LB-5300

Molecular detection of *Plasmodium* with Loop Mediated Isothermal Amplification (LAMP) and sensitivity comparison to PET-PCR assay

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INTRODUCTION

Molecular methods like polymerase chain reaction (PCR) can be used to increase the sensitivity of *Plasmodium* detection. However, practical deployment of molecular testing should address certain challenges such as simplification of sample preparation, reagent stability under ambient conditions, ease-of-use for the end-user and 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. The performance of the LAMP based ***illumi**gene*® malaria assay is compared with another real-time amplification based PET-PCR assay.

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 as described below. 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. For the real-time PET-PCR assay comparison, DNA extracted from the blood samples using QIAamp DNA Mini Kit (QIAGEN, CA) and performed the assay at Centers for Disease Control (CDC, Atlanta, GA) as described by Lucchi et al (PLOS ONE, Feb 13, Vol 8, issue 2, e56677).

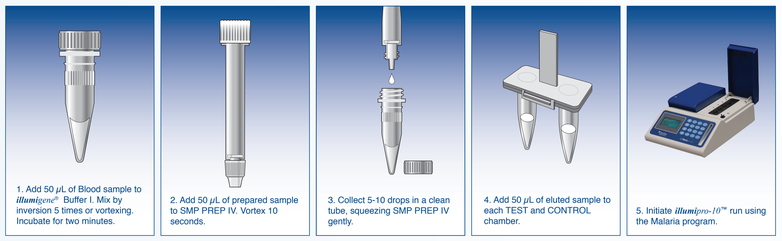
ASSAY WORKFLOWS

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.

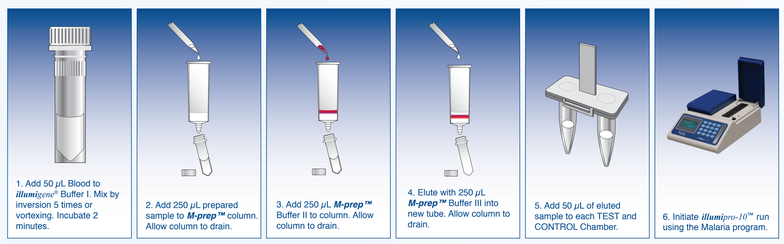
FIGURE 1
***illumi**gene*® Malaria simple filtration (SMP-Prep) workflow



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. The retrospective 50 positive and 5 negative samples are in complete agreement with both illumigene and PET-PCR methods. Only one negative blood sample showed as invalid (1%) with SMP-prep method. With repeat testing, the sample showed as negative.

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 and the pass rate are shown below.

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 Division of Parasitic Diseases and Malaria, Centers for Disease Control (CDC) along with negative blood samples collected from the donors at both Meridian Bioscience Inc., and Hoxworth blood center with the ***illumi**gene*® Malaria workflows. The 50 positive and 5 negative samples tested with both ***illumi**gene*® sample preparation methods are in 100% agreement with the PET-PCR method.

TABLE 3: ***illumi**gene*® Malaria sample preparation apparatus method or SMP-prep

***illumi**gene*® MALARIA SMP-PREP					
Reference method	**POS**	**NEG**	**Invalid**	**Total**	**Performance**
CDC PET-PCR Positive	50	0	0	50	100%
CDC PET-PCR Negative	0	5	0	5	100%
Negative Blood Donors	0	99	1*	100	99%

*The invalid result was negative after repeat testing

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

***illumi**gene*® MALARIA *M-prep*™					
Reference method	**POS**	**NEG**	**Invalid**	**Total**	**Performance**
CDC PET-PCR Positive	50	0	0	50	100%
CDC PET-PCR Negative	0	5	0	5	100%
Negative Blood Donors	0	100	0	100	100%

PET-PCR AND LAMP (***illumi**gene*®) COMPARISON:

Fifty five retrospective samples covering different species of *Plasmodium* including five negative samples tested with the two workflows of ***illumi**gene*® Malaria assay and compared with the CDC developed real-time PET-PCR assay using *Plasmodium* genus specific primers. The Ct values obtained for all the positive samples are below the positive cut-off value of 40.5

TABLE 5: Performance comparison of ***illumi**gene*® Malaria and real-time PET-PCR assay with retrospective clinical samples. The Ct values obtained with the *Plasmodium* genus specific primers are shown below along with the ***illumi**gene*® results.

S. No.	Sample ID	*Plasmodium* species/control	SMP-prep	*M-prep*™	PET-Genus
1	2014004527	Pf	Positive	Positive	22.63
2	2014004197	Pv	Positive	Positive	24.89
3	2014004841	Pf	Positive	Positive	25.2
4	2014004967	Pf	Positive	Positive	30.93
5	2014007904	Po	Positive	Positive	27.28
6	2014004968	Pf	Positive	Positive	23.8
7	3000002244	Pf	Positive	Positive	21.88
8	3000002392	Pf	Positive	Positive	22.97
9	3000024870	Pf	Positive	Positive	21.59
10	3000024871	Pf	Positive	Positive	25.23
11	2014007627	Pf	Positive	Positive	23.34
12	3000024879	Pf	Positive	Positive	23.58
13	3000024875	Pf	Positive	Positive	23.62
14	3000025371	Pf	Positive	Positive	20.83
15	3000027477	Pm	Positive	Positive	25.95
16	3000027036	Pv	Positive	Positive	21.13
17	3000025387	Pf	Positive	Positive	23.96
18	3000027479	Pf	Positive	Positive	26.4
19	3000045801	Pf	Positive	Positive	22.52
20	3000027655	Pf	Positive	Positive	27.35
21	2014007733	Pf	Positive	Positive	21.96
22	3000002790	Pf	Positive	Positive	26.06
23	2014007736	Pf	Positive	Positive	21.07
24	3000027146	Pf	Positive	Positive	22.87
25	3000027145	Pf	Positive	Positive	26.75
26	3000027148	Pf	Positive	Positive	28.15
27	3000027147	Pf	Positive	Positive	30.6
28	3000027143	Pv	Positive	Positive	27.8
29	3000046519	Pf	Positive	Positive	25.58
30	3000046520	Pv	Positive	Positive	34.25
31	3000045800	Pf	Positive	Positive	25.43
32	3000027480	Pv	Positive	Positive	26.36
33	3000046565	Pf	Positive	Positive	24.96
34	3000045799	Pv	Positive	Positive	24.89
35	3000046595	Pv	Positive	Positive	24.59
36	2013011785	Pf	Positive	Positive	24.3
37	2013011786	Pf	Positive	Positive	22.76
38	2013011903	Pf	Positive	Positive	23.12
39	2013011563	Pf	Positive	Positive	24.63
40	2013014739	Pf	Positive	Positive	26.88
41	2013014907	Pf	Positive	Positive	36.08
42	2013017286	Pv	Positive	Positive	32.51
43	2014004126	Pf	Positive	Positive	22.86
44	2014004203	Po	Positive	Positive	38.91
45	2014004204	Po	Positive	Positive	28.02
46	2014000290	Pf	Positive	Positive	26.9
47	300002270	Po	Positive	Positive	25.54
48	300002209	Po	Positive	Positive	28.31
49	300002971	Pv	Positive	Positive	23.83
50	3000002791	Pv	Positive	Positive	26.45
51	1001	Negative	Negative	Negative	No Ct
52	1002	Negative	Negative	Negative	No Ct
53	1003	Negative	Negative	Negative	No Ct
54	1004	Negative	Negative	Negative	No Ct
55	1005	Negative	Negative	Negative	No Ct
56	LAMP Positive	Positive	Positive	Positive	
57	LAMP Negative	Negative	Negative	Negative	
58	PET-PCR Positive	Positive			Positive
59	PET-PCR Negative	Negative			Negative

Pf: *P. falciparum*, Pv: *P. vivax*, Pm: *P. malariae*, Po: *P. ovale*

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. The performance of the ***illumi**gene*® assay is also comparable to the real-time amplification based PET-PCR assay. This provides a much needed alternative to the more complex molecular test for malaria diagnosis.

ACKNOWLEDGEMENTS

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