Validation of diagnostics to identify glucose-6-phosphate dehydrogenase activity in the US

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Short title: US G6PD Validation Study
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SEATTLE MALARIA CLINICAL TRIALS CENTER

PATH

Study title	Validation of a quantitative diagnostic to identify glucose-6-phosphate dehydrogenase activity in the US
Précis	Cross-sectional diagnostic accuracy study with up to 250 patient participants with a goal of obtaining 20 deficient and 20 intermediate samples. The clinic will recruit and consent study participants. Clinic staff will draw 3 EDTA whole blood tubes and obtain finger stick capillary blood. Clinic staff will perform the investigational SD Biosensor point-of-care (POC) test for G6PD deficiency and a HemoCue® hemoglobin test on finger stick capillary blood and on the EDTA anti-coagulated venous blood samples. Another EDTA anti-coagulated venous blood sample will be sent to a CLIA-certified lab for G6PD reference testing by the gold standard assay: G6PD measurement by spectrophotometry; this sample will also have a hemoglobin measurement by a hematology analyzer. Individuals identified as G6PD deficient with the reference test will be notified of their results by the clinic and referred to their physician for follow-up.
Objective	To assess the accuracy of a POC G6PD test as compared to a reference assay in detecting G6PD activity
Endpoints	• Sensitivity and specificity of SD Biosensor POC G6PD test compared to the Pointe Scientific test kit for identifying G6PD deficient individuals and women with intermediate G6PD activity • Accuracy between the SD Biosensor POC G6PD test measure of G6PD activity and the Pointe Scientific test kit • Accuracy between the SD Biosensor POC G6PD test measure of hemoglobin (Hb) and HemoCue 201+, a point of care reference haemoglobin assay • Comparison of the SD Biosensor POC G6PD test results for capillary and venous samples.
Population	250 male and female participants 18-65 years of age presenting at the clinic for care. Individuals who have received a blood transfusion within the last 3 months, according to self-report, will be excluded.
Study sites	Prevention Center, Fred Hutch Cancer Research Center University of Washington
Study duration	6 months (estimated).

Contents

Abbreviations

CE mark	European Conformity certification
CI	confidence interval
CLIA/CAP	Clinical Laboratory Improvement Amendments/ College of American Pathologists
CLSI	Clinical Laboratory Standards Institute
EDTA	ethylenediaminetetraacetic acid
FDA	Food and Drug Administration
FNTP	false negative true positive
FST	fluorescent spot test
G6PD	glucose-6-phosphate dehydrogenase
G6PDd	glucose-6-phosphate dehydrogenase deficiency
Hb	hemoglobin
IRB	institutional review board
IU	international unit
MTCT	Malaria Clinical Trials Center
POC	point-of-care
PQ	prequalification
P. vivax	*Plasmodium vivax*
QC	quality control
TPTN	test positive true negative
TTN	test and true negative
TTP	test and true positive
US FDA	United States Food and Drug Administration
UW	University of Washington
WHO	World Health Organization

1. Background and rationale for the study

Glucose-6-phosphate dehydrogenase (G6PD) is a critical housekeeping enzyme in red blood cells that supports protective systems against oxidative challenge by producing the reduced form of nicotinamide adenine dinucleotide phosphate [1, 2]. The most common human enzyme defect is G6PD deficiency, which affects more than 400 million people worldwide [3]. This deficiency can be severe, characterized by very low levels of G6PD activity or intermediate, characterized by moderately low levels of G6PD activity. Red blood cells are especially vulnerable to the effects of these mutations because they cannot replenish their supplies of the enzyme once they mature and enter the bloodstream. As a result, these cells are susceptible to hemolysis when subjected to oxidative stress, which can occur after therapy with the anti-malarial 8-aminoquinolines such as primaquine, a few antibiotics, and some anti-inflammatories. Hemolysis can also be activated by other exogenous agents, including foods (e.g., fava beans), henna, and some infections (e.g., hepatitis A or B, pneumonia, and typhoid fever). In newborns, G6PD deficiency is often first manifested as jaundice resulting from hyperbilirubinemia, which, if unchecked, can lead to kernicterus, a form of brain damage.

In 1989, the World Health Organization (WHO) working group on G6PD deficiency recommended that when possible, newborns should be screened for G6PD deficiency where G6PD deficiency is common [4]. However, this screening is not routinely done in the United States [5]. Screening for G6PD deficiency is included in the newborn screening programs of only two states and some facilities outside of those states may choose to adopt universal or targeted screening for G6PD independent of routine newborn screening programs [6,7]. While knowing the G6PD status of a patient is useful clinical information, access to testing for G6PD deficiency is very limited due to the price and complexity of the diagnostic products available for this condition, especially in and low-resource settings.

G6PD status is particularly relevant for the treatment and prevention of malaria. Malaria is prevented and treated using a variety of treatments, some of which pose a high risk to those with G6PD deficiency. The 8-aminoquinoline–based drugs for malaria treatment and prophylaxis, such as primaquine and tafenoquine, are the only ones with the capacity to prevent relapse and eliminate the liver stage parasites in *Plasmodium vivax* infections. Because of the risks associated to G6PD deficiency for primaquine exposure, WHO recommends that “the G6PD status of patients should be used to guide administration of primaquine for preventing relapse” [8].

Currently, the G6PD status of a patient is most often defined by the patient’s G6PD phenotype, characterized by analysis of total activity in blood lysate [3]. This method, considered the reference standard, is a costly and complex quantitative laboratory-based spectrophotometric test. The quantitative laboratory test can clearly identify subjects with all ranges of G6PD activities (including those with intermediate levels who may also be at risk of severe hemolysis), but these methods require an equipped laboratory and skilled personnel [9,10]. In field settings, the most commonly performed tests are qualitative devices such as the fluorescent spot test (FST) or the AccessBio, Inc. CareStart™ test [11,12] which can only discriminate gross deficiencies from all the other phenotypes. These are adequate for males who are either deficient or normal in G6PD status. Females, however, who carry two alleles of the G6PD gene, can present as deficient, intermediate, or normal for G6PD activity. Qualitative tests cannot discriminate an intermediate from a normal G6PD status. Quantitative tests are required to provide better case management for women, especially in anticipation of the availability of tafenoquine, which is indicated only for women and men with normal G6PD activity.

Given variety of clinical settings where G6PD status can be used to inform clinical care, point-of-care (POC) G6PD tests are required to support broader availability of primaquine and, in the future, tafenoquine. For treatment of women whose enzymatic activity is estimated to be normal by qualitative testing but is still too low for treatment of high-dose primaquine or tafenoquine, a quantitative portable device will be needed to obtain more accurate levels of G6PD activity to ensure appropriate and safe treatment [13].

PATH and the product developers have assessed the candidate POC test performance through laboratory evaluations using frozen specimens. Next steps in the validation of the POC test include an assessment of diagnostic accuracy using clinical samples in representative use cases.

2. Study objectives

The goal of this study is to contribute to a body of evidence that will support the submission of G6PD tests to the US Food and Drug Administration (FDA), WHO prequalification (PQ) process, and for product registration in target countries.

The primary objective is to assess the accuracy of the SD Biosensor STANDARD G6PD Analyzer in measuring G6PD activity when used by trained health care workers. This study aims to establish performance characteristics for SD Biosensor STANDARD G6PD Analyzer when performed from venous and capillary blood samples. Results from the SD Biosensor STANDARD G6PD Analyzer will be compared to results from an FDA-cleared quantitative G6PD assay and FDA-cleared hemoglobin assay: Pointe Scientific test kit.

3. Study design

This is a prospective cross-sectional diagnostic accuracy study. Study endpoints include:

- Sensitivity and specificity of SD Biosensor POC G6PD test compared to the Pointe Scientific test kit for identifying G6PD deficient individuals and women with intermediate G6PD activity
- Accuracy between the SD Biosensor POC G6PD test measure of G6PD activity and the Pointe Scientific test kit
- Accuracy between the SD Biosensor POC G6PD test measure of hemoglobin (Hb) and HemoCue 201+, a point of care reference haemoglobin assay
- Comparison of the SD Biosensor POC G6PD test results for capillary and venous samples.

3.1 Sample size

The sample size for this study is based on the expected prevalence of G6PD deficiency and on data requirements set by WHO through their process of prequalification of in vitro diagnostics [15]. This requires obtaining samples from participants with a range of G6PD activity levels. The WHO PQ process defines these levels as shown in Table 1.

Table 1. G6PD activity thresholds.

Sex	Level	Threshold	Estimated quantitative thresholds
Female	Deficient	G6PD activity <30% of the adjusted male median	<4 IU/ g Hb
Female	Intermediate	G6PD activity 30% to 80% of the adjusted male median	4-6 IU/ g Hb
Female	Normal	G6PD activity >80% of the adjusted male median	>6 IU/ g Hb
Male	Deficient	G6PD activity <30% of the adjusted male median	<4 IU/ g Hb
Male	Normal	G6PD activity >30% of the adjusted male median	>6 IU/ g Hb

Abbreviation: G6PD, glucose-6-phosphate dehydrogenase.

According to the target product profile, the novel POC G6PD will need to be at least 95% sensitive for detecting G6PD activity levels at 30% to 80% of normal enzyme activity [13]. Sample size calculations for this study are determined by a standard method, sometimes referred to as Buderer's equation, where study sample size requirements are calculated based on a clinically acceptable degree of precision, the hypothesized values of sensitivity and specificity, and the estimated prevalence of disease in the target population [14]. Assuming a sensitivity of 95%, with a confidence interval of 95%, and a 1% to 2% maximum marginal error, a minimum of 162 participants with deficient and intermediate G6PD activity will be needed for the analysis. To account for any possible device/diagnostic failures or compromised blood samples due to insufficient blood, signs of blood degradation, or contamination, the sample size is increased by 20% to approximately 200 participants with deficient activity and 200 participants with intermediate G6PD activity.

In this US, the estimated prevalence of G6PD activity at less than 30% of normal enzyme activity can be up to 12% in certain subpopulations [16,17]. By testing a minimum of 250 individuals, this study aims to include 20 deficient and 20 intermediate individuals.

In order to achieve the target number of deficient and intermediate samples (200), these data will be combined with data generated in other clinical evaluation in the US, Ethiopia and Brazil for regulatory submissions to the US FDA and/or WHO. The additional evaluations will include approximately 3,000 total participants with over 200 expected deficient and intermediate individuals.

3.2 Study sites

The Seattle Malaria Clinical Trials Center (MCTC) is an inter-institutional collaboration based primarily at the Fred Hutch Cancer Research Center and the University of Washington. For this study, clinic visits will be conducted at the Fred Hutch Prevention Center in the Arnold Building. The Prevention Center is a fully-equipped outpatient clinical research facility with exam rooms, a laboratory, pharmacy, dining area, and adequate office spaces. The center is staffed by a full-time staff capable of conducting all study procedures listed herein. Blood samples to be tested at the point of care will be tested in the Prevention Center clinical laboratory.

The University of Washington Department of Laboratory Medicine will provide reference laboratory services. Hemoglobin and G6PD reference testing will be performed at the Department's laboratories at the University of Washington Medical Center and/or Northwest Hospital. Mayo Medical Laboratories will serve as a backup send out laboratory for the G6PD test. All laboratories perform the Pointe Scientific G6PD assay and are CLIA/CAP certified.

Frozen blood samples not used by clinical or POC testing will be stored

4. Research participants

4.1 Characteristics of research participants

This study will involve healthy adult patients aged 18-65 years old capable of providing informed consent.

4.2 Inclusion and exclusion criteria

4.2.1 Patients

Criteria for inclusion of patients:

- Age 18-65
- Healthy volunteer by self-report
- Willingness to provide consent

Criteria for exclusion of patients:

- Blood transfusion in the past 90 days by self-report
- Pregnancy by self-report

5. Study procedures

Recruitment and screening at Seattle Malaria Clinical Trials Center

Up to 250 adult subjects meeting eligibility criteria will be included in this study. Subjects will be recruited from the greater Seattle area through a variety of Institutional Review Board (IRB)-approved messaging and outreach methods via the MC-002 general screening protocol (IR# 8366). Recruitment methods will target populations with expected

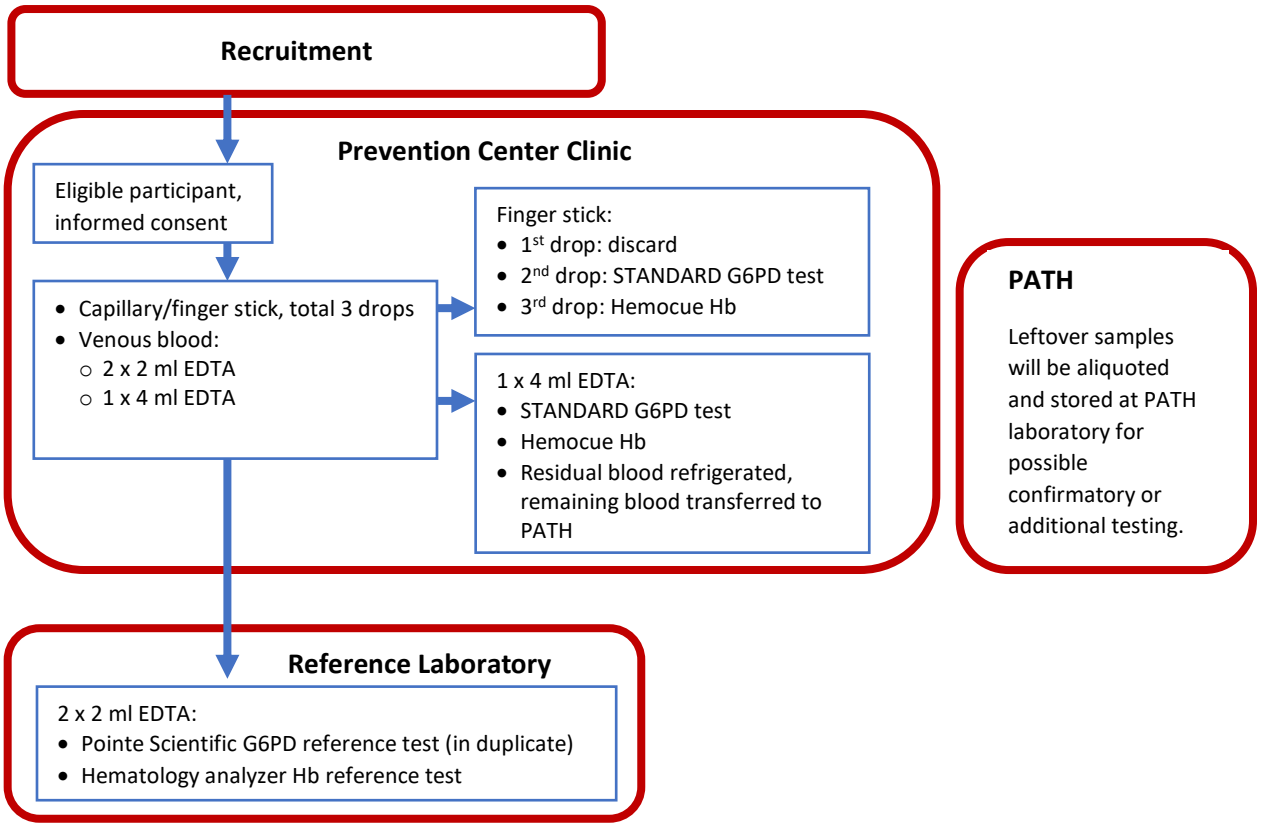
high prevalence of G6PD deficiency, including Southeast Asian populations and African-American populations. This will be achieved through outreach with community care facilities in the University of Washington network.

Screening procedures include the informed consent process and obtaining a brief history. The history will include questions regarding ethnicity, age, sex, and current health status. The screening/enrollment visit will take place at the Prevention Center clinic at the Fred Hutch Cancer Research Center. Subjects may be enrolled the same day that the informed consent procedure occurs. Subjects may also be invited to consent to be included in the database maintained for Seattle MCTC activities under the IRB-approved MC-002 general screening protocol and/or in the asymptomatic malaria infection study MC-005. The informed consent will include a provision that the reference laboratory G6PD result for subjects enrolled in MC-005 will be added to the MC-005 study database.

Sample testing at Seattle Malaria Clinical Trials Center and the UW Department of Laboratory Medicine

See Figure 1 for a summary of tests to be performed on the samples.

Figure 1. Diagnostic performance assessment process



Abbreviations: EDTA, ethylenediaminetetraacetic acid; G6PD, glucose-6-phosphate dehydrogenase; Hb, hemoglobin; ml, milliliter.

As noted in Table 3 below and Figure 1, volunteers will come to the clinic site, undergo the informed consent process, provide written informed consent, undergo blood draw and finger stick procedures.

Table 2. Summary of study procedures.

Participant group	List of study procedures	Estimated time	Appendices
Adults	• Written informed consent • Blood draw • POC tests	• 30 min	• Appendix A participant informed consent form • Appendix B case report form

5.1 Diagnostic performance assessment

5.1.1 Blood collection and testing procedures at the point of care

Following completion of the informed consent process, the study staff, who are trained in phlebotomy, will draw the following blood volumes:

- two 2-mL of ethylenediaminetetraacetic acid (EDTA)-anticoagulated blood
- one 4-mL tube of ethylenediaminetetraacetic acid (EDTA)-anticoagulated blood
- a finger stick sample of no more than 50 µl obtained with lancet

The tubes will be identified using a study subject number. At the clinic site lab, study staff will conduct the SD Biosensor POC G6PD test and the POC HemoCue Hb test on both finger stick blood and on blood from the 4-ml tubes of EDTA-treated venous blood. Results of the in-clinic performed POC tests will be recorded on data collection forms. If an invalid test result is obtained from the finger stick sample, a second finger stick sample will be obtained, and a second test will be run. If an invalid test result is obtained from a second finger stick, the results will be recorded as invalid. If the participant is found to be anemic based only on the HemoCue assay, the participant will be referred to a health care provider for follow-up and case management.

Once POC tests are completed on venous blood, the remaining blood in the 4-mL EDTA tube will be stored and transported as follows:

- Prevention center will transfer samples to PATH laboratories approximatly twice per week. Leftover samples will be aliquoted and stored at PATH laboratory for possible confirmatory or additional testing. Depending on availability of additional novel malaria tests, additional assays may be run on remaining samples. Samples will be aliquoted 6 x 0.5 ml of whole blood in cryogenic tubes and stored at –80°C in the freezer. Maximum storage time is 10 years after study end. This leftover sample will be stored for possible additional testing with the fluorescent spot test (FST). Appropriate permission for additional testing relating to malaria will be obtained through the consent process.
- The two 2-mL EDTA venous blood samples will be transported to the central reference lab by courier.

5.1.2 Testing procedures at the reference laboratory

At the reference laboratory, hemoglobin to normalize the G6PD values will be measured according to the G6PD reference assay instructions for use. The standard procedure for the reference laboratory is to measure hemoglobin for the Pointe Scientific G6PD test using a hematology analyzer. The Pointe Scientific G6PD reference assay will be performed in duplicate within 72 hours of collection. The temperature and humidity of the laboratory at the time of testing will also be recorded.

Test operators in the reference laboratory will be blinded to the results of the POC tests obtained and recorded at the clinic site. If there is a discordance between the replicates of the reference assay greater than allowed by the target product profile [13] (greater than 2 IU/ gram), testing with the reference test will be repeated using stored venous samples when possible. If there is discordance between the index test and the reference test, testing will be repeated using stored venous samples where possible to assess whether the discordance is reproducible. Performance characteristics and the results of additional testing of samples with discrepant results will be reported as per WHO guidelines [15].

5.2 Specimen collection, transport, and storage

As noted in Section 5.1.2 above, the clinic site staff will collect two 2-mL and one 4-mL EDTA tubes of whole blood as well as fingerstick capillary blood samples. Clinic staff will process the blood as in Section 5.1.1. Notably, immediately after collection, whole blood in EDTA will be stored in a refrigerator or a cooler box. As early as convenient and always within 18 hours, the two 2-ml EDTA samples will be transported to the reference laboratory.

5.3 Test result return

Results of HemoCue POC Hb testing will be returned immediately to participants who are anemic according to UW Hb reference ranges. Study staff will relay results and counsel participants who test positive for anemia according to the IRB-approved results return script. Participants will be notified at recruitment that the study team will tell them the results of the G6PD test after confirmation by the reference assay only if they are found to be G6PD intermediate or deficient by the reference assay. Participants who are found to be G6PD normal by the reference assay will not be notified of their result. Any participants found to be G6PD intermediate or deficient will be contacted via phone and notified of their result. The study team will recommend that the participant contact their care provider for any follow-up, including possible re-testing.

The study will make use of a study key that links participant ID, name, and contact information. This key will be populated at the time of consent and enrollment. This key will be accessible only by the MCTC Manager and will be kept in a secure location, separate from the study database. This key will enable the MCTC Manager to follow up with participants who are found to be G6PD deficient or intermediate by reference assay. The study staff involved in returning results will receive dedicated training aimed at providing the necessary information and delivering it in a way that facilitates comprehension. Once all laboratory testing and confirmatory testing is complete and all appropriate participants have been notified of their test results, the study key will be destroyed.

5.4 User proficiency

Study staff will be trained in the use of the assays. A proficiency panel of a characterized set of samples representative of varying levels of G6PD activity and Hb will be provided to ensure study personnel are able to perform the Pointe Scientific G6PD assay, the HemoCue Hb assay, and the SD Biosensor correctly. Proficiency testing will occur prior to the start of specimen testing on the reference methods and the investigational method. Users must pass proficiency before study testing can begin. Proficiency test results will be forwarded to PATH for review. Personnel who participate in further testing must obtain 90% or better to be deemed proficient.

5.5 Quality Control (QC) Testing

QC results must be within specifications for any test method result to be reported. QC testing on the Pointe Scientific G6PD assay, the HemoCue Hb assay, and the SD Biosensor will be performed each day of testing.

6. Consent process

The informed consent process encompasses all written or verbal study information the Seattle MCTC study staff provides to the subject, before and during the trial. All informed consent discussions will be documented by the study staff in the subject's source documentation. Consent discussions include, but are not limited to, background on G6PD and rationale for this study, an overview of the study design, study procedures, requirements for participation in the trial, and risks and benefits to the subject.

Written informed consent will be obtained only by Seattle MCTC study staff trained in the protocol and designated on the study signature log, using the protocol-specific Informed Consent Form approved by the local IRB and developed and administered in accordance with Seattle MCTC policies and procedures, local IRB/IEC requirements, federal guidelines 21 CFR 50.20, 45 CFR 46.116 and the ICH E6 Guidance Section 4.8.10.

Subjects will be provided with a copy of all consent forms that they sign. The original signed and dated copy will be kept on file in their study binder, stored in locked, limited-access cabinets within the Seattle MCTC.

7. Study products

7.1 SD Biosensor STANDARD G6PD Analyzer (investigational product)

The STANDARD G6PD Analyzer (Figure 2 on the following page) is designed to measure the quantitative determination of total Hb concentration and G6PD enzymatic activity in fresh human whole blood specimens based on reflectometry assays. The test is intended to aid in the identification of people with G6PD deficiency. The test is

currently not licensed for use in the US and is considered an investigational product. System components shall be labeled in accordance with regulatory requirements, including the following statement, “For Investigational Use Only. The performance characteristics of this product have not been established.”

Figure 2. SD Biosensor STANDARD G6PD Analyzer.

7.2 Pointe Scientific test kit (Reference Assay)

The Pointe Scientific test kit will serve as the reference assay to assess G6PD activity. Its intended use is for the quantitative, kinetic determination of G6PD in blood at 340 nm. It is designed for in vitro diagnostic use only.

- A spectrophotometer capable of measuring at 340 nm with a temperature-controlled cuvette compartment is required to perform the assay.
- To determine G6PD activity, which is reported in terms of grams of Hb or the number of red blood cells, the Hb or red blood cell count must be determined separately from performing the G6PD assay. Calculations are then performed to obtain the G6PD activity, normalized for the Hb level. For purposes of this study, the G6PD activity from the Pointe Scientific kit will be calculated in terms of grams of Hb as determined by hematology analyzer.
- US FDA cleared: k024006, Regulatory Class II, Product Code JBF.
- Performed at UW Medicine Northwest Hospital Laboratory (UW Department of Laboratory Medicine) with optional backup sent out to Mayo Medical Laboratories

7.3 HemoCue system

The HemoCue Hb 201+ system is designed for quantitative POC whole blood Hb determination in primary care using a specially designed analyzer, the HemoCue Hb 201+Analyzer, and specially designed microcuvettes, the HemoCue Hb 201+Microcuvettes. The HemoCue Hb 201+system is for in vitro diagnostic use only. It consists of a small portable analyzer (photometer) and plastic microcuvettes. The microcuvette serves both as a pipette and as a measuring cuvette. A blood sample is drawn into the cavity by the capillary action. The filled microcuvette is inserted into the HemoCue Hb 201+Analyzer. The measurement takes place in the analyzer, which measures the absorbance of whole blood at an Hb/HbO_2 isobestic point. The system is factory-calibrated and needs no further calibration. The HemoCue is available and registered for use at the study site.

7.4 Fluorescent Spot Test

The fluorescent spot test (FST) or TrinityBiotech G-6-PDH Screening test is widely used for in vitro diagnosis of G6PD deficiency using whole blood or dried blood spots. The G-6-PDH Screening test is a qualitative test performed by incubating a small amount of blood with glucose-6-phosphate and nicotinamide adenine dinucleotide phosphate (NADP). Drops of the mixture are removed at 5-minute intervals, spotted on filter paper and then viewed under long-

wave ultraviolet light. Fluorescence is clearly evident in mixtures prepared from normal blood, whereas deficient samples yield little or no fluorescence. The test is affordable and produces qualitative, visual results in minutes. This test will represent a qualitative standard care test that is currently available and cleared by the FDA. The test will be performed according to PATH's guide to fluorescent spot testing for G6PD deficiency.

7.5 Maintenance and Storage of Study Products

Commercial assays shall be stored at recommended storage conditions as provided in the product labeling. The STANDARD G6PD test shall be stored at ambient temperature. Control material for all assays shall be stored at recommended storage conditions as provided in the product labeling.

8. Data and data management

8.1 Statistical analyses

Data will be entered into a database with built-in validation rules to minimize data entry errors. Descriptive statistical analysis, including calculating point estimates, distribution, and frequencies, will be used to summarize and characterize the study population.

8.1.1 Diagnostic performance

For purposes of diagnostics for G6PD deficiency:

- A true positive: individuals who displays ≤ 30% (threshold) G6PD activity in circulating venous blood as determined by a quantitative assay and in this study specifically the Pointe Scientific Cat No. G7583. (see table 2).
- A true negative: individuals who display > 30% (threshold) G6PD activity in circulating venous blood as determined by a quantitative assay and in this study specifically the Pointe Scientific Cat No. G7583. (see table 2).

In order to investigate the performance of the assay to distinguish females with intermediate activity from female with normal activity, the sensitivity and specificity will be determined at G6PD activity thresholds of 70% and 80%. 70% refers to the threshold used in the clinical trials for tafenoquine as a cure to *P. vivax*, and 80% refers to the most recent WHO G6PD phenotype classification.

For the purposes of this study, an individual will be considered G6PD deficient (case) if they test positive by the Pointe Scientific assay. The primary success criterion will be focused on the ability to identify G6PD-deficient patients correctly, such that the SD Biosensor POC G6PD test on fingerstick blood and the Pointe Scientific assay on venous blood should both accurately identify all severely G6PD-deficient specimens (with <30% normal) as deficient.

The performance of the POC G6PD test against the Pointe Scientific assay will be determined by calculating the sensitivity and specificity. Sensitivity and specificity of the POC G6PD assay will be calculated as per Domingo et al. [19]. In summary, an adjusted male median will be calculated for both the POC G6PD and Pointe Scientific assay; from this median, the 30%, 70%, and 80% cutoff levels for the two tests will be used to categorically define G6PD-deficient cases.

Sensitivity will be determined using the following method:

- TTP = test and true positive (positive by reference assays according to case definition and positive by the POC G6PD test).
- FNTP = false negative true positive (positive by reference assays according to case definition and negative by the POC G6PD test).
- Sensitivity = TTP/(TTP+FNTP).

Specificity will be determined by the following method:

- TPTN = test positive true negative (negative by reference assays according to case definition and positive by POC G6PD test).
- TTN = test and true negative (negative by reference assays according to case definition and negative by POC G6PD test).
- Specificity = TTN/(TTN+TPTN).

Sensitivity and specificity results will be reported using 95% confidence intervals.

8.1.2 Accuracy between G6PD activity and hemoglobin methods

Quantitative agreement for both G6PD activity and Hb values between the SD Biosensor POC G6PD test and the Pointe Scientific assay will be graphically analyzed. Correlation graphs between the POC G6PD test and the Pointe Scientific assay will be plotted, and an R squared value will be determined. The following R squared values will be considered acceptable:

1. STANDARD G6PD test G6PD activity on capillary vs reference assay on venous blood > 0.8
2. STANDARD G6PD test G6PD activity on venous vs reference assay on venous blood > 0.85
3. STANDARD G6PD test hemoglobin concentration on capillary vs reference assay on capillary > 0.75
4. STANDARD G6PD test hemoglobin concentration on venous vs reference assay on capillary > 0.90

Plots, where differences between the G6PD POC test and the Pointe Scientific assay are plotted against the Pointe Scientific assay, will be used together with the 95% limits of agreement. Acceptable limits of agreement for Hb should be within +/-1.0 g/dL (based on a 6% estimate for allowable method bias) and for G6PD activity should be within +/-2.0 U G6PD/g Hb (based on a 15% estimate for allowable method bias).

All statistical analyses will be performed using Stata 13.0.

The data comparison for the analyses is outlined in Table 4 below.

Table 3. Comparison methods.

Index test by sample type	Reference method	
	G6PD normalized for Hb (U/g Hb)	Hemoglobin concentration (g/dL Hb)
Venous	Pointe Scientific G6PD normalized for hematology analyzer Hb result from venous specimen	HemoCue ® Hb from venous specimen
Capillary		HemoCue® Hb from finger stick

Abbreviations: G6PD, glucose-6-phosphate dehydrogenase; Hb, hemoglobin.

HemoCue is a registered trademark of HemoCue AB.

8.1.3 Interim analysis

An initial portion of this data will be used to support an FDA 510k submission. Per CLSI EP09-A3 (Measurement Procedure Comparisons and Bias Estimation Using Patient Samples; Approved Guideline – Third Edition) , it is recommended that measurement validation by a manufacturer include at least 100 patient samples spanning as much of the common measuring interval as feasible. This study proposes for the FDA submission from a G6PD perspective are at least 50 specimens in the G6PD severely deficient range, at least 50 specimens in the G6PD intermediate deficient range, and at least 100 specimens in the G6PD normal range. The data for this interim analysis will come from studies in Brazil and the US. Data collection for these studies are outlined in separate study protocols. When the numbers for FDA submission are reached during the trials, a data stop will occur, and the interim data will be cleaned and locked for submission purposes. Sites and investigators will be blinded to the results of the interim data analysis. The sites will keep enrolling until the numbers required for the WHO PQ are achieved. Should any results or observations occur during the continuation of the clinical study (following data cut-off for FDA

submission) which would impact our ability to demonstrate the safety, effectiveness, or equivalence to the predicate devices the FDA will be informed, and their consultation sought.

8.2 Data management

8.2.1 Data entry

The principal investigator is required to maintain original CRFs/source documents at the site. Participant data are entered on paper forms at the time the sample is taken and included with the samples sent back to the clinic site lab. All paper forms will be tracked by study ID number. Paper forms will be stored after the study ends in locked cabinets at the Seattle MCTC, after which time they will be destroyed following standard site procedures. Data from the paper forms will be entered into an electronic database system that is 21 CFR Part 11, FISMA, and HIPAA-compliant.

Specific procedures for transferring electronic instrument data will be described in study-specific Data Management Plan and training materials. All laboratory results will be entered into an electronic, password-protected database. Electronic study records will be deidentified upon completion of data collection. The electronic records will be maintained for at least 10 years in the databases and remain password-protected.

8.2.2 Data Access

The participants will be identified by a study identification number and the patient (clinical) identification number in the study database. The name and any other identifying detail will not be included in any study data electronic file. The database linking the participant's clinical identification number to the study identification number will be kept by the MCTC Manager and PATH will not have access to the link. All records will be kept locked and all databases will be password protected such that clinic staff and study staff will have access to their respective databases.

Direct access will be granted to authorized representatives from the sponsor, host institutions and the regulatory authorities to permit trial-related monitoring, audits and inspections.

8.2.3 Data Storage

The study team will maintain, and store securely, complete, accurate and current study records throughout the study. In accordance with regulations, study staff will retain all study records on site for at least 10 years after study closure. Study records will not be destroyed prior to receiving approval for record destruction from the sponsor. No study records will be destroyed while study specimens are still being stored. Applicable records include source documents, site registration documents and reports, informed consent forms, and notations of all contacts with participants.

8.2.4 Quality Control and Quality Assurance

The study will be conducted in accordance with the current approved protocol, International Conference on Harmonisation (ICH) good clinical practices (GCP), relevant regulations and standard operating procedures.

Regular monitoring will be performed according to ICH GCP. Data will be evaluated for compliance with the protocol and accuracy in relation to source documents. Study data will be aggregated into a database and an electronic monitoring report will be generated every month summarizing key indicators for study compliance. These indicators include but are not limited to the number of participants consented, the number of samples acquired, any deviations from study procedures, and corrective actions taken. Following written standard operating procedures, the monitors will verify that the clinical trial is conducted, and data are generated, documented and reported in compliance with the protocol, GCP and the applicable regulatory requirements. In addition, a PATH representative will conduct site monitoring visits as needed to ensure compliance with the protocol and relevant SOPs.

9. Benefit and risk considerations

9.1 Benefit to study participants

Participants in this study will have convenient access to an FDA-cleared Hb test. Participants who are found to be anemic will be counseled and referred to a health care provider for follow-up. Participants in this study will have the

opportunity to have their G6PD status tested by a reference assay. This information may inform the health care they receive in the future.

G6PD deficiency is a genetic condition that provides valuable clinical information for multiple clinical conditions beyond malaria treatment. If clinically relevant results are determined by the reference assay—that is, if G6PD activity is determined to be deficient or intermediate—participants will receive counseling regarding this information.

This research will also be advantageous for academic study and in the future for other people who will benefit from better G6PD tests and malaria treatment.

9.2 Risk and risk-mitigation considerations

The proposed study involves the use of an investigational product that has CE mark regulatory approval and no FDA determination has been made at this time.

The study will involve collection of venipuncture and capillary fingerstick blood. Only commercially available specimen collection products will be utilized, and the specimen collection methods will be those normally employed by a physician, clinic, or hospital. As such, study procedures do not represent significant risks to the participants beyond those that are associated with normal blood draws, such as pain, discomfort, feeling light-headed, fainting, and infection at the site of finger stick or venipuncture. The risks associated with blood draws will be mitigated through adherence to standard clinic procedures for infection control and using research staff who have been trained in best practices for blood collection. The volume of blood drawn as part of the study procedures is within the safety limits recommended by WHO and other organizations for both adults and children under 18 [20]. In the unlikely event of a research-related injury, cost of treatment will be covered by PATH. All decisions regarding clinical care will be made through referral to the local health care facilities.

There is a risk that the confidentiality of participant data will be compromised. The specimens will be inventoried and stored by patient codes established by the clinical site and will only be linked to patient identifiers and source documents through a study key. Individually identifiable protected health information or data will not be shared, except when required during audits by institutional and ethical review boards and regulatory agencies. All testing results will be filed and transferred to the study database as de-identified data. Results from the investigational test may not be used as a diagnostic procedure without confirmation of the diagnosis by another, medically established diagnostic product or procedure.

The study staff are at risk for exposure to blood-borne pathogens in the course of their work. All study team members will adhere to standard procedures for infection control. Study staff exposed to blood-borne pathogens during their study roles will follow their institutional guidelines for post-exposure prophylaxis.

10. Study safety and monitoring

We anticipate that this evaluation poses minimal risk to participants, as it does not involve any medical intervention and blood draw volumes are within acceptable ranges. No data safety monitoring board will be used. The study team will conduct necessary staff training on study procedures prior to initiating the evaluation. Only trained users who have been certified as proficient in the use of the test will be involved in blood collection. The information participants will provide in the context of this evaluation is not considered sensitive and sharing it will not pose any significant risk to them personally or professionally.

10.1 Adverse Events

10.1.1 Adverse Event Definitions

Adverse Event (AE): Any unfavorable and unintended sign (including an abnormal laboratory finding), symptom or disease in a subject that is temporally associated with the use of an investigational product or procedures, even if the event is not considered to be related to the study product or procedures.

Serious Adverse Event (SAE): An SAE is any AE occurring during study participation that results in any of the following outcomes:

- Death
- Life Threatening (refers to any event in which the subject was at risk of death at the time of the event; it does not refer to an event which hypothetically might have caused death if it were more severe)
- Hospitalization or prolongation of a hospital stay
- Persistent or significant disability or incapacitation (refers to any event which results in a substantial and/or permanent disruption of the subject's ability to carry out normal life functions)
- Required intervention to prevent permanent impairment/damage
- Congenital anomaly/birth defect
- Important medical event that may require intervention to prevent one of the preceding conditions.

Unanticipated Adverse Device Effect (UADE): Any serious adverse effect on health or safety or any life-threatening problem or death caused by, or associated with, a device, if that effect, problem, or death was not previously identified in nature, severity, or degree of incidence in the investigational plan or application (including a supplementary plan or application), or any other unanticipated serious problem associated with a device that relates to the rights, safety, or welfare of subjects. Refer to Protocol Section 8.2 (for a list of anticipated adverse events, signs or symptoms. (21CFR-812.3(s))

10.1.2 Adverse Event (AE) Management

Some reported or observed signs and symptoms are inherent to blood specimen collection and are likely to occur transiently for nearly all subjects in this study. Such signs or symptoms will not be considered AEs if they are mild (transient, easily tolerated, no interference with daily activities). The following will not be considered AEs:

- Mild, self-limited pain, swelling, bruising, or brief and minimal bleeding at the collection site

However, these signs and symptoms must be considered AEs and documented on the Adverse Event CRF should any of them occur in such a way that the extent or nature of the experience exceeds that normally associated with the procedure, as judged by the PI, or the event meets the criteria for a Serious Adverse Event (SAE).

10.1.3 Assessment of Adverse Events (AEs)

All AEs must be assessed for Seriousness, Severity, and Relationship. All AEs, regardless of classification, must be comprehensively documented in the CRF and on the SAE form, if applicable, and reported. This includes AEs related to marketed study products. The following information about the event is to be reported on the AE CRF:

- Seriousness, classified as: Non-Serious or Serious
- Severity, classified as:
    - Mild: Transient symptoms, easily tolerated, no interference with daily activities
    - Moderate: Marked symptoms, moderate interference with daily activities, tolerable
    - Severe: Considerable interference with daily activities, intolerable
- Relationship to the study product or study procedures, classified as:
    - Not Related: Evidence suggests absolutely no possible causal relationship between the event and the investigational study device (or procedures).
    - Unlikely Related: Evidence suggests that other possible causes or contributing etiological factors may have caused the event other than the investigational study device (or procedures).
    - Possibly Related: Evidence suggests a causal relationship between the event and the investigational study device (or procedures) cannot be ruled out
    - Related: Evidence suggests a reasonable causal relationship between the event and the device (or procedures) is likely

In addition, the following should be recorded for each AE:

- Action(s) taken to remedy the AE, including change in study treatment or participation, or medical/surgical treatments
- Duration of the AE from onset through resolution, as applicable

- Cause (including suspected product/procedure and/or other cause)
- Outcome of the event, including resolution and sequelae, as applicable

10.1.4 Additional Procedures for Assessing & Reporting Serious Adverse Events (SAE)
SAE criteria are specified in Section 9.1. All SAEs must also be assessed by the Investigator to determine whether an SAE is expected or unexpected. An adverse event will be considered unexpected or unanticipated if the nature, severity or frequency of the event is not consistent with the risk information previously described in the protocol, Informed Consent, or device instructions for use.

Any adverse event meeting the criteria for 'Serious', regardless of the Investigator's opinion of expectedness or relationship to the study product, must be reported to PATH within 24 hours. The Investigator or designee must report the event by telephone or email to PATH.

10.1.5 Reporting Obligations to IRB/EC and Health Authorities
The Investigator must report any adverse events which are serious, unanticipated/unexpected and probably or possibly related to the study product or procedures to the reviewing IRB/EC. This report must be submitted as soon as possible, but in no event later than 10 working days after the Investigator first learns of the event.

PATH will provide results of any evaluation of an unanticipated/unexpected adverse device effect to appropriate IRB/ECs within 10 working days after notification of the event.

10.2 Monitoring

The study team will be supervised by the local study lead. Study data will be entered into a database, and a monitoring report will be generated every two weeks, summarizing key indicators for study compliance. PATH and Fred Hutchison will hold bi-weekly data review calls to discuss data collection and data quality to date. These indicators include, but are not limited to, the number of participants consented, the number of samples acquired, any deviations from study procedures, and corrective actions taken. In addition, a member of the study team will conduct site-monitoring visits as needed to ensure compliance with the protocol and relevant standard operating procedures.

PATH will designate trained and qualified personnel to monitor the progress of this clinical study in accordance with Monitoring SOPs and the study-specific Monitoring Plan. Prior to study start, a study initiation visit will be conducted to provide training to site staff about the protocol, the completion of study documentation and data collection forms, the monitoring schedule, and all regulatory requirements. During the study, routine monitoring visits will be conducted to assure the site continues to adhere to the protocol, the investigator agreement, and regulations regarding conduct of clinical studies. Assessments will be made regarding the subjects' protection and safety, when relevant, as well as the quality, completeness, and integrity of the data.

11. Ethical considerations

11.1 Study conduct

The investigators will ensure that this study is conducted in full conformity with the current revision of the Declaration of Helsinki, or with the International Conference on Harmonisation Good Clinical Practice regulations and guidelines, whichever affords the greater protection to the subject. Additionally, the investigator assures that all activities of this protocol will be guided by the ethical principles of the Belmont Report and by US Department of Health and Human Services 45 US Code of Federal Regulations 46 and all of its subparts (A, B, C, and D). Investigators and study staff are trained in the protection of human subjects. Training in the principles of informed consent and in the study procedures for obtaining informed consent will be conducted before study initiation.

11.2 Informed consent

Study team members trained in the principles of informed consent and human subjects protection will obtain written informed consent from all participants.

11.3 Ethical review committees

The protocol, informed consent form, and recruitment materials will be submitted to the Fred Hutchison Cancer Center Institutional Review Board for review and approval.

11.4 Amendments

Amendments to the protocol will not be implemented without agreement from the Sponsor and prior submission to and written approval from the governing IRB/EC, except when necessary to eliminate an immediate hazard to the subject. Notice of an emergency modification shall be given to the reviewing IRB/EC as soon as possible, but in no event later than 5 working days after the emergency occurred. Protocol amendments may affect Informed Consent Forms for current and future subjects. Minor changes to the protocol, such as correction of typographical errors or changes in personnel names (other than the PI) or contact information will be processed as administrative changes. Administrative changes will be submitted to the governing IRB/EC but implementation of the administrative change may proceed without prior IRB/EC approval, unless so required by the IRB/EC or site SOPs.

11.5 Continuing review reports

The Principal Investigator will be responsible for submitting the required continuing review report and associated documents to the relevant IRBs, allowing sufficient time for review and continuation determination prior to the established continuing review date. A closeout report will be submitted at the end of five years, or upon completion of the study, whichever comes first.

11.6 Deviations

Protocol deviations are not permitted and should be implemented prospectively as a protocol amendment whenever practical or appropriate, unless required to protect the safety and well-being of the subject. Any deviation from the protocol that may have an impact on the safety or rights of the subject or the integrity of the study will be reported to the appropriate IRBs within 72 hours of when the deviation is identified. All other deviations will be reported in the annual continuing review report. Significant deviations may also need to be reported to the IRB/EC and local health authority. If the Investigator or their staff inadvertently deviates from the study plan, the Investigator should implement appropriate corrective and preventive procedures.

11.7 Unanticipated events

Any adverse events that are unanticipated, serious, and related or possibly related to participation in the research, any serious adverse events, or any incidents that suggest that the research places participants or others at risk, including breach of confidentiality, will be promptly reported to the appropriate IRBs within 10 days. A complete written report will follow the initial notification. Other incidents will be reported in the annual continuing review report.

11.8 Compensation

Subjects will be compensated for time and travel to participate in this trial. Subjects will receive $50 for the screening/enrollment visit. Information about compensation, including the amount and schedule of payment and applicable reporting to the IRS, will also be described in the informed consent form.

11.9 Genetic testing

G6PD is a genetic condition. The diagnostics used at the point of care and in the laboratory diagnose G6PD deficiency through a measurement of G6PD enzyme activity in the blood, not full genome sequencing.

12. Study Limitations

There are some limitations to this diagnostic evaluation. With regard to any diagnostic accuracy evaluation, there are opportunities for bias. This study will rely on the quantitative spectrophotometer assay as the reference test rather than genetic sequencing, and an imperfect reference test may lead to classification bias. Given the rates of G6PD prevalence in the Northwest United States and the data requirements in the WHO verification guidelines, some purposive sampling will be required, and we expect a significant number of the samples tested in Seattle to be G6PD normal.

13. Investigator responsibilities

The three project partners involved in this evaluation are Fred Hutchinson Cancer Research Center, PATH, and the University of Washington. Roles and responsibilities for each of the partners are listed below.

Table 4. Roles and responsibilities for study partners.

Task	*Fred Hutchison*	*PATH*	*UW*
Award oversight		*X*	
Study design and protocol development	*X*	*X*	*X*
Institutional review board submission and approval	*X*		
Evaluation of logistics arrangements	*X*		*X*
Procurement of all study supplies	*X*		*X*
Training on the use of study assays	*X*	*X*	*X*
Recruitment, consent, enrollment, and field data collection	*X*		
Laboratory-based data collection			*X*
Data entry and cleaning	*X*		*X*
Data analysis and reporting		*X*	
Site monitoring		*X*	

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Appendices

A. Consent form

B. Data collection forms

C. Recruitment materials