

# Detection of Falciparum Malaria Imported From Africa With a Highly Specific and Sensitive Loop-Mediated Isothermal Amplification (LAMP) Assay

**Xi Chen**

Kunming Medical University

**Maohua Pan**

Shanglin County People's Hospital

**Yaming Huang**

Kunming Medical University

**Pien Qin**

Shanglin County People's Hospital

**Siqi Wang**

Kunming Medical University

**Yu Si**

Kunming Medical University

**Xinxin Li**

Kunming Medical University

**Weilin Zeng**

Kunming Medical University

**Yanrui Wu**

Kunming Medical University

**Mengxi Duan**

Kunming Medical University

**xiaosong Li**

Kunming Medical University

**Xun Wang**

Kunming Medical University

**Hui Zhao**

Kunming Medical University

**Jiaqi Zhang**

Kunming Medical University

**Yucheng Qin**

Shanglin County People's Hospital

**Yanmei Zhang**

Kunming Medical University

**Zhaoqing Yang** (✉ [zhaoqingy92@hotmail.com](mailto:zhaoqingy92@hotmail.com))

Kunming Medical University

**Dominique Mazier**

Sorbonne University

**Zheng Xiang**

Kunming Medical University

**Qi Yang**

Kunming Medical University

---

## Research Article

**Keywords:** LAMP, malaria, molecular diagnosis, Plasmodium falciparum

**Posted Date:** March 17th, 2021

**DOI:** <https://doi.org/10.21203/rs.3.rs-288704/v1>

**License:**   This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

---

# Abstract

**Background:** Current malaria diagnostic tests, including microscopy and antigen-detecting rapid tests, cannot reliably detect low-density infections. Molecular methods such as polymerase chain reaction (PCR) are highly sensitive but remain too complex to be used in the field. To complement existing diagnostic methods, a new LAMP assay for the detection and identification of *Plasmodium falciparum* imported from Africa was developed.

**Methods:** A LAMP assay was developed to amplify the Actin I gene of *P. falciparum*. Microscopy, Nested PCR and the LAMP assay were conducted on 466 samples of suspected malaria patients imported from Africa to evaluate the new assay's sensitivity and specificity. A plasmid construct, cultured *P. falciparum*, and the clinical samples were used to evaluate the the limit of detection (LOD).

**Results:** Compared to Nested PCR, the sensitivity and specificity of the nove developed LAMP were 100 % (95% CI 98.54 - 100%) and 99.07 % (95% CI 96.68 - 99.89%) respectively. The LAMP assay was found to be highly sensitive, with the detection limit as low as  $10^2$  copies/ $\mu$ L and 10-fold higher detection limit than single PCR when performed on serial dilutions of the plasmid construct. The LAMP assay detected 0.01 parasites/ $\mu$ L, when cultured *P. falciparum* was used as template. The novel LAMP assay detected 1-7 parasites / $\mu$ L blood in clinical samples—which is more sensitively than the commercial product (Loopamp MALARIA Pan/Pf detection kit; Eiken Chemical Co., Tokyo, Japan) for clinical samples detaction of *P. falciparum*, which detected >20 parasites / $\mu$ L blood sample.

## Conclusion:

For the first time, the Actin I gene of *P. falciparum* was used as target for LAMP primers to detect imported *P. falciparum* from Africa imported to China. The novel, highly specific and sensitive LAMP assay is a practical and effective diagnostic method for detecting low-density *P. falciparum* infections imported from Africa. For the first time, such LAMP primers were evaluated by using a plasmid construct, cultured *P. falciparum* and clinical samples at same work plafom, which offer excellent means of assay validation and should be considered in future studies of this kind.

## Background

Malaria is a major cause of morbidity and mortality, leading up to 228 million cases and 405,000 deaths in 2018 (1). With increasing international trade, malaria infection among Chinese has grown. In 2017, 2,858 of 2,861 malaria cases (99.9%) were imported from abroad, of which 822 cases (63.7%) were caused by *Plasmodium falciparum* (2), which is responsible for most malarial cases in Africa (3). *Plasmodium vivax*, *Plasmodium malariae*, *Plasmodium ovale* and *Plasmodium knowlesi* are responsible for other cases. Because different species may require distinct treatment approaches, rapid and accurate diagnosis of *P. falciparum* is not only crucial for treatment, but also important for disease control, especially for elimination of malaria.

Microscopic examination of blood smears is the most widely used diagnostic approach in the field and remains the gold standard. This low-cost method allows parasitaemia to be evaluated and permits differentiation among *Plasmodium* species. Nevertheless, the limit of detection of microscopy is in the range of 10-100 parasites/ $\mu$ L, depending on the health professional's skill and the quality of the smear (4). Rapid diagnostic tests (RDT) (5, 6) provide a convenient diagnostic method because no special equipment or special skills are required, and the results can be easily obtained within a short time. However, microscopy and RDTs cannot reliably detect lower-density parasitaemia (<100 parasites/ $\mu$ L) (7). Molecular diagnostic tools employing DNA amplification have the advantage of high accuracy and high sensitivity; of these, nested PCR is considered sensitive and specific tool for malaria diagnosis(8). Nevertheless, this method is costly and requires trained personnel for its proper implementation.

Loop-mediated isothermal amplification (LAMP), originally developed by Notomi *et al*, is a very sensitive, easy and time saving method (9). The advantages of the LAMP method include high amplification efficiency under isothermal conditions (60-65°C), visual judgment based on the turbidity or fluorescence of the reaction mixture, and no need for expensive equipment (10). The LAMP method has detected as few as 100 copies of DNA template in blood samples (equal roughly to 5 parasites/ $\mu$ L) (11, 12). Currently, two commercial kits are available in the market: LoopAmp malaria (Pan/Pf) detection kit (Eiken Chemical Company, Tokyo, Japan) and Illumigene malaria LAMP assay (Meridian Biosciences, Cincinnati, USA). Each detects symptomatic malaria cases with high sensitivity and specificity (13-18). However, the evaluation of specificity and sensitivity of LAMP in large Chinese population in China has barely been reported (19-22), perhaps because commercialized LAMP malaria diagnostic kits are not available in China (<https://www.human.de/products/molecular-dx/malaria-lamp>).

Previous work established LAMP as more sensitive than microscopy or RDT to detect low-parasitemia infections (13-18, 23-26), but some showed negative results (10, 27-29). Assays targeting 18srRNA (13-18, 30) achieve sensitivity and specificity matching or exceeding PCR. Similar sensitivity and specificity are found targeting mitochondrial genes (11), apicoplast genome (31, 32), *Plasmodium* helical interspersed sub-telomeric superfamily (*PHIST*) (33), and *Pfdhfr* gene by Yongkiettrakul *et al.* (34), for *falciparum* malaria diagnosis as well as identified drug resistance associated genetic markers in *P. falciparum*, such as PfSNP-LAMP (35-40).

The aim of this study was to develop a novel LAMP assay for screening large numbers of clinical samples for *P. falciparum*, and to evaluate its specificity, sensitivity and clinical applicability for the imported malaria from Africa with low-density infections.

## Methods

### Sample collection and DNA extraction

3D7 clones of *P. falciparum* and a Strain H clone of *P. knowlesi* were obtained from the Malaria Research and Reference Reagent Resource Center (MR4). At least 3 samples of *Toxoplasma gondii* and *Schistosoma*

*japonicum* were obtained from the Department of Pathogen Biology and Immunology, Kunming Medical University.

Recruited patients were 22 to 68 years of age who presented with fever or history of fever within the last 48 h, having symptoms suggestive of malaria, and with travel history of Africa in two weeks. These samples were obtained at the Shanglin County People's Hospital, Guangxi, China, between 2016 and 2018. Malaria infections were diagnosed by microscopic examination of Giemsa-stained thick and thin blood films. Data of all cases were recorded, including gender, ethnicity, occupation, microscopic examination results and travel history. The human subject protocol for this study was approved by the Shanglin Hospital Institutional Review Board. Written informed consents were obtained from all included participants.

5mL venous blood was collected from every patient and 200uL was used for DNA extraction. DNA was extracted from the studied parasites and blood samples were obtained by using the High Pure PCR Template Preparation Kit (Roche) following the manufacturer's instructions.

### Microscopic diagnosis

Thick and thin blood smears were made and stained by Giemsa for observation. For thick smear, *Plasmodium* parasites were counted in fields totaling 200 WBCs (or 500 WBCs when the initial number of parasites was less than 99) on thick blood films. Parasite densities were recorded as the average value of parasites per 200 leukocytes as counted by 2 hospital laboratory staff (41). Parasite densities (parasite/ $\mu$ L whole blood) were estimated by following the World Health Organization (WHO) recommended method (number of parasites /WBC)  $\times$  8000 WBC count/ $\mu$ L.(42).

### Nested PCR assay

The species of malaria in a sample were determined by using a nested PCR assay. This assay targets the *Plasmodium* 18s rRNA gene, using previously published primers (43, 44). Briefly, for the first round, a 1 $\mu$ L DNA sample was used as template. For the second round, 1 $\mu$ L of PCR products of the first round was used as a template.

### LAMP primers designed and conditions of the LAMP reactions

The full length *P. falciparum* Actin I gene (GenBank accession no. XM\_001350811) is 1,131 bp; a 238 bp fragment (position 4 to 241) was selected as the target sequence for primer design and LAMP detection. Primer design was performed using LAMP Designer 1.15 (PREMIER Biosoft). The primers are listed in **Table 1** (patent applying no: 201910611312.9, China). Briefly, a reaction contained 1 $\mu$ L of DNA template, 40 pmol each of the FIP and BIP, 5 pmol each of the F3 and B3 primers, 20 pmol each of the LoopF and LoopB, 1 $\mu$ L of fluorescent detection reagent (Eiken), 1 $\mu$ L of *Bst* enzyme (Eiken), 12.5 $\mu$ L RM (Eiken) and DDW was added to make a 25  $\mu$ L reaction mix. In a thermostatic metal heating block, the mixture was incubated at 63°C for 45 min, and then the reaction was terminated by heating the mixture at 80°C for 5 min. After the reaction, the amplified products in the reaction tube were detected under visible light with

the naked eye according to the manufacturer's instructions, it changed from clear and orange to green and turbid; or under UV light the solution emitted fluorescent if it is the positive.

The assay results were observed independently by two experimenters who didn't know the PCR/microscopy results. When their evaluation conflicted, a third determination was solicited. This experiment was conducted in the laboratory of pathogen biology at Kunming Medical University.

### **Construction of positive control plasmid DNA**

PCR products obtained following amplification of 3D7 DNA with LAMP primers F3 and B3 (Table 1) were inserted in to pMD<sup>TM</sup>19 -T Vector (Takara). The PCR was performed at 94°C for 5 min for denaturation, followed by 35 cycles; 94°C for 30 s, 54°C for 30 s, 72°C for 1 min, and final extension 72°C for 5 min. PCR products were cloned into vector following its manufacturer's instructions. The recombinant pMD<sup>TM</sup>19 -T plasmid DNA cloned *P. falciparum* Actin I gene fragment was extracted by a Miniprep kit (TIANGEN) from *Escherichia coli* (DH5a strain), the concentration was measured by a spectrophotometer and copy numbers were calculated by following previously reported method (34).

### **Specificity and sensitivity of LAMP**

The specificity of the assay was tested using genomic DNAs (gDNAs) of various parasites (*T.gondii* and *S. japonicum*, *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale* and *P. knowlesi*).

For sensitivity testing, three DNA templates were prepared. 10-fold serial dilutions of constructed plasmid DNA using as the template, the LAMP reaction was compared with the single-PCR assay using F3 and B3 primers (described above), cultivated parasites prepared as previously described (45) and 10-fold serial dilutions of gDNAs of clinical malaria patients using as the templates, the comparison was performed between the LAMP and nested PCR. For comparison, patented LAMP primers (10, 46) (target gene 18srRNA) were also employed. Each sample was assayed at least 3 times.

### **Clinical applicability**

The clinical applicability of the LAMP was evaluated by using whole-blood samples collected from Shanglin County People's Hospital. Nested PCR was used as the reference as lab standard of sensitivity, specificity. Positive predictive value and Negative predictive value were calculated. Each sample was assayed at least 3 times.

### **Statistical analysis**

All data were analyzed with GraphPad Prism 6.0 and MEDCALP statistical software available at [https://www.medcalc.org/calc/diagnostic\\_test.php](https://www.medcalc.org/calc/diagnostic_test.php). Sensitivity, specificity, and positive and negative predictive values of LAMP were determined using nested PCR as the gold standard for diagnosis of malaria. The concordance response rate (percentage of responses with both positive and both negative results) and Kappa value (k) was determined to measure degree of agreements between two diagnostic

test results. Wilcoxon matched-pairs signed rank test and Chi-square test was performed. *P* value below 0.05 was considered statistically significant. LOD calculated by following the report(47).

## Results

### Evaluation of the specificity of LAMP primers

The specificity of the LAMP primers was investigated by using various parasites, including *Plasmodium* gDNAs as templates for LAMP. As shown in **Figure 1**, a green fluorescence was detected in *P. falciparum* gDNA (tube 1) but not when the template DNA derived from other parasite species.

### Evaluation of the sensitivity of LAMP primers

To examine the sensitivity of LAMP, three detection methods were used. First, the new LAMP assay was compared to the single-PCR assay using F3 and B3 primers by amplifying 10-fold serial dilutions of plasmid DNA. As shown in **Figure 2**, amplification by real-time LAMP was obtained in reaction tubes containing from  $10^9$  to  $10^2$  copies/ $\mu$ L of the DNA template in a 45-min reaction with SYBR Green, with the limit of detection (LOD) was  $10^2$ copies/ $\mu$ L. In contrast, the LOD for PCR using the F3 and B3 primers was  $10^3$ copies/ $\mu$ L. Therefore, the sensitivity of the LAMP was 10-fold greater than that of the single-PCR assay.

Secondly, using DNA from cultured parasites, the LOD of nested PCR and LAMP were both 0.01 parasites/ $\mu$ L. Thirdly, LAMP and nested PCR were evaluated by using DNA from 9 clinical samples, whole blood samples as templates (**Table S1**). The lowest rang and LOD of the nested PCR was 0.7-7 and 2.9 parasites/ $\mu$ L (95% CI 1.4 - 6.0) respectively. The lowest rang and LOD of LAMP primers were 1-7 and 3.8 (95% CI 2.0 - 7.0) parasites/ $\mu$ L respectively. Both showed similar LOD ( $P=0.3466$ ). The patent primers (Japan ) (10, 46) showed similar LOD ( $P=0.3466$ ).

### Comparison of the sensitivity and specificity among the LAMP, nested PCR, and microscopy for malaria diagnosis in the clinical samples

A total of 466 suspected malaria clinical samples were used to evaluate the performance of this set of primers for the detection of *P. falciparum* in clinical samples. Microscopy identified parasites with the range in parasite densities of the clinical samples 114-125240 parasite/ $\mu$ L blood. The foreign travel history of these individuals, and the malaria species these identified in by nested PCR, are listed in supplemental Table 1. Among the 466 samples, microscopy identified *P. falciparum* in 202, whereas nested PCR and the LAMP assay identified the parasite in 251 and 253, respectively (**Table 2**). Prevalence estimates derived from the two molecular amplification methods did not differ significantly from each other, but each was significantly greater than microscopy (nested PCR vs. Microscopy  $P= 0.0013$ ; LAMP vs. microscopy  $P= 0.0008$ ). The comparison of sensitivity and specificity, of microscopy, nested PCR and LAMP is shown in **Table 3a, 3b** and **3c**: all 251 nested PCR-positive samples for *P. falciparum* were also detected by the novel LAMP (sensitivity of 100%; 95% CI: 98.54 - 100%), Kappa = 0.99, 188 were detected

by microscopy (sensitivity of 74.90%; 95% CI: 69.06 - 80.14%), Kappa = 0.66. 213 nested PCR-negative samples for *P. falciparum* were also negative by the LAMP assay (specificity of 99.07%; 95% CI: 96.68 - 99.89%), by microscopy 198 were negative (specificity of 92.09%; 95% CI: 87.64 - 95.33%). LAMP successfully detected *P. falciparum* in the 40 mixed infections.

## Discussion

The risk of acquiring locally-transmitted malaria has been virtually eliminated in China, but imported malaria has grown. The diagnosis of malaria at primary clinics has traditionally been performed by microscopic examination of blood smears because of its ease and rapid application. Loop-mediated isothermal amplification (LAMP), a novel method developed by Notomi *et al.* is able to amplify DNA with great efficacy within an hour under isothermal conditions(9); it has been widely used as a diagnostic tool for several parasitic diseases(21, 48). Nevertheless, the LAMP method has not been implemented in a large Chinese clinical study (19-22). In this study, we have done so, and evaluated its specificity, sensitivity and clinical applicability for the detection of *P. falciparum* imported from Africa for the first time.

Validating practical assays for detecting imported malaria diagnosis is important for China to reach the goal of malaria elimination. We successfully targeted the Actin I gene of *P. falciparum* for species identification on a large sample of clinical isolates and found it to demonstrate high sensitivity and specificity. The *P. falciparum* Actin I gene was chosen as the target region, because it is a highly-conserved housekeeping gene and differs among the 5 species of *Plasmodium*.

Compared with the patent primers (Japan), targeting of 18sRNA, our assay demonstrated a comparable LOD ( $P=0.3466$ ); its sensitivity and specificity were 95% and 99%, respectively (45, 49). Our new assay appear more sensitive than those reported previously (19, 30). Our LAMP primers showed sensitivity of 1-7 parasites / $\mu$ L blood from clinical samples, a better performance in clinical patients than reported for the commercial kit, Eiken Loopamp™ MALARIA Pan/Pf detection kit (Eiken Chemical Co., Tokyo, Japan), reported LOD for that assay is >20 parasites / $\mu$ L blood for *P. falciparum* (30). The LOD of our LAMP was 100 copies, which was similar with Eiken Loopamp™ (10). Thus the present assay and Eiken Loopamp™ perform comparably when amplifying recombinant plasmid DNA.

Ponce *et al.* reported that the LOD of Malaria diagnostic kit (Illumigene®) was 0.1 Parasites/ $\mu$ L(44). They used serial dilutions of DNA extracted from cultured clones of the 3D7 and W2 strains of *P. falciparum*. We used the same dilution method with cultured parasites in the initial stage, and we also found it capable of detecting as few as 0.01 parasite/ $\mu$ L. Diluting cultured parasites to evaluate the sensitivity of the reaction does not, however, mirror actual conditions in clinical samples, because culture medium is likely to contain large amounts of DNA from parasites that have died. The concentration of DNA extracted, consequently, may be much higher than the concentration derived from low-parasitemia infections. Furthermore, red blood cell suspensions do not offer the same quantitative accuracy as do diluted DNA solutions.

This set of primers could reach the LOD of  $10^2$  copy/ $\mu$ L when tested with recombinant plasmid DNA, and higher than PCR with F3 and B3. Similar results were obtained by other teams (50). We believe that the recombinant plasmid DNA serves as a more precise means to define the sensitivity of such reactions. In our experiment, we used both the clinical samples and recombinant plasmid DNA as template to be used to evaluate the sensitivity; each showed similar results, high sensitivity and specificity. We strongly recommend use the two template types to aid inter-laboratory comparisons and validate clinical relevancy.

The LAMP assay in our study proved to be highly specific, and there was no nonspecific reaction to some common parasites or to other *Plasmodium* species. This set of primers presented 100% sensitivity (95% CI 98.54 - 100%) and 99.07 % specificity (95% CI 96.68 - 99.89%) compared to nested PCR ( $P = 0.3466$ ). This is as good or better than previously reported and commercially licensed assays (12, 50). Microscopy proved less reliable in cases of low parasitaemia. LAMP and PCR were discordant in two cases, wherein only the LAMP assay was positive. These two cases were confirmed by effective clinical antimalarial treatment, allowing us to conclude that these were true positives. Although this LAMP assay was carried out in the laboratory, it can be used in the field due to its simple equipment requirements and uncomplicated operating procedures. Compared with nested PCR, LAMP reaction time was only 45 minutes, which greatly improved the detection efficiency and provided timely evidence for clinical medication strategy.

Based on the results obtained, this LAMP assay offers an alternative for molecular diagnosis for *P. falciparum* infection in non-malaria-endemic regions, like China. This will strengthen the control and prevention of imported malaria in China.

## Declarations

### Availability of data and materials

The datasets during and/or analysed during the current study available from the corresponding author on reasonable request.

### Acknowledgments

First and foremost, we thank all the patients that donated blood for volunteering to participate in the study. We thank laboratory staffs for culturing *P. falciparum* 3D7. The authors thank Dr. Benjamin M. Rosenthal for his English editing and all the patients for volunteering to participate in the study.

### Funding

This study was supported by the National Science Foundation of China (31860604 and U1802286), Major science and technology projects of Yunnan Province (2018ZF0081) and International Science and Technology Cooperation-Yunnan International Science and Technology Cooperation Base

(202003AE140004). XC, ZX and YZ were under sponsoring from the Yunnan Applied Basic Research Projects-Union Foundation (2018FE001-190 and 2019FE001-015 and 2015FB034 respectively). YW was supported by the Hundred-Talent Program of Kunming Medical University (60117190439), Foundation of the Education Department of Yunnan Province (2018JS151), and the Innovation Experiment Project of Yunnan Province (202010678064) and Kunming Medical University (2020JXD014). YQ was supported by Guangxi Zhuang Autonomous Region Health Commission of Scientific Research Project (Z20190892).

### **Ethics approval and consent to participate**

We used clinical samples obtained from the Department of Infectious Diseases in Shanglin Hospital, Shanglin County, Guangxi Province, China and from the Malaria surveillance clinic, Tengchong County, Yunnan Province, China. The clinical subjects were Chinese migrant workers returned from Africa or the China-Myanmar border. This study was originally approved by the Institutional Review Board of Shanglin Hospital and Institutional Review Board of Kunming Medical University. A written informed consent was obtained from the participants. All methods were carried out in accordance with relevant guidelines and regulations/declaration of Helsinki.

### **Potential conflicts of interest.**

All authors: No reported conflicts of interest.

### **Consent for publication**

Not applicable.

### **Availability of data and materials**

All data generated or analyzed during this study are included in this published article and its supplementary information file. The datasets of clinical malaria samples used and analyzed during the present study are available from the corresponding author on reasonable request.

### **Authors' contributions**

Jiaqi Zhang: Conceptualization, Investigation, Writing - Original Draft, Visualization

Xi Chen: Methodology, Formal analysis, Supervision

Maohua Pan: Investigation, Methodology, Supervision

Yucheng Qin: Validation, Investigation

Hui Zhao: Validation, Data Curation

Siqi Wang: Validation, Data Curation

Pien Qin: Investigation, Resources

Yu Si: Resources, Validation

Qi Yang: Resources, Validation

Xinxin Li: Resources, Validation

Weilin Zeng: Supervision

Zheng Xiang: Data Curation

Yanrui Wu: Visualization, Supervision

Mengxi Duan: Resources, Validation

Xiaosong Li: Resources, Validation

Xun Wang: Resources, Validation

Dominique Mazier: Writing - Review & Editing

Yanmei Zhang: Conceptualization, Data Curation

Yaming Huang: Resources: Project administration, Conceptualization

Zhaoqing Yang: Conceptualization, Methodology, Funding acquisition

## References

1. WHO. WORLD MALARIA REPORT 2018. 2018.
2. Zhang L FJ, Zhang S, Xia Z, Zhou S. The progress of national malaria elimination and epidemiological characteristics of malaria in China in 2017. *Chin J Parasitol Parasit Dis.* 2018;36:201-9.
3. Wongsrichanalai C, Barcus MJ, Muth S, Sutamihardja A, Wernsdorfer WH. A review of malaria diagnostic tools: microscopy and rapid diagnostic test (RDT). *The American journal of tropical medicine and hygiene.* 2007;77(6 Suppl):119-27.
4. Guthmann JP, Ruiz A, Priotto G, Kiguli J, Bonte L, Legros D. Validity, reliability and ease of use in the field of five rapid tests for the diagnosis of *Plasmodium falciparum* malaria in Uganda. *Trans R Soc Trop Med Hyg.* 2002;96(3):254-7.
5. WHO. Malaria rapid diagnostic test performance, Results of WHO product testing of malaria RDTs (2009). 2010.
6. Banoo S, Bell D, Bossuyt P, Herring A, Mabey D, Poole F, et al. Evaluation of diagnostic tests for infectious diseases: general principles. *Nat Rev Microbiol.* 2006;4(12 Suppl):S20-32.

7. Milne LM, Kyi MS, Chiodini PL, Warhurst DC. Accuracy of routine laboratory diagnosis of malaria in the United Kingdom. *Journal of clinical pathology*. 1994;47(8):740-2.
8. Notomi T, Okayama H, Masubuchi H, Yonekawa T, Watanabe K, Amino N, et al. Loop-mediated isothermal amplification of DNA. *Nucleic Acids Res*. 2000;28(12):E63.
9. Mori Y, Nagamine K, Tomita N, Notomi T. Detection of loop-mediated isothermal amplification reaction by turbidity derived from magnesium pyrophosphate formation. *Biochem Biophys Res Commun*. 2001;289(1):150-4.
10. Han ET, Watanabe R, Sattabongkot J, Khuntirat B, Sirichaisinthop J, Iriko H, et al. Detection of four *Plasmodium* species by genus- and species-specific loop-mediated isothermal amplification for clinical diagnosis. *J Clin Microbiol*. 2007;45(8):2521-8.
11. Polley SD, Mori Y, Watson J, Perkins MD, González IJ, Notomi T, et al. Mitochondrial DNA targets increase sensitivity of malaria detection using loop-mediated isothermal amplification. *J Clin Microbiol*. 2010;48(8):2866-71.
12. Liu H, Feng G, Zeng W, Li X, Bai Y, Deng S, et al. A more appropriate white blood cell count for estimating malaria parasite density in *Plasmodium vivax* patients in northeastern Myanmar. *Acta Trop*. 2016;156:152-6.
13. Hopkins H, González IJ, Polley SD, Angutoko P, Ategeka J, Asiimwe C, et al. Highly sensitive detection of malaria parasitemia in a malaria-endemic setting: performance of a new loop-mediated isothermal amplification kit in a remote clinic in Uganda. *J Infect Dis*. 2013;208(4):645-52.
14. Polley SD, González IJ, Mohamed D, Daly R, Bowers K, Watson J, et al. Clinical evaluation of a loop-mediated amplification kit for diagnosis of imported malaria. *J Infect Dis*. 2013;208(4):637-44.
15. Mohon AN, Lee LD, Bayih AG, Folefoc A, Guelig D, Burton RA, et al. NINA-LAMP compared to microscopy, RDT, and nested PCR for the detection of imported malaria. *Diagn Microbiol Infect Dis*. 2016;85(2):149-53.
16. Lucchi NW, Gaye M, Diallo MA, Goldman IF, Ljolje D, Deme AB, et al. Evaluation of the Illumigene Malaria LAMP: A Robust Molecular Diagnostic Tool for Malaria Parasites. *Sci Rep*. 2016;6:36808.
17. Rypien C, Chow B, Chan WW, Church DL, Pillai DR. Detection of *Plasmodium* Infection by the illumigene Malaria Assay Compared to Reference Microscopy and Real-Time PCR. *J Clin Microbiol*. 2017;55(10):3037-45.
18. Perera RS, Ding XC, Tully F, Oliver J, Bright N, Bell D, et al. Development and clinical performance of high throughput loop-mediated isothermal amplification for detection of malaria. *PLoS One*. 2017;12(2):e0171126.
19. Zhang Y, Yao Y, Du W, Wu K, Xu W, Lin M, et al. Development of loop-mediated isothermal amplification with *Plasmodium falciparum* unique genes for molecular diagnosis of human malaria. *Pathog Glob Health*. 2017;111(5):247-55.
20. Mao R, Ge G, Wang Z, Hao R, Zhang G, Yang Z, et al. A multiplex microfluidic loop-mediated isothermal amplification array for detection of malaria-related parasites and vectors. *Acta Trop*. 2018;178:86-92.

21. Tao ZY, Zhou HY, Xia H, Xu S, Zhu HW, Culleton RL, et al. Adaptation of a visualized loop-mediated isothermal amplification technique for field detection of *Plasmodium vivax* infection. *Parasit Vectors*. 2011;4:115.
22. Lu F, Gao Q, Zhou H, Cao J, Wang W, Lim CS, et al. Molecular test for vivax malaria with loop-mediated isothermal amplification method in central China. *Parasitol Res*. 2012;110(6):2439-44.
23. Charpentier E, Benichou E, Pagès A, Chauvin P, Fillaux J, Valentin A, et al. Performance evaluation of different strategies based on microscopy techniques, rapid diagnostic test and molecular loop-mediated isothermal amplification assay for the diagnosis of imported malaria. *Clin Microbiol Infect*. 2020;26(1):115-21.
24. Imai K, Tarumoto N, Misawa K, Runtuwene LR, Sakai J, Hayashida K, et al. A novel diagnostic method for malaria using loop-mediated isothermal amplification (LAMP) and MinION™ nanopore sequencer. *BMC Infect Dis*. 2017;17(1):621.
25. Vásquez AM, Vélez G, Medina A, Serra-Casas E, Campillo A, Gonzalez IJ, et al. Evaluation of highly sensitive diagnostic tools for the detection of *P. falciparum* in pregnant women attending antenatal care visits in Colombia. *BMC Pregnancy Childbirth*. 2020;20(1):440.
26. Buates S, Bantuchai S, Sattabongkot J, Han ET, Tsuboi T, Udomsangpetch R, et al. Development of a reverse transcription-loop-mediated isothermal amplification (RT-LAMP) for clinical detection of *Plasmodium falciparum* gametocytes. *Parasitol Int*. 2010;59(3):414-20.
27. Ocker R, Prompunjai Y, Chutipongvivate S, Karanis P. MALARIA DIAGNOSIS BY LOOP-MEDIATED ISOTHERMAL AMPLIFICATION (LAMP) IN THAILAND. *Rev Inst Med Trop Sao Paulo*. 2016;58:27.
28. Kollenda H, Hagen RM, Hanke M, Rojak S, Hinz R, Wassill L, et al. Poor Diagnostic Performance of a Species-Specific Loop-Mediated Isothermal Amplification (LAMP) Platform for Malaria. *Eur J Microbiol Immunol (Bp)*. 2018;8(4):112-8.
29. Paris DH, Imwong M, Faiz AM, Hasan M, Yunus EB, Silamut K, et al. Loop-mediated isothermal PCR (LAMP) for the diagnosis of falciparum malaria. *Am J Trop Med Hyg*. 2007;77(5):972-6.
30. Piera KA, Aziz A, William T, Bell D, González IJ, Barber BE, et al. Detection of *Plasmodium knowlesi*, *Plasmodium falciparum* and *Plasmodium vivax* using loop-mediated isothermal amplification (LAMP) in a co-endemic area in Malaysia. *Malar J*. 2017;16(1):29.
31. Oriero EC, Okebe J, Jacobs J, Van Geertruyden JP, Nwakanma D, D'Alessandro U. Diagnostic performance of a novel loop-mediated isothermal amplification (LAMP) assay targeting the apicoplast genome for malaria diagnosis in a field setting in sub-Saharan Africa. *Malar J*. 2015;14:396.
32. Oriero CE, van Geertruyden JP, Jacobs J, D'Alessandro U, Nwakanma D. Validation of an apicoplast genome target for the detection of *Plasmodium* species using polymerase chain reaction and loop mediated isothermal amplification. *Clin Microbiol Infect*. 2015;21(7):686.e1-7.
33. Zhang YJ, Sun B, Shen HF, Wu K, Song LJ, Shen S, et al. [Establishment and evaluation of loop-mediated isothermal amplification based on *Plasmodium falciparum* unique genes coding PHIST proteins]. *Zhongguo Xue Xi Chong Bing Fang Zhi Za Zhi*. 2016;28(1):39-44, 50.

34. Yongkiettrakul S, Jaroenram W, Arunrut N, Chareanchim W, Pannengpetch S, Suebsing R, et al. Application of loop-mediated isothermal amplification assay combined with lateral flow dipstick for detection of *Plasmodium falciparum* and *Plasmodium vivax*. *Parasitol Int.* 2014;63(6):777-84.
35. Mohon AN, Menard D, Alam MS, Perera K, Pillai DR. A Novel Single-Nucleotide Polymorphism Loop Mediated Isothermal Amplification Assay for Detection of Artemisinin-Resistant *Plasmodium falciparum* Malaria. *Open Forum Infect Dis.* 2018;5(4):ofy011.
36. Chahar M, Mishra N, Anvikar A, Dixit R, Valecha N. Establishment and application of a novel isothermal amplification assay for rapid detection of chloroquine resistance (K76T) in *Plasmodium falciparum*. *Sci Rep.* 2017;7:41119.
37. Yongkiettrakul S, Kampeera J, Chareanchim W, Rattanajak R, Pornthanakasem W, Kiatpathomchai W, et al. Simple detection of single nucleotide polymorphism in *Plasmodium falciparum* by SNP-LAMP assay combined with lateral flow dipstick. *Parasitol Int.* 2017;66(1):964-71.
38. Imai K, Tarumoto N, Runtuwene LR, Sakai J, Hayashida K, Eshita Y, et al. An innovative diagnostic technology for the codon mutation C580Y in kelch13 of *Plasmodium falciparum* with MinION nanopore sequencer. *Malar J.* 2018;17(1):217.
39. Chahar M, Anvikar A, Dixit R, Valecha N. Evaluation of four novel isothermal amplification assays towards simple and rapid genotyping of chloroquine resistant *Plasmodium falciparum*. *Exp Parasitol.* 2018;190:1-9.
40. Chahar M, Anvikar A, Valecha N. Development and Evaluation of a Novel HNB Based Isothermal Amplification Assay for Fast Detection of Pyrimethamine Resistance (S108N) in *Plasmodium falciparum*. *Int J Environ Res Public Health.* 2019;16(9).
41. Johnston SP, Pieniazek NJ, Xayavong MV, Slemenda SB, Wilkins PP, da Silva AJ. PCR as a confirmatory technique for laboratory diagnosis of malaria. *J Clin Microbiol.* 2006;44(3):1087-9.
42. WHO. Basic Malaria Microscopy, Learner's guide, 2010. 2010.
43. Calderaro A, Piccolo G, Perandin F, Gorrini C, Peruzzi S, Zuelli C, et al. Genetic polymorphisms influence *Plasmodium ovale* PCR detection accuracy. *J Clin Microbiol.* 2007;45(5):1624-7.
44. Ponce C, Kaczorowski F, Perpoint T, Mialhes P, Sigal A, Javouhey E, et al. Diagnostic accuracy of loop-mediated isothermal amplification (LAMP) for screening patients with imported malaria in a non-endemic setting. *Parasite.* 2017;24:53.
45. Poon LL, Wong BW, Ma EH, Chan KH, Chow LM, Abeyewickreme W, et al. Sensitive and inexpensive molecular test for falciparum malaria: detecting *Plasmodium falciparum* DNA directly from heat-treated blood by loop-mediated isothermal amplification. *Clin Chem.* 2006;52(2):303-6.
46. Lau YL, Meganathan P, Sonaimuthu P, Thiruvengadam G, Nissapatorn V, Chen Y. Specific, sensitive, and rapid diagnosis of active toxoplasmosis by a loop-mediated isothermal amplification method using blood samples from patients. *J Clin Microbiol.* 2010;48(10):3698-702.
47. Mohon AN, Getie S, Jahan N, Alam MS, Pillai DR. Ultrasensitive loop mediated isothermal amplification (US-LAMP) to detect malaria for elimination. *Malar J.* 2019;18(1):350.

48. Fernández-Soto P, Gandasegui J, Carranza Rodríguez C, Pérez-Arellano JL, Crego-Vicente B, García-Bernalt Diego J, et al. Detection of *Schistosoma mansoni*-derived DNA in human urine samples by loop-mediated isothermal amplification (LAMP). *PLoS One*. 2019;14(3):e0214125.
49. Tegegne B, Getie S, Lemma W, Mohon AN, Pillai DR. Performance of loop-mediated isothermal amplification (LAMP) for the diagnosis of malaria among malaria suspected pregnant women in Northwest Ethiopia. *Malar J*. 2017;16(1):34.
50. Iseki H, Kawai S, Takahashi N, Hirai M, Tanabe K, Yokoyama N, et al. Evaluation of a loop-mediated isothermal amplification method as a tool for diagnosis of infection by the zoonotic simian malaria parasite *Plasmodium knowlesi*. *J Clin Microbiol*. 2010;48(7):2509-14.

## Tables

**Table 1 LAMP Primers used in this study (patent applying no. 201910611312.9)**

| Primer      | Sequence (5' → 3')                            |
|-------------|---|
| F3          | GGAGAAGAAGATGTTCAAGC                          |
| B3          | CCCAATTCGTAACAATACCATG                        |
| FIP(F1c+F2) | AACGGAACGAGGTGCATCAT-TTGTTGACAACGGATCAGG      |
| BIP(B1c+B2) | AGTAGGAAGACCAAAGAATCCAGGA-TGTGCTTCATCACCAACAA |
| LoopF       | CTCCTGCAACTCCTGCTT                            |
| LoopB       | GTTGGTATGGAAGAGAAAGATGC                       |

**Table 2. The positive outcome of 466 clinical susceptible malaria cases diagnosed by three methods**

| Species                     | Microscopy (M)    | Nested PCR (N)     | LAMP (L)          |
|-----------------------------|-------------------|--------------------|-------------------|
| <i>P. f.</i>                | 202               | 218                | 253               |
| <i>P. v.</i>                | 14                | 18                 |                   |
| <i>P. m.</i>                | 3                 | 5                  |                   |
| <i>P. o.</i> (curtisi)      | 130               | 64                 |                   |
| <i>P. o.</i> (wallikeri)    |                   | 46                 |                   |
| Mix                         | 4                 | 40 (33)*           |                   |
| Negative                    | 113               | 75                 | 213               |
| Total                       | 466               | 466                | 466               |
| <i>P</i> value <sup>1</sup> | $P_{M\&N}=0.0013$ | $P_{N\&L}= 0.8954$ | $P_{M\&L}=0.0008$ |

*P. f.* : *Plasmodium falciparum*, *P. v.*:*Plasmodium vivax*, *P. m.* :*Plasmodium malariae*,

*P. o.* (curtisi) : *Plasmodium ovale* (curtisi),

*P. o.* (wallikeri) : *Plasmodium ovale* (wallikeri)

\* Mixed infections contained 33 *P. falciparum*

<sup>1</sup> Chi-square in Diagnosis of *P. f.*

M&N Microscopy and Nested PCR

N&L Nested PCR and LAMP

M&L Microscopy and LAMP

**Table 3a. Comparison of diagnostic accuracy of LAMP and nested PCR for 466 suspected malaria samples.**

|                           | PCR +                           | PCR - | Total |
|---------------------------|---------------------------------|-------|-------|
| LAMP +                    | 251                             | 2     | 253   |
| LAMP -                    | 0                               | 213   | 213   |
| Total                     | 251                             | 215   | 466   |
| Kappa = 0.99              |                                 |       |       |
| Sensitivity               | 100 % (95% CI 98.54 - 100%)     |       |       |
| Specificity               | 99.07 % (95% CI 96.68 - 99.89%) |       |       |
| Positive predictive value | 99.21 % (95% CI 97.17 - 99.9%)  |       |       |
| Negative predictive value | 100 % (95% CI 98.28 - 100%)     |       |       |

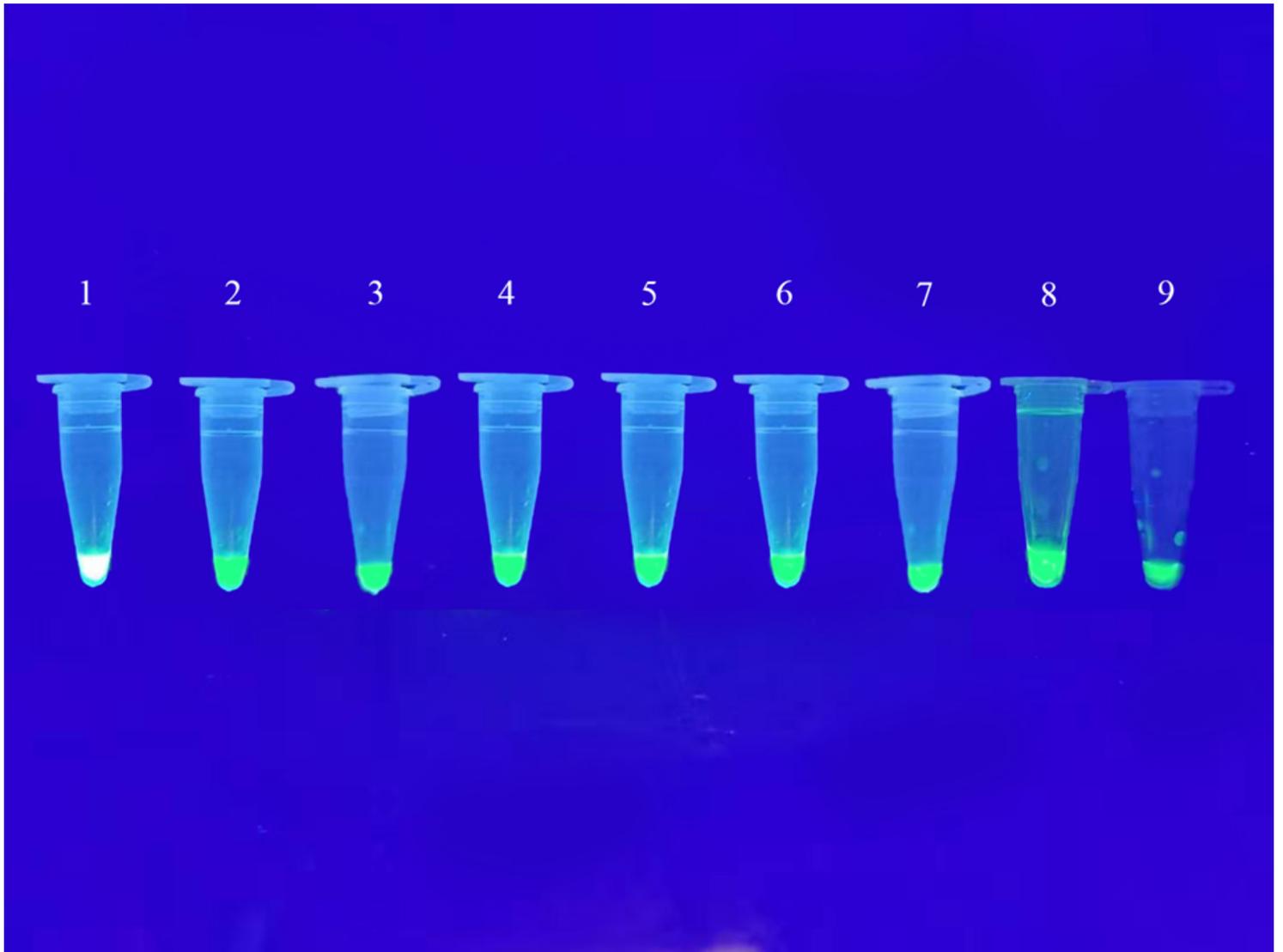
**Table 3b. Comparison of diagnostic accuracy of microscopy and nested PCR for 466 suspected malaria samples.**

|                           | PCR +                           | PCR - | Total |
|---------------------------|---------------------------------|-------|-------|
| microscopy +              | 188                             | 17    | 205   |
| microscopy -              | 63                              | 198   | 261   |
| Total                     | 251                             | 215   | 466   |
| Kappa = 0.66              |                                 |       |       |
| Sensitivity               | 74.90 % (95% CI 69.06 - 80.14%) |       |       |
| Specificity               | 92.09 % (95% CI 87.64 - 95.33%) |       |       |
| Positive predictive value | 91.71 % (95% CI 87.05 - 95.10%) |       |       |
| Negative predictive value | 75.86 % (95% CI 70.20 - 80.92%) |       |       |

**Table 3c. Comparison of diagnostic accuracy of LAMP and microscopy for 466 suspected malaria samples.**

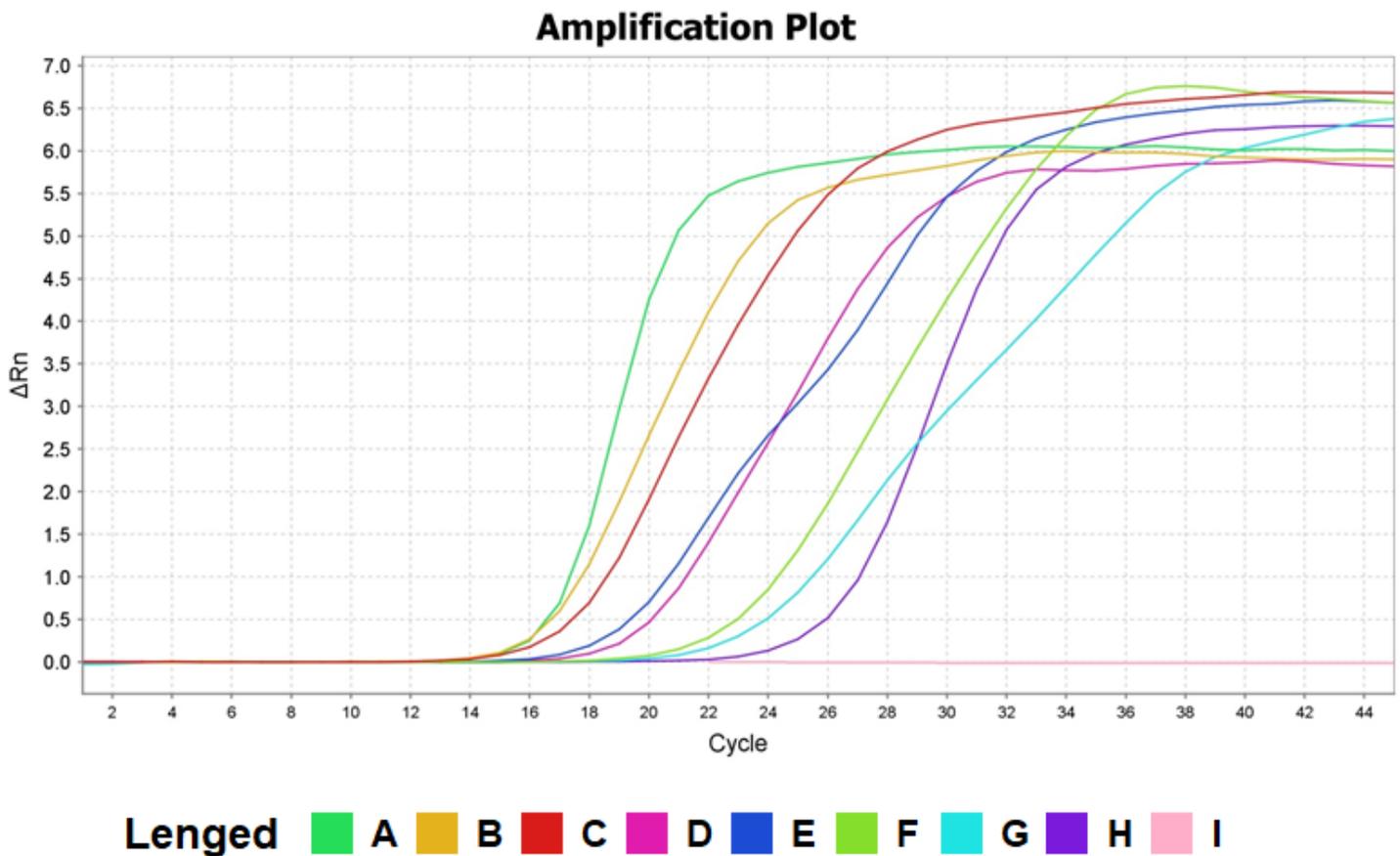
|                           | microscopy +                    | microscopy - | Total |
|---------------------------|---------------------------------|--------------|-------|
| LAMP +                    | 189                             | 64           | 253   |
| LAMP -                    | 16                              | 197          | 213   |
| Total                     | 205                             | 261          | 466   |
| Kappa = 0.66              |                                 |              |       |
| Sensitivity               | 92.20 % (95% CI 87.63 - 95.47%) |              |       |
| Specificity               | 75.48 % (95% CI 69.80 - 80.57%) |              |       |
| Positive predictive value | 74.70 % (95% CI 68.88 - 79.94%) |              |       |
| Negative predictive value | 92.49 % (95% CI 88.09 - 95.65%) |              |       |

## Figures



**Figure 1**

The LAMP results of various parasites and Plasmodium gDNAs. 1: *P. f.* (3D7), 2: *P. v.*, 3: *P. m.*, 4: *P. o.* (*curtisi*), 5: *P. o.* (*wallikeri*) 6: *P. k.* (Strain H) 7: *Toxoplasma gondii* 8: *Schistosoma japonicum* 9: DDWater



**Figure 2**

Real-time LAMP assay monitored by real-time measurement of SYBR Green. A: 109, B: 108, C: 107, D: 106, E: 105, F: 104, G: 103, H: 102, I: 101 unit: copy/ $\mu$ L, Each cycle lasts 1 minute.

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SupplementalTable1.docx](#)