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# Validation of an apicoplast genome target for the detection of *Plasmodium* species using polymerase chain reaction and loop mediated isothermal amplification

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## Abstract

The genome of the *Plasmodium* apicoplast, which has a higher copy number compared with current targets for molecular diagnosis of malaria, appears to be a suitable target for detection of submicroscopic infections that are capable of sustaining transmission. Novel primers targeting a conserved segment of the apicoplast (PFC10_AP|0010:rRNA) were designed and used in a number of different high throughput platforms such as single-step PCR (ssPCR), nested PCR (nPCR) and loop-mediated isothermal amplification (LAMP) for parasite detection. Replicates of ten-fold serial dilutions of *Plasmodium falciparum* 3D7 DNA, with equivalent parasite density ranges of 200 000 to 0.2 parasites/μL, were used to determine the limit of detection and repeatability of each assay. A panel of 184 archived DNA samples extracted from either EDTA whole blood or dried blood spots, from across West Africa and South East Asia was used to determine the diagnostic performance of the assays. All assays amplified the 2 parasites/μL dilution except the ssPCR, which amplified two of the three replicates. Using an 18S rRNA PCR as reference, the sensitivity was 98% (95% CI 93–100%) for the LAMP assay, 87% (95% CI 79–93%) for ssPCR and 100% (95% CI 97–100%) for nPCR. Specificity was 91% (95% CI 83–96%) for LAMP, 82% (95% CI 72–90%) for ssPCR and 66% (95% CI 54–76%) for nPCR. The apicoplast genome-based nPCR detected more positive samples overall than the reference method. Discrepant samples were confirmed as true positives using a probe-based real-time quantitative PCR assay. The results show that the apicoplast genome is a suitable target for molecular diagnosis of malaria.



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## Introduction

In 2012, compared with the year 2000, mortality attributed to malaria decreased by 45% worldwide and by 49% in Africa, saving an estimated 3.3 million lives. However, malaria remains a global health burden with an estimated 627 000 malaria deaths worldwide in 2012 [1]. In 2010, the WHO recommended prior treatment confirmation of clinical malaria by microscopy or a rapid diagnostic test [2]. Although diagnostic capabilities have recently been scaled up in the public sector, there is still a significant gap to meet the global target of universal access to malaria diagnosis in both private and public sectors and at community level by 2015 [3].

Asymptomatic carriers contribute significantly to transmission and commonly have low parasite densities [4]. However, the commonly used malaria diagnostic methods do not reliably detect such low parasite densities. As malaria declines, the number of samples to be screened to identify infected individuals and to better target intervention measures will



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increase substantially [5]. This is crucial for malaria elimination programmes implementing active case detection with the aim of reducing the human reservoir [6]. Identification of malaria parasites in peripheral blood samples can be most reliably performed by detecting parasite DNA, which has transformed the possibilities for diagnosis and investigation of malaria epidemiology [7]. Polymerase chain reaction, the most widely used molecular method for malaria diagnosis, has been modified and optimized to improve diagnosis and accuracy of species identification [8–12], but a major disadvantage of PCR methods is that they are almost impossible to perform at peripheral health centres or in the field. Hence, isothermal amplification methods that do not require multiple temperature cycles and highly purified DNA template have been developed to address these shortcomings [13–16].

Molecular diagnosis of malaria involves targeted amplification and detection of parasite genes, the most common being the conserved small subunit ribosomal RNA (rRNA) 18S locus [8,10,12,16]. Unlike many other eukaryotes with hundreds to thousands of copies of the rRNA gene, *P. falciparum* has only seven copies [17]. Several targets have been amplified in different PCR and isothermal assays, including the mitochondria [18,19] particularly the *Cytochrome b* gene [20], the *stevor* genes, metabolizing enzyme genes and repeat regions within the genome [21–23]. The apicoplast is a plastid organelle, homologous to the chloroplasts in plants and found in apicomplexan parasites. It is semi-autonomous with its own genome and expression machinery. Its genome copy number has been estimated to be approximately 15 for *P. falciparum* [24,25]. The apicoplast is therefore an obvious target for a diagnostic test aiming to detect low-density infections because of the high copy number but it has not been investigated for diagnostic purposes. This study reports the optimization and validation of the apicoplast of *P. falciparum* as a target for molecular diagnosis of malaria using different molecular assays.

## Materials and methods

### Samples

Replicates of ten-fold serial dilutions of laboratory cultures of *P. falciparum* clone 3D7 prepared as described elsewhere [26] were used to determine the limit of detection of the three novel assays. A total of 184 DNA samples from different studies archived at the Medical Research Council Unit, The Gambia were analysed to test the utility of the novel target for malaria diagnosis with three different molecular methods, namely a single step PCR (ssPCR), nested PCR (nPCR) and loop-mediated isothermal amplification (LAMP). Samples were selected to cover a wide geographical range, sample type and parasite species; and comprised 80 samples from across three West African countries, 88 samples from South East Asia, six laboratory strains analysed in duplicates (3D7, W2, K1, Dd2, T994 and HB3) and four negative controls (see Supporting information, Table S1). All DNA samples had been extracted with the QIAxtractor® robot (Qiagen GmbH, Hilden, Germany) according to the manufacturers' protocol and stored at −20°C for a median period of 19 months (range 1–36 months). Ethical approval was obtained from the Joint Gambian Government/MRC Ethics Committee.

### Primer design and assay optimization

*Plasmodium falciparum* apicoplast sequences from 15 Gambian isolates and eight laboratory clones were aligned against the PlasmoDB reference sequence (ID: emb|X95275.2|) (Fig. 1). Primers for both PCR and LAMP assays were designed from a

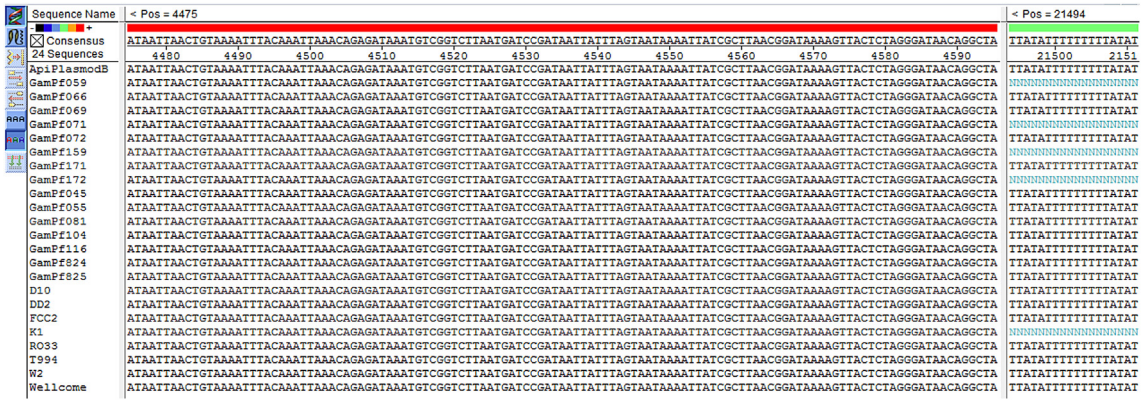


**FIG. 1.** Conserved region of aligned apicoplast sequences of field isolates (GamPf059, GamPf066, GamPf069, GamPf071, GamPf072, GamPf059, GamPf171, GamPf172, GamPf045, GamPf055, GamPf081, GamPf104, GamPf116, GamPf824 and GamPf825) and laboratory strains (D10, DD2, FCC2, K1, RO33, T994, W2 and Wellcome) against the reference sequence (ApiPlasmoDB) on Lasergene® MegAlign™ (DNASTAR Inc.).

conserved region of the consensus sequence, a 1.5-kb segment of a ribosomal RNA gene (PFC10_AP|0010:rRNA) encoding on the apicoplast genome. PCR primers were designed using PRIMER EXPRESS® and LAMP primers were designed using PRIMER EXPLORER V4. The primers were validated *in silico* with a web tool, THE SEQUENCE MANIPULATION SUITE by Bioinformatics Organization, Inc. (Hudson, MA, USA). All assays were optimized for temperature and concentration of primers, deoxyribonucleotides (dNTPs) as well as magnesium chloride ($MgCl_2$) using triplicates of three laboratory strains and two negative controls. After optimization, an experienced laboratory technician independently repeated the assays using the optimal conditions to assess repeatability. Table 1 shows the list of successful primers.

### Amplification conditions

*Nested PCR*. Final amplification reaction mixture for the outer PCR contained 0.2 μM each of both forward and reverse primers, 1× Thermopol buffer, 1 Unit of *Taq* DNA polymerase (New England Biolabs, (UK) Ltd. Hitchin, Hertfordshire.), 0.2 mM of each dNTP and 2 μl of DNA per sample. Amplification conditions were 95°C for 5 min, 94°C for 30 s, 50°C for 30 s, 72°C for 30 s, repeated for 24 cycles, 72°C for 5 min. One microlitre of the outer PCR product was used for the inner PCR with similar conditions except annealing at 55°C and repeated for 29 cycles. End product was determined by agarose gel electrophoresis stained with ethidium bromide or by an automated QIAxcel electrophoresis system (Qiagen GmbH). The primers amplified a 138-bp fragment after the second round of amplification.

*Single-step PCR*. The outer LAMP primers were used to amplify a 205-bp fragment in a single-step amplification reaction. The final amplification reaction mixture contained 0.3 μM each of the forward and reverse LAMP primers, 1× Thermopol buffer, 1 Unit of *Taq* DNA polymerase (New England Biolabs), 0.2 mM of each dNTP and 2 μl of DNA sample. The cycling conditions were 95°C for 5 min, 93°C for 30 s, 56°C for 30 s, 72°C for 1 min, repeated for 34 cycles, 72°C for 5 min. End product was determined same as the nPCR above.

*LAMP amplification conditions*. Final amplification reaction mixtures contained 1.6 μM each of the inner primers (FIP and BIP), 0.2 μM each of the outer primers F3 and B3c, 0.8 μM each of the loop primers (LPF and LPB), 1× Isothermal Amplification Buffer, 1 Unit of Bst 2.0 WarmStart™ DNA polymerase (New England Biolabs), 1.0 mM of each dNTP, 1.0 mM $MgCl_2$ and 2 μl of DNA sample. The reaction was performed in a clear 96-well plate at 65°C for 60 min in a water bath. End product was determined both visually by naked eye, observing colour changes after adding 2 μL of 1000× SYBR® Green I (Life Technologies, Grand Island, NY, USA) and electrophoresis and scored independently by two observers. The LAMP primers amplified the expected loop-structured fragments and specificity was confirmed with a restriction digest using *Fok*I (New England Biolabs) (Fig. 2).

### Data analysis

Sensitivity and specificity of the optimized assays were calculated using a species-specific 18S rRNA nested PCR assay (18S rRNA PCR) [10] as the reference standard. Cohen's κ (κ coefficient) was calculated to assess the degree of agreement between the assays. The precision of the estimates was determined by calculating 95% CI (rounded up to the nearest whole number) for each test statistic. All statistical analysis was performed with STATA 12 (StataCorp, College Station, TX, USA).

### Discrepant results

Genus-specific real-time quantitative PCR (qPCR) primers and probe targeting the 18S rRNA locus of *Plasmodium* spp [12]. was used to amplify samples with discrepant results to assess the possibility of false-negative results with the reference method. In this assay, samples were run in duplicates and intra-assay coefficient of variation (CV) was calculated for the Ct

**TABLE 1. List of successful primers used for loop-mediated isothermal amplification (LAMP), nested PCR and single-step PCR assays**

| Name | 5′ to 3′ sequence |
|---|---|
| LAMP primers | |
| ApF3 (outer forward) | CGGATAAAAGTTACTCTAGGGATA |
| ApB3 (outer reverse) | TTATATTAGATATGGACCGAACTG |
| ApLF (forward loop) | GAGGTGCCAAACCTTTT |
| ApLB (reverse loop) | ATTAAAGCGATACGTGAGCTGG |
| ApFIP (ApFIc + ApF2; forward inner) | GGATGCGATAAGCCGACATCTTTTCCGAGAGTCCATATTGAC |
| ApBIP (ApBIc + ApF2; reverse inner) | TTAAGGGTAAGTCTGTTCGCCTTCTCACGACGTTCTGAAC |
| Nested PCR primers | |
| Api 1736 (outer forward) | AAATGTCGGTCTTAATGATCC |
| Api 1976 (outer reverse) | TATGGACCGAACTGTCTCACG |
| Api 1808 (inner forward) | GGATAACAGGCTAATCTTTTCC |
| Api 1940 (inner reverse) | CTCACGTATCGCTTTAATAGG |
| Single-step PCR Primers (Same as the LAMP outer primers) | |
| ApF3 (forward) | CGGATAAAAGTTACTCTAGGGATA |
| ApB3 (reverse) | TTATATTAGATATGGACCGAACTG |

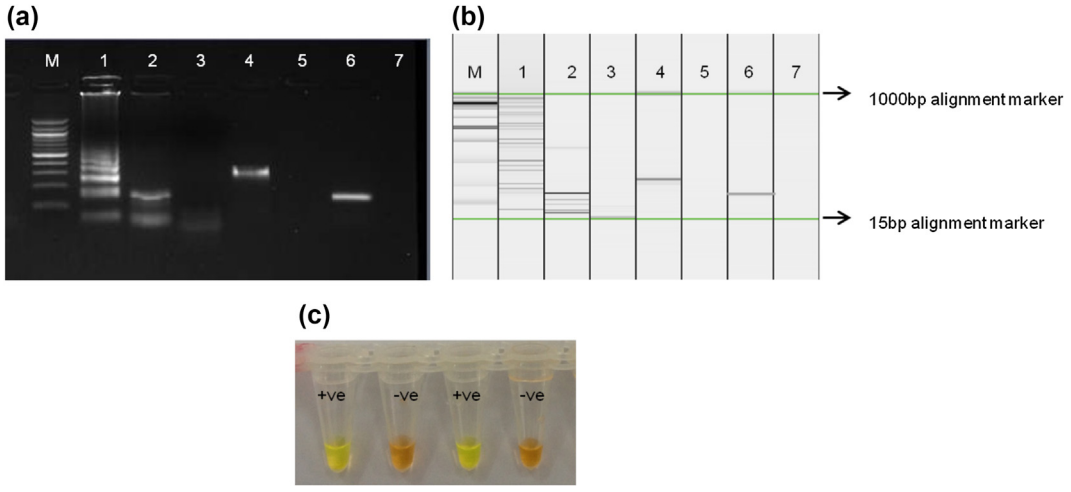


**FIG. 2.** Images of amplified DNA products on agarose gel electrophoresis (a); an automated electrophoresis system, QIAxcel (b); and LAMP visualization by naked eye after addition of SYBR GreenI (c). Lane M, 100-bp DNA ladder; lane 1, loop-mediated isothermal amplification (LAMP) +ve; lane 2, LAMP + ve *Fok*I digest; lane 3, LAMP −ve; lane 4, single-step PCR (ssPCR) +ve; lane 5, ssPCR −ve; lane 6, nested PCR (nPCR) +ve; lane 7, nPCR −ve.

values with a cut-off of 10%. The qPCR assay was used to resolve discrepancies and to provide absolute parasite densities to determine the impact of low parasite density on specificity.

## Results

### Limit of detection and repeatability

Ten-fold serial dilutions of 3D7 DNA, with parasite density ranging from the equivalent of 200 000 to 0.2 parasites/μL were run in triplicate for each assay to determine the limit of detection. All the assays amplified the 2 parasites/μL dilution except ssPCR, which amplified in only two of the triplicates (see Supporting information, Table S2). In addition, the nPCR detected two of three replicates at aten-fold higher dilution of 0.2 parasites/μL, whereas LAMP and the reference method both amplified only one of the three replicates. There were no significant differences in results obtained when the assays were independently repeated.

### Comparison using archived DNA samples

Out of the total 184 samples, 105 (57%) were detected as positive by the reference 18S rRNA PCR assay, 82% of which were *P. falciparum* mono-infections, 7% were *Plasmodium vivax* mono-infections, 1% were *Plasmodium malariae* mono-infections, and 10% were mixed infections of *P. falciparum* and *P. vivax*. The test positivity was 60% (110/184) for LAMP, 57% (105/184) for ssPCR and 72% for nPCR. With samples positive for all methods, including the reference assay, the test positivity was 48% (89/184) (Fig. 3). Of the 79 samples negative by the reference assay, six were positive by all three methods, eight by nPCR and ssPCR, one by nPCR and LAMP and 12 by nPCR alone. There were no discrepancies between scores of the LAMP assay by the two independent observers, regardless of method of end-point determination. The nPCR assay had the highest sensitivity, followed by LAMP and then ssPCR with only 87%. LAMP had the highest specificity, followed by ssPCR and then nPCR (Table 2).

### Degree of agreement

The κ coefficient assessing the degree of agreement (after correcting for agreement due to chance) with the reference method was 0.9 for LAMP, 0.7 for ssPCR and 0.7 for nPCR (Table 2). Comparing the LAMP assay with either ssPCR or nPCR gave a κ coefficient of 0.7.

### Other parameters

Using McNemar's chi square to test for marginal homogeneity, there were no significant differences in the performances of LAMP and ssPCR compared with the reference method. However, there was a significant difference with nPCR (McNemar's $\chi^2$ = 27.00; Exact significance probability <0.01). Similarly, both LAMP and ssPCR showed no significant differences by either geographic origin or sample type whereas with nPCR there was a significant difference by sample type, with more discrepancies in the dried blood spot samples (McNemar's $\chi^2$ = 23.00; Exact significance probability <0.01), but not by geographic origin.

### Discrepant results

Discrepancies occurred significantly more with the dried blood spot (85%, 23/27) compared to whole blood samples (15%, 4/

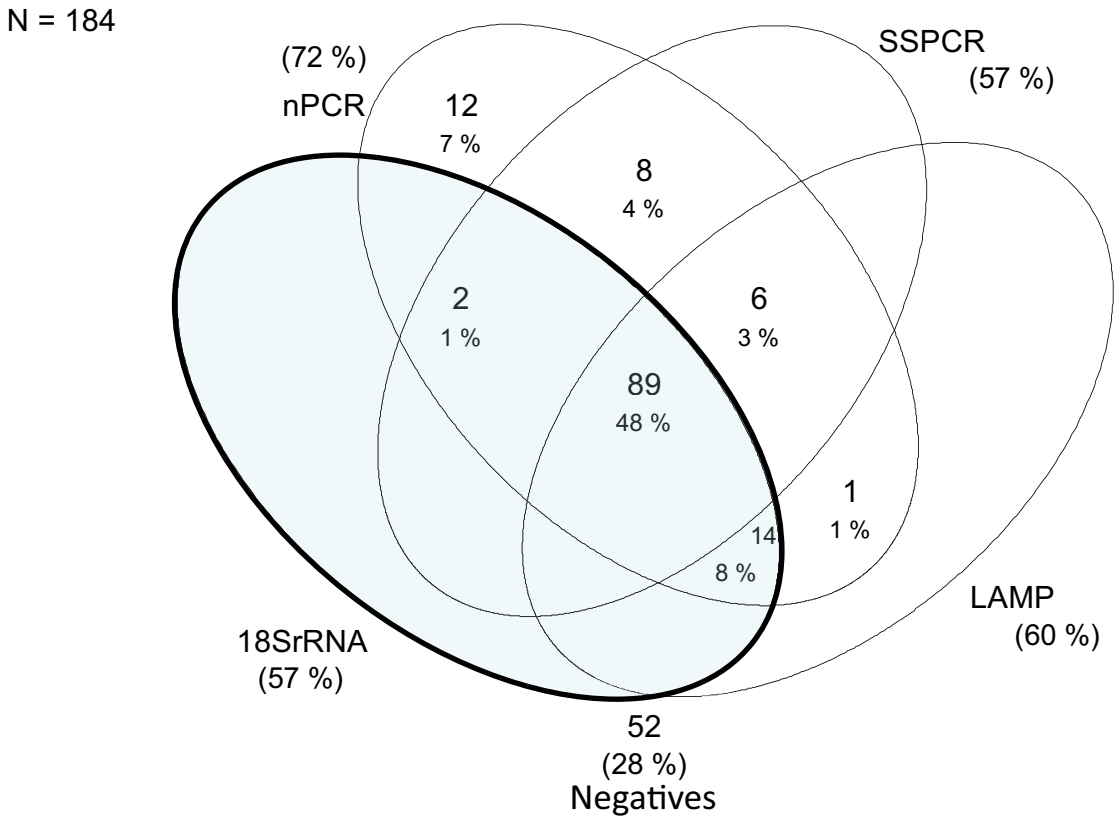


**FIG. 3.** Venn diagram showing distribution of samples detected as positive by each of the methods. Proportions (%) refer to the total of 184 samples.

**TABLE 2. Comparison of the optimized assays with the reference method (classifying 105 positive and 79 negative samples)**

| | 18S rRNA + ve | 18S rRNA –ve | Sensitivity | Specificity | Cohen's κ coefficient |
|---|---|---|---|---|---|
| LAMP + ve | 103 | 7 | 98% (95% CI 93–100%) | 91% (95% CI 83–96%) | 0.9 (95% CI 0.8–1.0) |
| LAMP −ve | 2 | 72 | | | |
| ssPCR + ve | 91 | 14 | 87% (95% CI 79–93%) | 82% (95% CI 72–90%) | 0.7 (95% CI 0.6–0.8) |
| ssPCR −ve | 14 | 65 | | | |
| nPCR + ve | 105 | 27 | 100% (95% CI 97–100%) | 66% (95% CI 54–76%) | 0.7 (95% CI 0.6–0.8) |
| nPCR −ve | 0 | 52 | | | |

Abbreviations: LAMP, loop-mediated isothermal amplification; nPCR, nested PCR; ssPCR, single-step PCR.

27). The qPCR analysis of these samples indicated low parasite densities (<35 parasites/μL), with the amplification curve crossing the detection threshold after cycle 35, which corresponds to parasite densities <20 parasites/μL in the standard curve.

### Usability of assays

All assays were high throughput, easy to use with optimized protocols and were performed in a 96-well plate format. Turnaround time for 88 samples with positive and negative controls that could be analysed per 96-well plate was approximately 300 min with the reference PCR method and nPCR, 150 min for ssPCR and 90–120 min for LAMP, depending on whether end point determination was by naked eye or agarose gel electrophoresis.

## Discussion

The diagnosis of malaria has evolved rapidly within the last few years. New and improved screening tools and strategies are being developed, as currently available tools cannot promptly detect low-density infections in the field. Consequently, nucleic acid tests are optimized for increased sensitivity and simplified for field deployability. Using a novel target with higher copy number, the apicoplast genome, we validate its suitability for

detection of low-grade malaria infections in a range of molecular assays, including the LAMP assay that is potentially usable at peripheral health facilities.

Although the primers were designed from aligned *P. falciparum* sequences, they amplified non-*falciparum* species as well, indicating that the target is possibly conserved across the genus (see Supporting information, Fig. S1). Generally, the test positivity by either LAMP or ssPCR assays targeting the apicoplast genome was similar to the reference 18S rRNA PCR method, although the LAMP assay had better agreement. The apicoplast genome-based nPCR assay detected all samples classified as positive by the other methods as well as 12 additional samples that were confirmed to be low-density infections by a very sensitive, probe-based qPCR assay. This explains the lower specificity of nPCR as it detected an important number of positive samples—confirmed by the probe-based qPCR assay—among samples considered negative by the reference method. As expected, the majority (85%) of discrepancies occurred with dried blood spot samples, probably because the amount of blood screened per assay (approximately 4 μL of blood in one 3-mm punch of dried blood spot) [27] would have been much lower than in whole blood samples. The impact of the sampling method on assay sensitivity is likely to be more pronounced in low-density infections as parasite DNA would be less efficiently extracted from dried filter paper blood spots compared with whole blood samples.

The nPCR targeting the apicoplast genome was more sensitive than the reference method, particularly for samples with a parasite density <2 parasites/μL. Similar to other laboratory-based PCR assays, its routine use in the field would be challenging, though the use of real-time screening PCR in a mobile laboratory has been recently reported [28]. Hence, the LAMP assay that is less complex was developed alongside. Overall, the sensitivity and specificity of the apicoplast genome-based LAMP assay was similar to previously described LAMP assays targeting the 18SrRNA gene or the mitochondria [13,15]. End-product determination of the LAMP assay by observing colour changes after addition of SYBR® Green I is practical for field use though it may be a problem for individuals with colour blindness [29]. Colour charts of positive and negative results may be needed, with systematic double reading.

A major limitation of this study is the unavailability of either microscopy or rapid diagnostic test results for most of the tested samples. As these are the currently used diagnostic tools for malaria, it would have been interesting to evaluate the new assays against standard diagnostic methods. Also, running replicates of the discrepant samples could have increased the sensitivity of the reference PCR method. In a recent study by Hopkins et al., overall sensitivity of the reference nested PCR assay was increased by repeating in three wells all samples that tested negative in the first run with any amplification of the expected fragment size scored as positive [30].

All the assays are high throughput and able to process samples in a 96-well format. This is very important for the potential use of field-based molecular assays for mass screening and treatment campaigns as the fast turn-around-time will enable infected subjects to be treated within a short time. The faster turn-around time of the LAMP assay and comparable sensitivity with the reference 18S rRNA method would support further assessment to determine its performance under field conditions. As more endemic regions begin to consider the possibility of malaria elimination, field-based molecular assays are poised to be the favoured option for surveillance and targeted interventions [31]. More sensitive targets are currently being explored for potential field-based molecular tests and the apicoplast genome appears to be a suitable candidate.

## Transparency declaration

The authors declare that they have no conflict of interest.

## Acknowledgements

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## Appendix A. Supporting information

Supporting information related to this article can be found at http://dx.doi.org/10.1016/j.cmi.2015.02.025.

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