ID25

Detection of *Plasmodium* parasites with Loop Mediated Isothermal Amplification (LAMP) using simple sample preparation methods

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INTRODUCTION

Current malaria diagnostic tests rely on parasite detection by microscopy methods or antigen-based rapid diagnostic tests (RDT). Molecular methods like polymerase chain reaction (PCR) can be used to increase the sensitivity of detection. However, practical deployment of molecular testing should address certain challenges such as 1) simplification of sample preparation from blood, 2) reagent stability under ambient conditions, 3) ease-of-use for the end-user and 4) affordable pricing. Loop mediated isothermal amplification (LAMP) is a highly sensitive, rapid molecular method which can be used to detect *Plasmodium* DNA. We report on the performance of a simplified malaria assay in an easy to use LAMP platform using pre-dispensed, ambient temperature stable, lyophilized reagents.

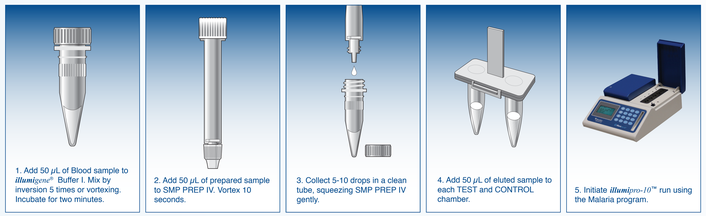
METHODS

The Meridian ***illumi**gene*® Malaria DNA Amplification Assay (Research Use Only (RUO), Not Cleared for use in USA) uses LAMP to detect *Plasmodium* parasite at the genus level. During LAMP amplification, an increase in turbidity occurs due to the magnesium-pyrophosphate built up as a by-product. The change in turbidity is measured by the Meridian ***illumi**pro-10*™ instrument and a result is determined. Two methods were designed to extract DNA from whole blood collected in EDTA as anticoagulant: simple filtration (Meridian SMP-prep) or 7-10 minutes gravity-driven gel filtration column (Meridian ***M-prep***™). Both procedures rely on chemical lysis and produce amplifiable DNA for LAMP amplification. Both methods were evaluated using five well characterized *Plasmodium falciparum* standards (strains US05FPH1, US05FFC27/A3, US05FBeninI, US05FSantaLucia, US08F NigeriaXII) and 206 clinical specimens consisting all the five human-infecting *Plasmodium* species (*P. falciparum, P. vivax, P. ovale, P. malariae and P. knowlesi*). A blood sample was mixed with lysis buffer and the lysate transferred to either SMP-prep or ***M-prep***™. The collected eluates from each device were directly added to the ***illumi**gene*® Malaria test device containing lyophilized Malaria test and control reagents.

ASSAY WORKFLOWS

Two workflows are shown below for the ***illumi**gene*® Malaria assay, a simplified workflow with Sample prep device (SMP-prep IV) and with ***M-prep***™ device.
Simple filtration (SMP-prep) workflow: Fifty microliters of whole blood sample or external control mixed with 320 µl of lysis buffer. After a 2 minute incubation, fifty microliters of the lysate added to a simple sample device with filter containing 900 µl of reaction buffer. Five to ten drops of squeezed filtrate from the device collected and added 50 µl each to both test and control devices and loaded the device onto ***illumi**pro-10*™ reader. The workflow is outlined in Figure 1 below.

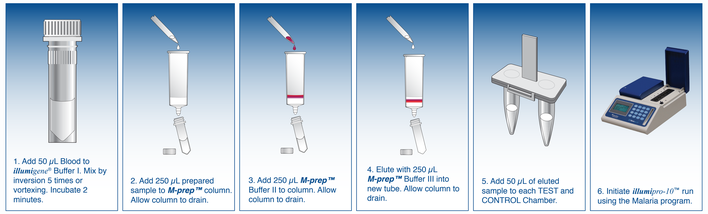
FIGURE 1
***illumi**gene*® Malaria simple filtration (SMP-prep) workflow



M-PREP Workflow

Fifty microliters of whole blood sample or external control mixed with 320 µl of lysis buffer. After 2 minutes incubation, 250 µl of the lysate added to ***M-prep***™ gel filtration column followed by gravity based wash and elution steps. Fifty microliters of eluted sample added to both test and control devices and loaded the device onto ***illumi**pro-10*™ reader. The workflow is outlined in Figure 2 below.

FIGURE 2.
illumi**gene*® Malaria ***M-prep™ workflow



RESULTS

The Limit of Detection (LoD) of *P. falciparum* was determined by Probit modelling to be 2.02 parasites/µl of blood with the SMP-prep method and 0.29 parasites/µl with ***M-prep***™ method. The assay also detected all the five human-infecting *Plasmodium* species. Sensitivity for both sample preparation methods was determined to be 99.0%, and the specificity to be 100% by 206 microscopy, RDT or PCR positive, retrospective samples, and 100% sensitive after resolving with in-house PCR method. No cross-reactivity was observed with human genomic DNA and other organisms tested.

ANALYTICAL SENSITIVITY

At least twenty replicates representing five different *Plasmodium falciparum* strains (US05F PH1, US05F Santa Lucia, US08F Nigeria XII, US05F FC27/A3, and USF Benin I) were tested at different concentrations with both SMP-prep (Table 1) and ***M-prep***™ methods (Table 2). A minimum of 20 valid results per dilution were used to determine the LoD using a statistically-based methodology which allows for the determination of LoD with a 95% confidence interval. LoD was calculated using probit analysis that assesses the relationship between the probability of the response and the parasite concentration.

TABLE 1: Parasite levels and the total number of replicates from all the five strains and the pass rate are shown below.

Parasites/ µl	LAMP-Reps	LAMP-Pass	% Pass
4	30	30	100
2	45	42	93.3
1	30	24	80.0
0.5	30	13	43.3

Probit Analysis: 95% = 2.02 Parasites/ µl with 95% CI 1.51 - 3.53

TABLE 2: Parasite levels and the total number of replicates from all the five strains

Parasites/ µl	LAMP-Reps	LAMP-Pass	% Pass
0.8	20	20	100
0.4	20	19	95
0.2	20	19	95
0.1	20	12	60

Probit Analysis: 95% = 0.29 Parasites/ µl with 95% CI 0.19 - 1.0

CLINICAL SENSITIVITY

To confirm the presence of *Plasmodium* target sequence in clinical samples, retrospective clinical samples were tested with the two ***illumi**gene*® Malaria workflows. Retrospective malaria blood specimens obtained from both Discovery Lifesciences and Centers for Disease Control (CDC) were tested along with blood samples collected at both Meridian Bioscience Inc., and Hoxworth blood center with the ***illumi**gene*® Malaria workflows.

TABLE 3: ***illumi**gene*® Malaria: SMP-prep Method

SMP-prep	REFERENCE METHOD: Microscopy, PET-PCR, and negative blood donors		
	POS	NEG	Total
POS	99	0	99
NEG	1	105	106
INV	0	1*	1
Total	100	106	206

*The invalid result was negative after repeat testing
50 positives and 5 negatives were tested in parallel with PET-PCR; 100% agreement
50 positives confirmed by Microscopy: 1 false negative

TABLE 4: ***illumi**gene*® Malaria: ***M-prep***™ method

M-prep™	REFERENCE METHOD: Microscopy, PET-PCR, and negative blood donors		
	POS	NEG	Total
POS	99	0	99
NEG	1	106	107
INV	0	0	0
Total	100	106	206

50 positives and 5 negatives were tested in parallel with PET-PCR ; 100% agreement
50 positives confirmed by Microscopy: 1 false negative

CROSS REACTIVITY

Based on the *Plasmodium* target DNA sequence alignments, the LAMP targets selected for ***illumi**gene*® Malaria are highly conserved within the *Plasmodium* genus. No DNA sequence homology was noticed with the selected targets other than the *Plasmodium* sequences using BLAST sequence analysis software at NCBI against entire content of GenBank non-redundant sequences database. No cross-reactivity was observed with human genomic DNA and with other organisms tested in the cross-reactivity experiments, Table 5.

TABLE 5: List of the organisms tested for ***illumi**gene*® Malaria Cross-reactivity

Sample No.	Name	Concentration	Result
1	*Kingella kingae*	1.2E+08 cfu/mL	Negative
2	*Klebsiella pneumoniae*	1.2E+08 cfu/mL	Negative
3	*Staphylococcus aureus*	1.2E+08 cfu/mL	Negative
4	*Streptococcus pneumoniae*	1.2E+08 cfu/mL	Negative
5	*Vibrio parahaemolyticus*	1.2E+08 cfu/mL	Negative
6	*Bordetella pertussis*	1.2E+08 cfu/mL	Negative
7	Human DNA	200 ng/ µl	Negative
8	*Babesia microti*	8 ng/ µl	Negative
9	*Leptospira interrogans*	10.9 ng/ µl	Negative
10	*Leishmania donovani*	10.4 ng/ µl	Negative
10	*Leishmania donovani*	10.4 ng/ µl	Negative

CONCLUSIONS

The RUO LAMP based ***illumi**gene*® Malaria assay is capable of detecting the *Plasmodium* species at genus level with analytical sensitivity recommended by WHO (2 parasites/µl), while using an extremely simple procedure in less than one hour. This provides a much needed alternative to the more complex molecular test for malaria diagnosis.

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