigators who will benefit from the availability of this deep sequencing results:

Dr. David A. Fidock, Ph.D. Professor of Microbiology & Immunology and Medical sciences, Columbia University, New York, NY.

Dr. Sangeeta Bhatia, MD., PhD. Professor and Director, Laboratory of Multiscale Regenerative Technologies, MIT, Cambridge, MA

Dr. Stefan Kappe, PhD. Professor and Malaria Program Director, Seattle Biomedical Research Institute, Seattle, WA.

Dr. Ana Rodriguez, Ph.D. Associate Professor, Department of Microbiology and Parasitology, NYU Medical center, New York, NY.

Dr. Maria M. Mota, Ph.D. Group Leader, Malaria Research Program, Lisbon's Instituto de Medicina Molecular, University of Lisbon, Portugal.

Sanaria will produce all sporozoites and axenically cultured liver stage parasites necessary for deep sequencing. JCVI will be responsible for making high quality RNA, labeling, deep sequencing, analysis, annotation and submission of data to the public data bases.

Collaborators and their role:

Sanaria: Project Co-coordination in collaboration with JCVI Project manager, providing sequencing-quality parasite materials, manuscript preparation.

JCVI: Overall project coordination and management, sample processing and quality control, deep sequencing of RNA, data analysis, data submission to relevant repositories and co-leading manuscript writing.

6. Availability & Information of Strains:

H. *Indicate availability of relevant laboratory strains and clinical isolates. Are the strains/isolates of interest retrospectively collected, prepared and ready to ship?*
Note: If samples are prospectively prepared the GSC can provide protocols and

recommendation based on the Centers past experiences. The samples must however meet

7. Compliance Requirements:

7a. Review

NIAID supports rapid data and reagent release to the scientific community for all sequencing and genotyping projects funded by NIAID GSC

4. Epstein JE, Tewari K, Lyke KE, Sim BK, Billingsley PF, Laurens MB, Gunasekera A, Chakravarty S, James ER, Sedegah M, Richman A, Velmurugan S, Reyes S, Li M, Tucker K, Ahumada A, Ruben AJ, Li T, Stafford R, Eappen AG, Tamminga C, Bennett JW, Ockenhouse CF, Murphy JR, Komisar J, Thomas N, Loyevsky M, Birkett A, Plowe CV, Loucq C, Edelman R, Richie TL, Seder RA, and Hoffman SL. Live attenuated malaria vaccine designed to protect through hepatic CD8 T cell immunity. *Science* 334: 475-480, 2011.
5. Lopez-Barragan MJ, Lemieux J, Quinones M, Williamson KC, Molina-Cruz A, Cui K, Barillas-Mury C, Zhao K, and Su XZ. Directional gene expression and antisense transcripts in sexual and asexual stages of Plasmodium falciparum. *BMC Genomics* 12: 587, 2011.
6. Levin JZ, Yassour M, Adiconis X, Nusbaum C, Thompson DA, Friedman N, Gnirke A, and Regev A. Comprehensive comparative analysis of strand-specific RNA sequencing methods. *Nat Methods* 7: 709-715, 2010.

Investigator Signature:

Investigator Name: **Date**