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Article

Development and Clinical Validation of Iso-IMRS: A Novel Diagnostic Assay for *P. falciparum* Malaria

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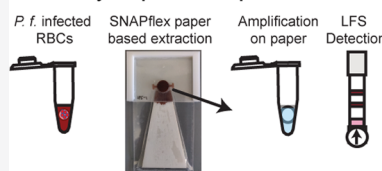ACCESS | Metrics & More | Article Recommendations | Supporting Information

ABSTRACT: In many countries targeting malaria elimination, persistent malaria infections can have parasite loads significantly below the lower limit of detection (LLOD) of standard diagnostic techniques, making them difficult to identify and treat. The most sensitive diagnostic methods involve amplification and detection of *Plasmodium* DNA by polymerase chain reaction (PCR), which requires expensive thermal cycling equipment and is difficult to deploy in resource-limited settings. Isothermal DNA amplification assays have been developed, but they require complex primer design, resulting in high nonspecific amplification, and show a decrease in sensitivity than PCR methods. Here, we have used a computational approach to design a novel isothermal amplification assay with a simple primer design to amplify *P. falciparum* DNA with analytical sensitivity comparable to PCR. We have identified short DNA sequences repeated throughout the parasite genome to be used as primers for DNA amplification and demonstrated that these primers can be used, without modification, to isothermally amplify *P. falciparum* parasite DNA via strand displacement amplification. Our novel assay shows a LLOD of ~1 parasite/μL within a 30 min amplification time. The assay was demonstrated with clinical samples using patient blood and saliva. We further characterized the assay using direct amplicon next-generation sequencing and modified the assay to work with a visual readout. The technique developed here achieves similar analytical sensitivity to current gold standard PCR assays requiring a fraction of time and resources for PCR. This highly sensitive isothermal assay can be more easily adapted to field settings, making it a potentially useful tool for malaria elimination.

Forward and Reverse primers bind in multiple loci for *P. falciparum* genome

Multiple amplicons using only two primers

Assay adoption to the point of care

P. f. infected RBCs | SNAPflex paper based extraction | Amplification on paper | LFS Detection

Assay Highlights
30 minute amplification
Visual readout
NGS characterization
Demo with clinical samples (blood, saliva)

INTRODUCTION

According to the 2018 World Malaria report, there were 217 million reported malaria cases in 2017, resulting in 435,000 deaths; this represents an increase of about 3.6 million cases from the previous year. The majority of the disease burden for malaria falls in resource-limited countries in Africa and Asia, and of the five species of malaria parasites, *P. falciparum* remains the most prevalent.[1] In countries with a high disease burden, the need for diagnostics is clear: malaria is a treatable disease, and sensitive diagnostics would enable early intervention, saving many lives, particularly those of children.[2] Although some countries have seen a slowdown in progress, many countries are getting closer to elimination, showing a significant decline in cases annually. In countries approaching elimination, where malaria prevalence is low, parasite loads are also extremely low, often <100 parasites/μL.[3] In these countries, sensitive diagnostics are necessary to monitor and treat asymptomatic malaria cases, which could result in parasite transmission to mosquitos, furthering the spread of the disease. Continuous surveillance in elimination-phase countries will enable targeted treatment to eliminate low parasite load infections. According to the WHO, diagnostics designed for this purpose must be able to detect parasite loads <10 parasites/μL blood.[4]

The current gold standard for malaria diagnosis remains the microscopic examination of patient blood; the typical limit of detection of 100 parasites/μL[5−7] is insufficient to detect low parasite load infections. In countries with low average parasitemia levels, it is recommended that tests are repeated every 12−24 h for up to 3 days in patients with febrile symptoms to adequately rule out malaria infection.[8] This testing is time-consuming and labor-intensive. Antigen-based rapid diagnostic tests (RDTs) have been accepted as an alternative to clinical microscopy. Although they are cheaper and faster than microscopy, RDTs suffer from poor sensitivity,

with a limit of detection similar to clinical microscopy.[5−7] Although recent efforts have focused on making RDTs more sensitive,[9] initial demonstrations indicate that ultrasensitive RDTs still lack the sensitivity to identify low-density infections.[10]

Nucleic acid amplification tests (NAATs), particularly PCR-based tests, are widely considered the most sensitive methods for malaria diagnosis.[11] To increase sensitivity, several NAATs have been developed to amplify and detect multicopy genes[12−21] or tandem repeat sequences[22−24] in the *P. falciparum* genome parasite DNA with high analytical sensitivity. Recently, we demonstrated a novel method to computationally identify short, identical multirepeat sequences in the *P. falciparum* genome (IMRS) for use as PCR primers. These primers yielded significantly improved sensitivity compared to existing PCR methods amplifying multicopy genes or tandem repeat sequences.[25]

Although PCR assays are highly sensitive, they are difficult to deploy in resource-limited settings due to the need for DNA extraction steps and precise and expensive thermal cycling equipment. Isothermal NAATs have emerged as alternatives to PCR; because they require only a single temperature for amplification, they are easier to deploy, requiring only a single stable heat source. Several isothermal methods, including loop-mediated isothermal amplification (LAMP),[26−34] helicase dependent amplification (HDA),[35] and recombinase polymerase amplification (RPA),[36,37] have been developed for *P. falciparum*, and many of these methods have been adapted to point-of-care compatible devices (Table S1). Currently, two commercially available methods utilize LAMP for sensitive diagnosis of *P. falciparum*[26,27] but require specialized equipment to detect products.

Here, we show a novel method for isothermal amplification of *P. falciparum* DNA using a single pair of repeated primers binding to multiple loci across the *P. falciparum* genome, which we computationally identified using the IMRS algorithm we previously developed. Using the IMRS-identified primer pair, we developed a 30 min isothermal amplification assay, which we call "iso-IMRS," with similar analytical sensitivity to qPCR and demonstrated successful DNA amplification from parasite-infected red blood cells and clinical samples. We also developed a room-temperature, centrifuge-free DNA extraction method and a lateral flow strip-based visual readout method to simplify adaptation of the iso-IMRS assay to point of care (POC).

■ EXPERIMENTAL SECTION

Identification of Iso-IMRS Primers. We utilized the Identical Multi-Repeat Sequence (IMRS) algorithm,[25] with modified input constraints to enable isothermal amplification. Briefly, using the published, annotated genomic sequence for *P. falciparum* (3D7 strain, GeneDB, version 2013-03-01) as the input, the IMRS algorithm identifies identical, repetitive sequence substrings from multiple loci within the annotated genome sequence and generates a library of unique repeat sequences that can be used as amplification primers. The algorithm identified repeated sequences in which repeats of the same sequence and orientation were <30 base pairs apart and primers of opposite orientation (i.e., forward−reverse) were located within an amplifiable region <3000 base pairs apart to enable (1) strand displacement and (2) isothermal amplification. The repeat pairs were evaluated using the NIH's Basic Local Alignment Search Tool (BLAST) to ensure that they

were specific to the pathogen genome. These resulting primer pairs amplify several fragments of DNA of different sizes due to the distribution of the primers throughout the genome.

From this screen, the primer pair resulting in the most amplicons was selected for assay development, as this pair amplifies the largest amount of DNA, leading to the best possible analytical sensitivity. The resulting primer pair consisted of a forward primer that repeats in 52 locations (5′-CTGGAGGTCAGTTCACAGTACCC-3′) and a reverse primer that repeats in 55 locations (5′-CTCTACAATCCG-TAGAGTTACTGG-3′); both primers occur in four regions on chromosomes 6, 10, and 11.

Iso-IMRS Amplification Assay. The iso-IMRS amplification assay uses one forward and one reverse primer, which bind to 52 and 55 sites, respectively. The 25 μL reaction mixture uses 640 U/μL *Bst* 2.0 polymerase (New England Biosciences), with 1× isothermal amplification buffer, 3.2 mM forward primer, and 1.6 mM reverse primer (Integrated DNA Technologies) combined with 10 mM dNTPs (Agilent), 0.4 M Betaine (Sigma-Aldrich), and 2% molecular-biology-grade Ficoll-400 (Sigma-Aldrich). The sample (5 μL) is included in each 25 μL reaction. For real-time amplification reactions, 0.2× EvaGreen intercalating dye and 30 mM ROX reference dye are included. Amplification is carried out at 56 °C for 40 min, followed by a melting curve analysis for real-time amplification. Amplified products were visualized by gel electrophoresis on 10% acrylamide gels.

Lower Limit of Detection. To assess the lower limit of detection (LLOD) in the buffer, varying concentrations of genomic DNA (gDNA) isolated from the 3D7 lab strain of *P. falciparum* (BEI Resources, NIAID, NIH) were prepared in Tris−HCl elution buffer, pH 8.5 (QIAGEN Buffer EB). The DNA was amplified using (1) qPCR targeting the 18S rRNA gene[38] and (2) the novel iso-IMRS assay. DNA was diluted to several concentrations, both amplification assays were performed on each sample, and the amplification products were visualized by fluorescence-based real-time amplification and gel electrophoresis. To determine the LLOD (the concentration at which the sample is detected with 95% confidence) of the *P. falciparum* 18S rRNA qPCR assay and iso-IMRS assay, probit analysis was performed in MATLAB using the ratio of successful reactions to the total number of reactions performed for each assay.

P. falciparum Parasite Culture. *P. falciparum* (3D7 strain, BEI Resources, MRA-102) parasites were cultured in human red blood cells isolated from freshly collected whole blood (Research Blood Components, Boston, MA) using standard culture procedures for malaria parasites.[39] A culture of early ring-stage *P. falciparum* parasites was maintained in 5% human red blood cells in complete RPMI medium and grown at 37 °C, 5% CO_2, and 3% O_2. Parasites were synchronized with 5% D-sorbitol once per week. The culture was maintained at high parasitemia (>10% infected red blood cells) to allow for subsequent dilution. To monitor parasitemia, 2 × 3 μL of each individual culture was collected into a thin blood smear sample on glass slides, fixed with methanol, and stained with Giemsa stain to assess the parasitemia each day. The samples were visualized with 100× oil immersion microscopy, parasitemia levels were determined for 10 separate fields of view for each slide, and the average parasitemia from 20 fields of view was determined. For experiments with simulated patient samples, each culture was serially diluted in human whole blood to final concentrations of 10, 5, 1, and 0.1% parasitemia. Samples were

collected at the ring stage, trophozoite stage, and schizont stage.

Parasite DNA Extraction from Infected Red Blood Cells. For standard DNA extraction experiments, 100 μL of the samples diluted in whole blood were extracted and purified using standard protocols for the blood and tissue extraction kit (QIAGEN).

For SNAPflex extraction experiments, a custom lysis buffer containing 5 M guanidine thiocyanate, 0.1 M MOPS, 0.5% *N*-laury lsarcosine, and 2-mercaptoethanol was used. The sample (100 μL, infected red blood cells diluted in whole blood) was lysed with 200 μL of complete lysis buffer (68% (v/v) custom lysis buffer, 29% (v/v) 10% nonyl phenoxypolyethoxylethanol (Sigma-Aldrich), and 3% (v/v) GlycoBlue (15 mg/mL blue glycogen, Applied Biosystems)). After 15 min incubation at room temperature, 129 μL of 1-butanol was added and the sample was mixed by inversion. The lysed samples were applied to the capture membrane of the SNAPflex device. After sample application, the membrane was washed with 400 μL of "prewash" buffer containing 12.5% custom lysis buffer, 70% ethanol, 200 μL of 70% ethanol, and 100 μL of 95% ethanol. The capture membranes were dried for 10 min at room temperature.

18S rRNA TaqMan qPCR Assay. A previously reported TaqMan qPCR assay was used for quantitative analysis of extracted *P. falciparum* DNA.[40] Gene-specific primers for the 18S rRNA gene initiated amplification, and a HEX and Black Hole Quencher dual-labeled species-specific probe was included for fluorescence quantification. Purified *P. falciparum* genomic DNA was used for standard curve analysis with input concentration ranging from 1E1fg/μL to 1E5fg/μL. The sample (5 μL) was added to 20 μL of Master Mix for a final reaction volume of 25 μL (final concentrations: 1× SureStart, 10× buffer, 3 mM $MgCl_2$, 0.3 μM forward primer, 0.3 μM reverse primer, 0.2 μM probe, 0.8 mM dNTPs, 30 nM ROX reference dye, 0.025 U/mL Taq polymerase). Samples were incubated for an initial denaturation step at 95 °C for 10 min and 45 cycles at 95 °C for 15 s and 60 °C for 1:00.

Clinical Sampling. Eleven symptomatic malaria infected patients (by microscopy) at the outpatient clinic at Bungoma County Referral Hospital, western Kenya provided matched samples (dried blood spot, saliva, and urine) between January and March 2018. The samples were stored at 4 °C and transported at ambient temperature to central labs at Mount Kenya University, where genomic DNA extraction was carried out using the QIAamp DNA Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions and then aliquoted for iso-IMRS amplification. The sampling was authorized by the Ethical Review Committee of Mount Kenya University and carried out in accordance with the approved guidelines.

Characterization. After iso-IMRS amplification, products were size-separated by gel electrophoresis on a 3.5% agarose gel. Bands of 100, 150, 250, and 300 bp were extracted using a gel extraction kit (QIAGEN), and the products were sequenced by Illumina Direct Amplicon Sequencing (Genewiz, Boston, MA). Raw reads were processed through FastQC to generate a set of quality metrics and subsequently quality-trimmed with cutadapt with a quality score of 20 as the cutoff. Trimmed reads were then aligned to the reference genome with BWA and sorted and indexed with SAMtools. The sorted BAM alignment was converted to a. tdf file of coverage using the count function of IGVTools. The alignment was visualized

with the GenomeView genome browser. For each product size, the sequences were aligned to *P. falciparum* and *P. vivax* reference genomes available from NCBI.

Iso-IMRS Visual Readout. For the Iso-IMRS visual readout, lateral flow strips containing a test line with anti-FAM antibodies were used (U-Star). The forward primers included in the reaction mixture were modified: 1 mM forward primers modified with the FAM antigen were used in combination with 2.2 mM unmodified forward primers (Integrated DNA Technologies, Coralville, IA). Biotin dUTP (0.04 mM, Thermo Scientific) was added to the reaction mixture for biotin modification of the amplified products. After amplification, 20 μL of postamplification products were precipitated with 2.5 M ammonium acetate (Sigma-Aldrich) and 50% (v/v) isopropanol (Thermo Fisher). The solution was filtered through a 0.7 μm pore size glass fiber membrane (Millipore) cut into 1/4″ circles to remove unincorporated dNTPs and primers from the solution. The paper was washed with 60 μL of 70% ethanol and 30 μL of 95% ethanol, dried at room temperature, and inverted onto the gold nanoparticle conjugate pad of the lateral flow strip. Running buffer was applied to the glass fiber pad to elute products onto the lateral flow strip.

SNAPflex Point-of-Care DNA Extraction from Infected Red Blood Cells. Infected red blood cells were extracted using SNAPflex as described above. For quantification experiments, after SNAPflex extraction, the capture membrane was submerged in 100 μL of standard elution buffer (QIAGEN Buffer EB) for 10 min at 50 °C. The eluted DNA was used in iso-IMRS and qPCR extraction as described. For on-paper amplification experiments, the parasite-infected red blood cell samples were extracted with SNAPflex as described above. The capture membrane containing parasite DNA was transferred directly to an amplification reaction tube. The iso-IMRS reaction mixture (75 μL) was added to the tube (the sample volume was substituted with nuclease-free water). After amplification, 20 μL of products were collected and used for the iso-IMRS visual readout protocol described.

■ RESULTS AND DISCUSSION

Proposed Isothermal Amplification Method. We utilized the IMRS genome mining algorithm previously reported[25] to design a pair of short primer sequences to isothermally amplify DNA. The algorithm identified two unique primers, a forward primer binding in 52 independent loci and a reverse primer binding in 55 independent loci across four chromosome regions in the *P. falciparum* genome (Figure S1). The proposed amplification scheme takes advantage of individual primers annealing to multiple genome locations. The primer binding sites are distributed in close proximity (<30 bp) to each other. A strand displacing polymerase initiates amplification from each primer. As amplification proceeds, single strands are displaced from multiple locations and serve as templates for further amplification (Figure 1). The distribution of binding sites in several loci enables the generation of multiple products, increasing assay sensitivity without additional primers or enzymes for DNA denaturation like most isothermal methods.[41] Our proposed amplification schema is similar to multiple displacement amplification (MDA).[42−45] However, while MDA utilizes random hexamers, our longer, exact-match primers enable specificity to the *P. falciparum* genome. Unlike most isothermal amplification methods, iso-IMRS requires only two primers of 24−25 base
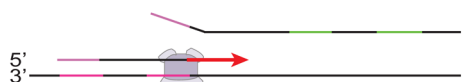
52 Forward and 55 Reverse primer binding sites are distributed throughout the genome.
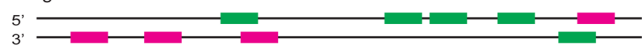
After primers anneal, DNA polymerization initiates from multiple sites with Bst 2.0 strand displacing polymerase.

As DNA synthesis occurs from upstream sites, downstream products get displaced. Displaced strands become templates for further amplification.

Due to primer distribution, several products can be formed with only two primers, increasing assay sensitivity without additional complexity.

Multiple amplicons of varying sizes

Legend:
Bst 2.0
Forward primer binding site
Reverse primer binding site
Genome

Figure 1. Proposed amplification method using 52 forward and 55 reverse primer binding sites enabling multiple product formation.

pairs, reducing the possibility of nonspecific amplification due to primer dimer formation caused by high concentrations of longer primers.[46] The primers are specific to the *P. falciparum* genome, and due to their wide distribution, we expect that they can bind *P. falciparum* DNA from field isolates despite possible heterogeneity. Although we did not cover this for this publication, the algorithm can also identify repeated sequences to use as primers for other species of *Plasmodium* as well as for other organisms.

Iso-IMRS Shows Comparable Analytical Sensitivity to qPCR with Purified DNA and *In Vitro* Cultured Parasites in Whole Blood. The novel assay we proposed, called "iso-IMRS", utilizes one forward and one reverse primer sequence and a standard *Bst* 2.0 strand displacing polymerase. Across

several DNA concentrations, the assay produces consistent products of several sizes, visualized by both gel electrophoresis and real-time amplification using an intercalating dye (Figures 2a and S2). Because these products can be detected with an intercalating dye, we hypothesize that many of the products generated by iso-IMRS are double-stranded.

We first determined the analytical sensitivity of the iso-IMRS assay using purified *P. falciparum* genomic DNA as a test material. Iso-IMRS had comparable analytical sensitivity to a qPCR assay amplifying the multicopy 18S rRNA gene[38] (Figure 2b). Because iso-IMRS does not amplify a single target locus, generating a target-specific TaqMan probe for this method was not possible, and readout could be done with only nonspecific intercalating dye. We chose the representative qPCR assay shown in Figure 2 to directly compare iso-IMRS to an assay using an intercalating dye as the fluorescence readout. Using probit analysis, the lower limit of detection (LLOD) for iso-IMRS was calculated as 24.5 fg/μL genomic DNA, approximately 1 parasite/μL based on the average genome size for *P. falciparum*.[47] This was similar to the LLOD of qPCR for the 18S rRNA gene, which was calculated to be 33.3 fg/μL, or approximately 1.4 parasites/μL. Notably, iso-IMRS achieves this sensitivity within 30 min and at a single temperature, representing a significant reduction in resource needs compared to qPCR without sacrificing analytical sensitivity. The iso-IMRS assay also showed improved sensitivity compared to an isothermal LAMP assay for *P. falciparum*[51] (Figure S3). These data support the potential of the proposed method to detect asymptomatic infections <10 parasites/μL.

We next tested iso-IMRS with simulated patient samples. Using synchronized *P. falciparum* parasites cultured *in vitro* in human red blood cells and diluted into whole blood, we tested iso-IMRS with several parasite concentrations at varying growth stages. DNA was extracted using a silica spin-column extraction kit and parasite DNA was quantified with a TaqMan qPCR assay for *P. falciparum*.[40] The extracted DNA was also analyzed with real-time iso-IMRS amplification, and the threshold time (time to exponential amplification) was determined. Across several concentrations, iso-IMRS consistently showed exponential amplification of parasite DNA within 30 min (Figure 2C). As the parasite DNA concentration approached the LLOD of the assay, amplification between
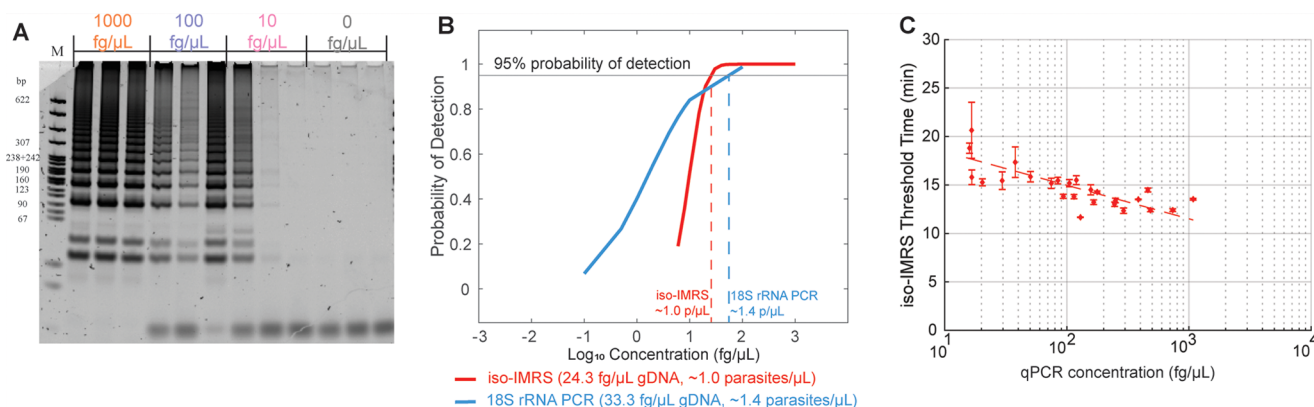

Figure 2. (A) Iso-IMRS-amplified products on 10% acrylamide gel show multiple amplified products across input P. falciparum gDNA concentration ranging from 100 to 10 fg/μL. (B) Probit analysis of iso-IMRS (re)demonstrates similar LLOD to the 18S rRNA PCR assay (blue). (C) DNA extracted from simulated patient samples (*in vitro* cultured P. falciparum parasites in whole blood) amplifies with iso-IMRS within 30 min.

technical replicates was more stochastic, resulting in increased variability in the iso-IMRS threshold time at lower concentrations. However, in all cases, there was successful iso-IMRS amplification of extracted parasite DNA. We also observed highly stochastic but successful DNA amplification at concentrations <10 fg/μL; however, these data were not shown as the samples fall outside of the qPCR standard curve and could not be reliably quantified.

Iso-IMRS Successfully Amplifies *P. falciparum* DNA from Patient Blood Samples and Patient Saliva Samples. To assess the utility of iso-IMRS in field settings, we analyzed whole blood samples from 18 febrile patients tested for *Plasmodium* by microscopy. Ten patients were *Plasmodium*-positive and eight patients had febrile symptoms but were *Plasmodium*-negative. An additional sample from a healthy donor was *Plasmodium*-negative. For all 19 samples, DNA extracted from patient blood was amplified with iso-IMRS. The sample results (Table 1) showed good

Table 1. Iso-IMRS Analysis of Patient Whole Blood Samples

	microscopy diagnosis	
	Plasmodium positive	*Plasmodium* negative
iso-IMRS positive	9	1
iso-IMRS negative	1[a]	8

[a]Further analysis by nested PCR showed that the sample was *P. falciparum*-negative.

concordance between the clinical diagnosis and the iso-IMRS assay: nine out of ten positive samples were iso-IMRS-positive, and eight out of nine negative samples were iso-IMRS-negative. Two samples were not in concordance with the clinical diagnosis.

One sample positive by microscopy was diagnosed negative by iso-IMRS. However, when DNA from this sample was further analyzed with a nested PCR assay specific for *P. falciparum*, it was found to be *Plasmodium*-positive but negative for *P. falciparum*. Therefore, the results of the iso-IMRS assay were consistent with the expected results for this sample. There was one false-positive result (diagnosed *Plasmodium*-negative but amplified by iso-IMRS).

We also assessed the potential for iso-IMRS diagnosis using noninvasive patient saliva samples. Previous publications have found that while cell-free DNA is present in significantly lower concentrations in saliva compared to those in peripheral blood, *P. falciparum* DNA is detectable in saliva.[48,49] We hypothesized that our highly sensitive assay would be capable of detecting even low levels of DNA in saliva. DNA was extracted from the saliva of ten patients diagnosed *Plasmodium*-positive by microscopy and analyzed by nested PCR using universal primers to amplify *Plasmodium* DNA and using the amplified product as a template for the second PCR assay specific for *P. falciparum* DNA. By the nested PCR method, eight samples were *Plasmodium*-positive and *P. falciparum*-positive and two samples were *Plasmodium*-positive and *P. falciparum*-negative. The same extracted DNA was analyzed by iso-IMRS, and the results from all samples were consistent with the nested PCR results (Table 2).

Iso-IMRS Assay Characterization with Next-Generation Sequencing Confirms Assay Specificity. The iso-IMRS assay was further characterized by comparing the products acquired by gel electrophoresis (Figure S4) to product sizes predicted *in silico*. Expected product sizes were

Table 2. Iso-IMRS Analysis of Patient Saliva Samples

	universal primer positive	
	P. falciparum positive	*P. falciparum* negative
iso-IMRS positive	8	0
iso-IMRS negative	0	2

determined with the original IMRS algorithm, which predicted an excess of products <1000 bp. Comparing this prediction to products visualized by gel electrophoresis (Figure S2), most of the expected products are represented on the gel except for products of ~200 bp. It is possible that ~200 bp products were produced in lower concentrations than the others, resulting in a band that was not visible on the gel.

To further characterize amplified products, Illumina next-generation sequencing (NGS) was performed on four amplified products. After amplification, products were size-separated and four individual bands of 100, 150, 250, and 300 bp were extracted and analyzed by NGS. The resulting NGS forward and reverse reads were aligned to the *P. falciparum* genome and *P. vivax* genome to confirm specificity.

All four products aligned to the *P. falciparum* genome in the expected regions predicted by the algorithm, with minimal alignment (<5 reads in either orientation) in regions outside of the predicted areas (Table 3). Additionally, the product

Table 3. Predicted Amplification Regions Correlate with NGS Alignment to *P. falciparum* Genome Regions

chromosome	predicted amplification region, IMRS algorithm	NGS alignment to *P. falciparum*
6	57,296−59,909	57,220−59,935
6	1,305,863−1,309,145	1,305,840−1,309,117
10	1,582,798−1,583,926	1,582,797−1,583,849
11	94,873−96,754	94,897−96,805

sequences showed minimal alignment (<5 reads in either orientation) to the *P. vivax* genome, indicating that the products are specific to *P. falciparum*.

Although the four products sequenced were of different sizes, we observed that the NGS data showed alignment to the same region of the *P. falciparum* genome for all four products (Figure S5). To explain this observation, we analyzed the predicted products *in silico* and found that the highest-concentration NGS reads aligned with short products <200 bp predicted by the algorithm (Figures S6−S8).

Based on these observations, the longer aligned regions likely consist of a range of different overlapping shorter products. In other words, for example, the 100 bp band that was isolated and sequenced likely consists of different 100 bp products that aligned in such a manner of overlaps to finally appear as a 300 bp product in the NGS alignment. Notably, the NGS results show that all sequenced products align with the regions predicted by the algorithm and do not show nonspecific alignment outside of the predicted regions (Figures S6−S8).

Adapting Iso-IMRS to the Point of Care with Instrument-Free DNA Extraction and Visual Readout. One impediment to deploying NAAT methods to the POC is the need for resource-intensive DNA extraction techniques, such as centrifugal methods. To address this issue, we developed a paper-based, centrifuge-free device to extract nucleic acids from whole blood, called the flexible System for

Nucleic Acid Preparation (SNAPflex).[50] Whole blood is lysed at room temperature and the extracted DNA is precipitated and purified in the SNAPflex device on a paper membrane by passive wicking (Figure 3A). Captured nucleic acids can be either used directly on paper or eluted and analyzed with NAATs.
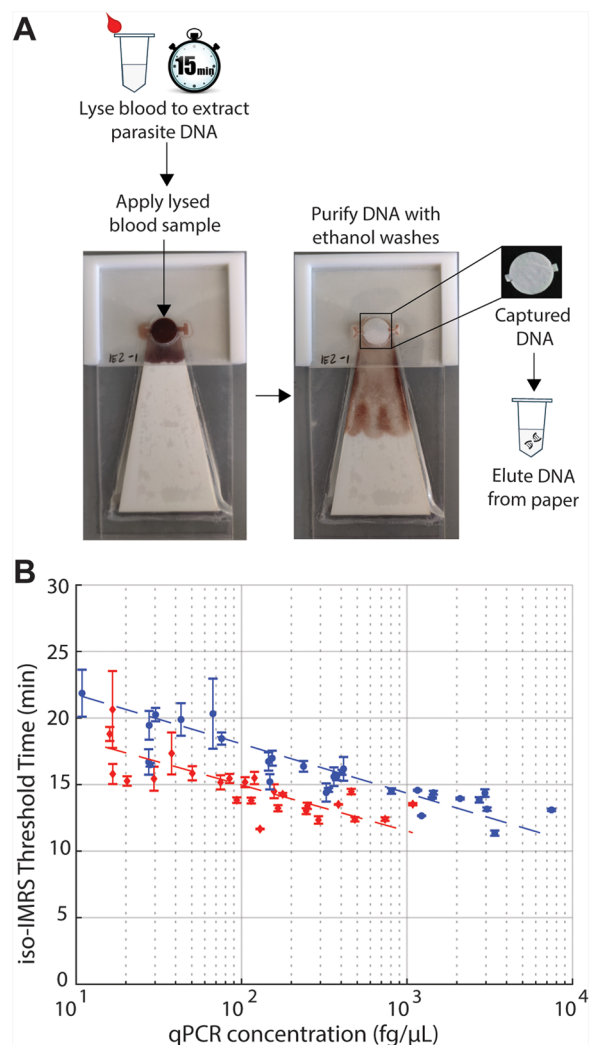

Figure 3. (A) SNAPflex centrifuge-free DNA extraction and elution from whole blood (B) iso-IMRS amplification of *in vitro* cultured parasites extracted with SNAPflex (blue) compared to spin-column extraction kits (red).

For this study, DNA from SNAPflex-extracted *P. falciparum* simulated patient samples (infected red blood cells in whole blood) was eluted into a standard elution buffer. As a control, samples were also extracted with standard centrifuge-based silica membrane extraction kits (data also presented in Figure 2). Parasite DNA was quantified using both qPCR and real-time iso-IMRS. The results showed successful amplification of SNAPflex-extracted samples across several parasite concentrations, demonstrating that iso-IMRS is compatible with the POC extraction method (Figure 3B). While all SNAPflex-extracted samples were successfully amplified by iso-IMRS, the resulting threshold times shifted later for SNAPflex-extracted samples (blue) compared to the control samples (red). We hypothesize that residual lysed blood components or salts may have impacted the iso-IMRS amplification efficiency. However,

although amplification occurred later for SNAPflex-extracted samples, the data show successfully that amplified sample concentrations commensurate with asymptomatic infections (<10 parasites/μL, ~240 fg/μL).

The iso-IMRS assay was further adapted to a visual "YES/NO" readout using nitrocellulose lateral flow strips (LFSs) with an anti-FAM antibody-coated "test line" and a biotin-coated "control" line. A fraction of biotinylated dNTPs were included in the iso-IMRS assay mix so that amplified products would contain biotin to bind to streptavidin-coated gold nanoparticles. The assay mixture also included a fraction of FAM antigen-tagged forward primers to enable product binding to the "test line". The "test" line captures antigen- and gold nanoparticle-tagged amplicons, appearing red, while the "control line" captures streptavidin-coated gold nano-particles only, demonstrating successful flow (Figure 4a).
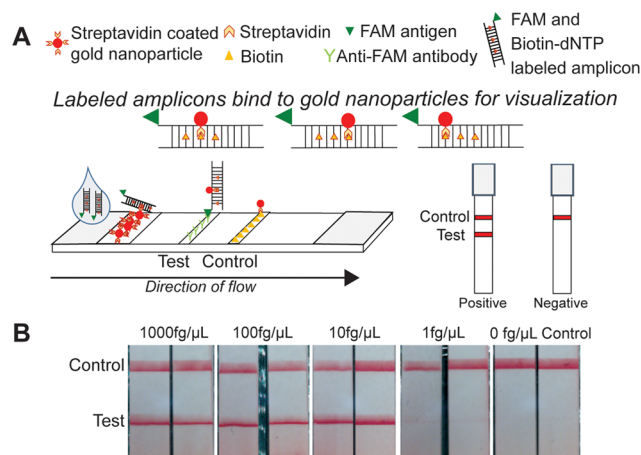

Figure 4. (A) Schematic for LFS visualization with biotin-dNTPs and anti-FAM-labeled LFSs. (B) Iso-IMRS amplified products visualized on LFSs show similar LLOD to real-time amplification.

Prior to applying the sample to the LFS, a mixture of ammonium acetate and isopropanol was added to the iso-IMRS products to precipitate larger, amplified DNA products while leaving unincorporated dNTPs and primers in solution. The solution was then filtered through a glass fiber membrane to filter out the unincorporated dNTPs, ensuring that these would not saturate the gold nanoparticles. The glass fiber membrane with amplified products was placed directly onto the lateral flow strip and products were eluted with a running buffer onto the conjugate pad to bind gold nanoparticles and flow across the strip (Figure S9). In moving this method to a true POC technique, further optimization of biotin-labeled dNTPs is required to eliminate these additional steps for dNTP removal.

Adapting iso-IMRS to "YES/NO" readout on LFS showed similar sensitivity to fluorescence-based readout (Figure 4b). Similar to the fluorescence-based results, amplification was stochastic at concentrations <10 fg/μL. Including FAM-tagged forward primers was sufficient for LFS capture and visual-ization. Further optimization studies showed that incorporat-ing FAM-tagged reverse primers in addition to forward primers may potentially improve the analytical sensitivity of LFS and is a further direction to explore (Figure S10).

Finally, iso-IMRS amplification of parasite DNA directly on paper from extraction with the SNAPflex POC extraction device was demonstrated. Using simulated patient samples

(infected red blood cells in whole blood), DNA from several parasite cultures was isolated onto paper using the SNAPflex device. After extraction, the capture membrane was placed directly into an iso-IMRS reaction to amplify captured parasite DNA. The results were then visualized using the lateral flow strip method. The samples were also extracted with standard silica column-based extraction kits, and parasite DNA was analyzed by qPCR to determine the expected parasite concentration. These experiments showed that, across several DNA concentrations, direct amplification and detection on paper is possible. As expected, parasite DNA concentration >10 fg/μL showed a strong signal on LFS (Figure 5). We also
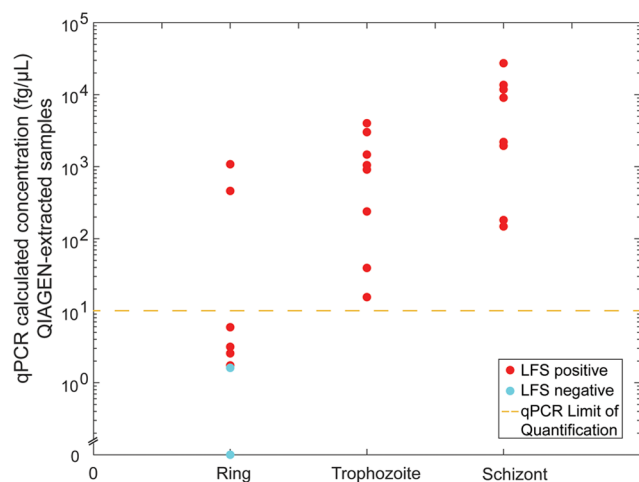

Figure 5. *In vitro* cultured parasite samples at three growth stages were extracted with SNAPflex, amplified by iso-IMRS, and detected positive (red) or negative (blue) on LFS.

observed the lateral flow detection of several samples with DNA concentration below the expected LLOD for iso-IMRS, including samples that were below the qPCR limit of quantification. These results indicate that iso-IMRS LFS detection may be more sensitive than fluorescence readout in this case, potentially due to the detection of partial amplified products. Notably, there were no false-positive LFS results from whole blood samples without parasites because the LFS binding reaction adds stringency to the assay by selecting for specific amplification products. These results further support that the iso-IMRS LFS readout of samples with <10 fg/μL DNA likely represents a true positive result.

CONCLUSIONS

Here, we developed a novel isothermal assay for the amplification and detection of *P. falciparum* DNA. The iso-IMRS assay uses only two primers, each distributed in >50 locations across the genome, to generate several DNA products of multiple sizes. Products can be detected either by traditional methods (i.e., with fluorescent intercalating dyes) or with visual "YES/NO" readout (i.e., lateral flow strips). The iso-IMRS assay shows excellent analytical sensitivity comparable to qPCR (~1 parasite/μL) and successfully amplifies DNA from simulated patient samples consisting of parasite-infected red blood cells in whole blood. Iso-IMRS successfully amplified DNA from clinical samples, suggesting good initial clinical sensitivity and specificity for both patient blood and saliva samples.

We have further demonstrated the assay with SNAPflex, a centrifuge-free room-temperature DNA extraction device. Future integration of iso-IMRS with SNAPflex and translation to a lyophilized format will enable its use at the point of care for malaria elimination.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.analchem.0c03847.

> Primer distribution, real-time amplification curves, LOD comparison to the LAMP isothermal assay, predicted amplicon product sizes, amplicon alignment to the *P. falciparum* genome, LFS optimization, and iso-IMRS troubleshooting (PDF)

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Author Contributions

S.R.L. conceived the concept of iso-IMRS and designed the IMRS algorithm. Experimental work for iso-IMRS development and demonstration and manuscript authorship was performed by N.K. under the guidance of C.M.K. and M.C. and with experimental assistance from M.Z. S.K. and S.R.L. provided additional scientific guidance. NGS analysis was performed by P.L. under the guidance of J. Galagan. J. Gitaka conducted clinical sampling, laboratory assays on the clinical samples, and data analysis. M.K. conducted laboratory assays on the clinical samples. All authors have given approval to the final version of the manuscript.

Notes

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