

Molecular-based isothermal tests for field diagnosis of malaria and their potential contribution to malaria elimination

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In countries where malaria transmission has decreased substantially, thanks to the scale-up of control interventions, malaria elimination may be feasible. Nevertheless, this goal requires new strategies such as the active detection and treatment of infected individuals. As the detection threshold for the currently used diagnostic methods is 100 parasites/ μL , most low-density, asymptomatic infections able to maintain transmission cannot be detected. Identifying them by molecular methods such as PCR is a possible option but the field deployment of these tests is problematic. Isothermal amplification of nucleic acids (at a constant temperature) offers the opportunity of addressing some of the challenges related to the field deployment of molecular diagnostic methods. One of the novel isothermal amplification methods for which a substantial amount of work has been done is the loop-mediated isothermal amplification (LAMP) assay. The present review describes LAMP and several other isothermal nucleic acid amplification methods, such as thermophilic helicase-dependent amplification, strand displacement amplification, recombinase polymerase amplification and nucleic acid sequence-based amplification, and explores their potential use as high-throughput, field-based molecular tests for malaria diagnosis.

Keywords: malaria elimination, isothermal amplification, PCR, tHDA, LAMP, NASBA, SDA, RPA

Introduction

Detecting *Plasmodium* parasites in peripheral blood, in both febrile patients and asymptomatic carriers, is essential for any malaria control programme aiming at decreasing local transmission and achieving the pre-elimination status.¹ The WHO recently launched a global initiative known as T3 ('Test. Treat. Track. '), aimed at supporting malaria-endemic countries in their efforts to achieve universal coverage with diagnostic testing, antimalarial treatment and surveillance.² Though malaria has been eliminated from Europe and North America with no other diagnostic tool than microscopy,³ this seems more difficult in tropical countries, including sub-Saharan Africa, because of the substantial number of individuals harbouring a malaria infection at extremely low densities, undetectable by microscopy.⁴ The currently available tools for diagnosing malaria include microscopy, parasite antigen/enzyme detection kits [commonly referred to as rapid diagnostic tests (RDTs)] and molecular tools (discussed in Cordray and Richards-Kortum⁵), each of them with specific advantages and limitations and with the potential of being deployed at different levels of the health system (microscopy and RDTs more peripherally than molecular tools).^{5,6} Indeed, microscopy and RDTs remain the only feasible options at health facility or lower level, e.g. community case management of malaria.⁶ These two diagnostic tools have limitations. For example, the absence of the *pfhrp-2* gene (encoding the HRP-2 protein, to which most antigen

detection tests are directed) in *Plasmodium falciparum* populations from some endemic areas resulted in a substantial proportion of false negatives.⁷ Moreover, both microscopy and RDTs cannot detect parasite densities <100 parasites/ μL , particularly in field conditions, while asymptomatic carriers have a much lower parasite density.⁸ The latter can be identified by nucleic acid amplification tests (NAATs), often by PCR, one of the most widely used molecular methods for the detection and identification of infectious diseases. The detection limit of PCR, which is ~ 1 parasite/ μL depending on the assay type,^{9,10} is lower than either RDTs or microscopy and PCR is therefore able to detect asymptomatic malaria carriers who may be targeted for treatment.¹¹ In some settings, asymptomatic sexual and asexual stages of *P. falciparum* infections may persist and sustain transmission at very low parasite densities, below the threshold of detection by microscopy or RDTs and in reach only of molecular methods such as PCR.¹²⁻¹⁴ However, PCR-based assays are the least feasible to perform in field settings as they are prone to contamination, heavily influenced by the purity of the sample nucleic acid content thus relying on purified extracts and requiring cold storage facilities for reagents.^{5,15,16} Therefore, until recently, molecular diagnostic methods required specialized equipment and personnel and could not be carried out outside reference facilities. The possibility of amplifying DNA at isothermal temperatures without the need of a thermocycling apparatus has created the opportunity of performing molecular diagnostic tests at a

more peripheral level¹⁷ and thus of improving the management of infectious diseases, especially in resource-limited settings.^{15,16,18} Recent guidelines published by WHO recommend that diagnostic devices for resource-limited settings should be ASSURED: Affordable, Sensitive, Specific, User-friendly, Rapid and robust, Equipment-free and Deliverable to end users.^{19,20} This paper reviews some molecular isothermal amplification techniques (with the ASSURED criteria in mind) with the aim of determining their possible deployment for high-throughput detection of asymptomatic *P. falciparum* carriers in the field, e.g. in mass screening and treatment campaigns.

Sample preparation for field-deployable molecular assays

Preparing the sample for molecular assays is a bottleneck for NAATs because it involves lengthy processes, often performed manually.^{18,21,22} DNA extraction for molecular assays is a critical step, with different methods resulting in different yield and quality of the nucleic acid.^{23,24} One of the major problems, particularly when processing large quantities of samples, is the inhibition of the PCR by the haem (from red blood cells) obtained from the crude DNA extraction.²⁵ Ideally, the DNA extraction method should have the following characteristics: rapid preparation and high throughput; high reliability; production of good-quality DNA for long-term storage; avoidance of cross-contamination; and reasonable costs.²³

Several isothermal technologies may fulfil these requirements as they appear less affected by the inhibitory effect of blood products and have similar sensitivity and specificity as standard PCR when DNA is extracted using crude methods such as heat treatment.^{18,26,27}

PCR adaptations for detection of malaria parasites in the field

New approaches for simplifying PCR for field settings, including assays that are less prone to inhibition, have been explored.^{28–30} The palm-held PCR device by Aham Biosystems³¹ features three stages of specially structured heat blocks that are maintained at temperatures suitable for each of the three PCR steps and the sample is circulated across the high- and low-temperature zones inside the sample tube. Field PCR units and portable real-time PCR systems with freeze-dried reagents have been developed by Biofire Diagnostics for mobile analytical laboratories and field hospitals for pathogen detection.³² Lab-on-chip, point-of-care diagnostic assays are also being designed for the diagnosis of disease pathogens.³³ However, these assays are still relatively expensive for resource-limited settings and are not entirely infrastructure-free. Post-amplification detection is also being simplified with the development of PCR–nucleic acid lateral-flow immune assays.³⁴

Isothermal amplification of nucleic acids

Based on new findings on DNA/RNA synthesis, non-PCR-based methods of nucleic acid amplification involving the use of accessory proteins and mimicking *in vitro* nucleic acid amplification have been developed.³⁵ Isothermal approaches can facilitate rapid target amplification through single-temperature incubation,

reducing the system complexity compared with PCR-based methods. Established isothermal amplification methods have different degrees of complexity (multiple enzymes or primers) and also varying sensitivity and specificity.³⁶ A major advantage of isothermal amplification methods is the simplicity of endpoint determination, often by visual observation, which allows assays to be run in a closed system, thus reducing the risk of post-amplification contamination.^{20,37,38} The choice of which reaction to use is primarily driven by the target of interest to be amplified. Amplification time, reaction temperature, tolerance to substances in biological samples, length of the target, initial heat denaturation and complexity (i.e. the number of enzymes and primers required) are all factors to be considered when choosing an appropriate method.³⁹ In the past two decades, several isothermal amplification methods for detecting pathogens, including malaria parasites, have been used (Table 1).

Isothermal amplification methods currently used in malaria diagnosis

Loop-mediated isothermal amplification (LAMP)

LAMP, first described in 2000,⁴⁰ can be performed with simplified and inexpensive specimen processing, under isothermal conditions in a simple heating block or water bath. Furthermore, it can be formatted for visual detection without the need for instrumentation, which is a major advantage for its field deployment.^{41,42} LAMP is a one-step amplification reaction that employs self-recurring strand-displacement synthesis primed by a specially designed set of primers identifying six distinct sequences on the target DNA.⁴⁰ Detailed reviews (principles, primer set-up and design) are available elsewhere.^{37,38,41–43} At costs ranging from USD <1.00/test (using ready-made reaction mixture prepared with individual reagents) to USD 5.31/test (using commercially available reaction mixture),³⁸ LAMP assays proffer to be cheaper than PCR assays. However, one major short-fall of the LAMP assay is the complexity in primer and assay design and optimization (Table 2).

The use of LAMP for the diagnosis of various diseases, including human malaria, has been investigated extensively.^{6,25,26,44–57} A lower limit of detection in the range of 5–10 parasites/ μ L has been reported,^{25,46} while sensitivity and specificity, when compared with an 18S rRNA gene PCR assay using crude DNA extracts, were 95% and 99%, respectively (Table 1).²⁶ However, a subsequent study using the same PCR and LAMP protocols reported much lower estimates of sensitivity and specificity, i.e. 76% and 90%, respectively.⁴⁵ Taking microscopy as the gold standard and with different primer sets targeting the same gene, the sensitivity and specificity were 96% and 94%, respectively.⁴⁶ In 130 field samples collected in Thailand and using a composite reference diagnosis for each sample, i.e. two out of three tests [microscopy, nested PCR (nPCR) and LAMP] giving the same result, LAMP had 100% sensitivity and specificity for *P. falciparum* infection.⁵⁸ With primers targeting the mitochondrial genome and nPCR assay as the reference test, the sensitivity and specificity were 93% and 100%, respectively.²⁵ In a recent study in Uganda, the sensitivity and specificity of LAMP *P. falciparum* primers were 98% and 98%, respectively, and 97% and 99% for the genus primers.⁵⁹ In a recent comparison of an optimized LAMP protocol against a highly sensitive three-well nPCR reference assay (in

Table 1. Isothermal molecular-based tests for diagnosis of pathogens

	Isothermal amplification assay	Pathogen	Isothermal condition	Gold standard/ref test	Sensitivity or limit of detection	Specificity	Reference
1	tHDA	<i>Clostridium difficile</i>	65°C for 60 min	PCR	100%	100%	79
2	tHDA	herpes simplex virus	64°C for 60 min	ELVIS shell vial assay	100%	96.30%	78
3	tHDA	<i>H. pylori</i>	95°C for 2 min; 60°C for 1 h	culture; histology	90%; 96.6%	95.7%; 96.8%	74
4	tHDA	<i>H. pylori</i>	95°C for 2 min; 60°C for 1 h	culture; histology	92.5%; 100%	95.4%; 98.8%	75
5	tHDA	<i>Staphylococcus aureus</i> ; MRSA	95°C for 5 min; 60°C for 1 h	biochemical and genotyping methods	100%; 100%	100%; 98%	76
6	tHDA	Ebola virus	65°C for 120 min	ND	0.2 pg of total human RNA for GAPDH detection; 3.2 copies of Ebola virus-armoured RNA	ND	73
7	tHDA	HIV	65°C for 75 min	ND	50 copies/assay	ND	77
8	tHDA	<i>N. gonorrhoeae</i>	65°C for 60 min	Abbott CT/GC kit	100%	100%	115
9	tHDA	<i>Plasmodium</i> spp.; <i>P. falciparum</i> ; <i>P. vivax</i>	64°C for 90 min	microscopy/NASBAMT	97%	100%	81
10	LAMP	<i>P. falciparum</i>	60°C for 120 min	PCR	95%	99%	26
11	LAMP	<i>P. falciparum</i>	65°C for 120 min	PCR	76%–79%	58%–89%	45
12	LAMP	<i>Plasmodium</i> spp.	60°C for 100 min; inactivation 80°C for 2 min	microscopy	98.50%	94.30%	46
13	LAMP	<i>P. falciparum</i>	63°C for 90 min	microscopy	97.80%	85.70%	44
14	LAMP	<i>Plasmodium</i> spp.	63°C for 90 min	microscopy; PCR	96.7%; 98.9%	91.7%; 100%	6
15	LAMP	<i>Plasmodium</i> spp.	60°C for 100 min; inactivation 80°C for 2 min	microscopy	98.30%	100%	116
16	LAMP	<i>Plasmodium</i> spp.; <i>P. falciparum</i>	65°C for 40 min; inactivation 80°C for 5 min	PCR	93.9%; 93.3%	100%; 100%	25
17	LAMP	<i>P. falciparum</i> ; <i>P. vivax</i>	60°C for 60 min	composite ref (LAMP, Mx, PCR)	100%; 100%	100%; 100%	58
18	LAMP	<i>Plasmodium</i> spp.; <i>P. falciparum</i>	65°C for 40 min; inactivation 80°C for 5 min	nPCR	98.4%; 97%	98.1%; 99.2%	59
19	LAMP	<i>P. falciparum</i>	65°C for 90 min; inactivation 80°C for 5 min	RT-PCR	100%; 100%	98.1%; 100%	47
20	NASBA	<i>Trypanosoma brucei</i>	65°C for 2 min; 41°C for 92 min	microscopy	10 parasites/mL	100%	117
21	NASBA	<i>Leishmania</i>	65°C for 2 min; 41°C for 92 min	microscopy	93.3%; 98.6%	100%; 100%	118
22	NASBA	<i>Plasmodium</i> spp.	65°C for 4 min; 41°C for 2 h	microscopy	97.40%	80.90%	67
23	NASBA	<i>Plasmodium</i> spp.	65°C for 4 min; 41°C for 2 h	microscopy	100%	94%	68
24	NASBA	astrovirus	65°C for 5 min; 41°C for 95 min	RT-PCR			66
25	NASBA	<i>P. falciparum</i>	65°C for 4 min; 41°C for 2 h	microscopy			119
26	NASBA	<i>P. falciparum</i>	65°C for 4 min; 41°C for 2 h	real-time QT-PCR			69
27	NASBA	<i>M. tuberculosis</i>	42°C for 1 h	culture	85.70%	95.50%	120
28	NASBA	hepatitis A virus	65°C for 5 min; 40±1°C for 150 min	ND	1 pfu	ND	121
29	RPA	MRSA	37°C for 60 min	ND	2 copies/rxn	ND	87
30	RPA	MRSA	39°C for 1 h	ND		ND	88
31	RPA	RVFV	42°C for 20 min	ND			102

32	SDA	<i>M. tuberculosis</i>	95°C for 2 min; 40°C for 2 h	culture and smear at positive threshold of 2.4 and 15.5 <i>M. tuberculosis</i> organisms per rxn	100%; 95%	84%; 96%	122
33	SDA	<i>M. tuberculosis</i>	BD ProbeTec™ ET System	culture	96.10%	100%	111
34	SDA	<i>E. coli</i>	95°C for 2 min; 53°C for 35 min; 95°C for 5 min	PCR	4.3 cfu/5 µL	100%	110
35	SDA	<i>N. gonorrhoeae</i> ; <i>C. trachomatis</i>	BD ProbeTec™ ET System	composite ref (PCR and LCR)	90%; 95%	100%; 100%	123
36	SDA	<i>N. gonorrhoeae</i> ; <i>C. trachomatis</i>	52.5°C for 1 h	culture or composite ref of any two molecular methods (PCR, SDA, LCR)	100%; 92%	100%; 99%	124
37	SDA	<i>N. gonorrhoeae</i> ; <i>C. trachomatis</i>	BD ProbeTec™ ET System	composite ref of two or three (PCR, SDA, TMA)	97.1% or 100%; 92.2% or 100%	98.8% or 96%; 96.47% or 89.6%	125
38	SDA	<i>N. gonorrhoeae</i> ; <i>C. trachomatis</i>	BD ProbeTec™ ET System	culture	100%; 95.3%	99.7%; 99.3%	126
39	SDA	<i>M. tuberculosis</i>	BD ProbeTec™ ET System	microscopy	77.80%	97.70%	109
40	SDA	<i>N. gonorrhoeae</i>	BD ProbeTec™ ET System	culture	98.10%	100%	127
41	SDA	<i>N. gonorrhoeae</i> ; <i>C. trachomatis</i>	BD ProbeTec™ ET System	culture	96.2%; 92%	98.4%; 94.9%	128

GADPH, glyceraldehyde-3-phosphate dehydrogenase; LCR, ligase chain reaction; Mx, microscopy; NASBAMT, NASBA malaria test; ND, not determined; rxn, reaction.

which amplification in any well of three replicates per sample was counted as positive), LAMP showed a sensitivity of 90% compared with 51% for microscopy.⁶⁰ LAMP has also been reported for the detection of other *Plasmodium* species including *Plasmodium vivax*,^{46,61} *Plasmodium malariae*, *Plasmodium ovale*,⁴⁶ human *Plasmodium knowlesi* infection^{62,63} and gametocytes by reverse transcription (RT-LAMP),⁴⁷ showing the ease of adaptability of this method.

Nucleic acid sequence-based amplification (NASBA)

NASBA, first described by Kievits *et al.*⁶⁴ in 1991, is a homogeneous, isothermal nucleic acid amplification method that is particularly suited to RNA targets in a double-stranded DNA background. A cocktail of three enzymes (reverse transcriptase, T7 RNA polymerase and RNase H) acting in concert allows the rapid amplification of target sequences by >10⁸-fold without the use of expensive thermal-cycling equipment, the end product being a single-stranded RNA antisense to the original RNA template.⁶⁵ Since there is no DNA denaturation step in NASBA, contaminating genomic or proviral DNA (the precursor or latent form of a virus integrated into the genetic material of a host cell) is not amplified. However, the extent of the reaction cannot be controlled by adjusting the number of cycles and the likelihood of non-specific interactions is increased because the amplification temperature cannot exceed 41°C without the risk of enzymatic denaturation.⁶⁶

The reliability of the NASBA process has been tested by sequencing the RNA product directly from a NASBA reaction, with 90% of the sequences readable, an excellent result considering the AT richness of the *P. falciparum* genome.⁶⁵ For detection and semi-quantification of malaria parasites and species identification, NASBA, compared with microscopy, had a sensitivity of 97% and specificity of 81%.⁶⁷ Another study detecting products of NASBA amplification by electrochemiluminescence reported a sensitivity of 100% and specificity of 94%.⁶⁸ Schneider *et al.*⁶⁹ reported a significant correlation between parasite quantification results by real-time quantitative NASBA (QT-NASBA) and real-time quantitative PCR (QT-PCR). QT-NASBA has also been used to determine gametocyte carriage (prevalence and density) with the ability of detecting gametocyte densities as low as 0.02–0.1/µL.^{69,70} The cost of NASBA assays has not been determined. However, considering that NASBA requires similar consumables and infrastructure as PCR, it is expected that assay costs would be similar (Table 2).

Thermophilic helicase-dependent amplification (tHDA)

In this system, which was first described in 2004,⁷¹ strands of duplex DNA are separated by a DNA helicase and coated by single-stranded DNA-binding proteins (SSBs). Sequence-specific primers hybridize to each border of the target DNA and DNA polymerases extend the primers annealed to the templates to produce a double-stranded DNA (Figure 1). The two newly synthesized double-stranded DNA products are subsequently used as substrates by DNA helicases, entering the next round of the reaction. Thus, a simultaneous chain reaction proceeds, resulting in the exponential amplification of the selected target sequence.⁷¹ tHDA amplifies nucleic acid targets efficiently at 65°C and requires fewer protein components than the mesophilic HDA platform,

Table 2. Comparison between established PCR and novel isothermal amplification assays

	PCR	LAMP	NASBA	tHDA	RPA	SDA	
1	sample processing prior to amplification	nucleic acid extraction required	amplification from crude samples possible	nucleic acid extraction required	amplification from crude samples possible	nucleic acid extraction required	amplification from crude samples possible
2	cost	USD 7–8 per sample ¹²⁹	USD <1–5.3 ³⁸	equivalent to PCR	relatively cheaper than PCR	relatively cheaper than PCR	relatively cheaper than PCR
3	ease of use/simplicity of operation	complex	relatively easier to set up than PCR	relatively easier to set up than PCR	relatively easier to set up than PCR	relatively easier to set up than PCR	relatively easier to set up than PCR
4	skill/training required	high	moderate	high	moderate	moderate	moderate
5	stability of reagents	cold chain required for enzymes	cold chain required for enzymes	cold chain required for enzymes	cold chain required for enzymes	reagents available as dry pellets	cold chain required for enzymes
6	amplification time	~2 h	30–60 min	~2 h	~60 min	~60 min	30–60 min
7	simplicity of design	complex primer design and assay optimization	complex primer design and assay optimization	complex primer design and assay optimization	complex primer design and assay optimization	complex primer design and assay optimization	complex primer design and assay optimization
8	principle	high temperature and thermostable polymerase	thermophilic strand displacement polymerase	reverse transcription and strand displacement polymerase	helicase and thermophilic strand displacement polymerase	recombinase–polymerase complex	thermophilic strand displacement polymerase
9	test temperature	varying	65°C	41°C	60–65°C	37°C	40°C
10	risk of contamination	potential risk minimized by proper set up	potential risk minimized by proper set up	potential risk minimized by proper set up	potential risk minimized by proper set up	potential risk minimized by proper set up	potential risk minimized by proper set up
11	quality assurance control	possible	possible	possible	possible	possible	possible
12	post-amplification detection	electrophoresis; fluorescence detection	naked eye; turbidity measurement; electrophoresis	fluorescence detection	lateral-flow strip; electrophoresis; fluorescence detection	fluorescence detection; electrophoresis	fluorescence detection; electrophoresis
13	sensitivity	high	high	high	high	high	high
14	specificity	high	high	high	high	high	high
15	limit of detection (<i>Plasmodium</i>)	1–5 parasites/ μ L	1–5 parasites/ μ L	<1 parasite/ μ L	unknown	unknown	unknown
16	<i>Plasmodium</i> species identification	yes	yes	yes	yes	possible	possible
17	identification of sexual and asexual forms	yes	yes	yes	possible	possible	possible
18	high throughput	yes	yes	yes	yes	yes	yes
19	instrumentation requirement	thermocycler	heating block or water bath	real-time cycler	heating block or water bath	real-time cycler; heating block or water bath	real-time cycler; heating block or water bath
20	infrastructure requirement	electricity	electricity and exothermal chemical devices	electricity	electricity	electricity	electricity
21	field tested	no	yes	yes	yes	no	no
22	product developer	various	Eiken Group, Japan	Cangene Corporation, Canada	Biohelix Corporation, USA	TwistDx, USA	Becton Dickinson and Co., USA

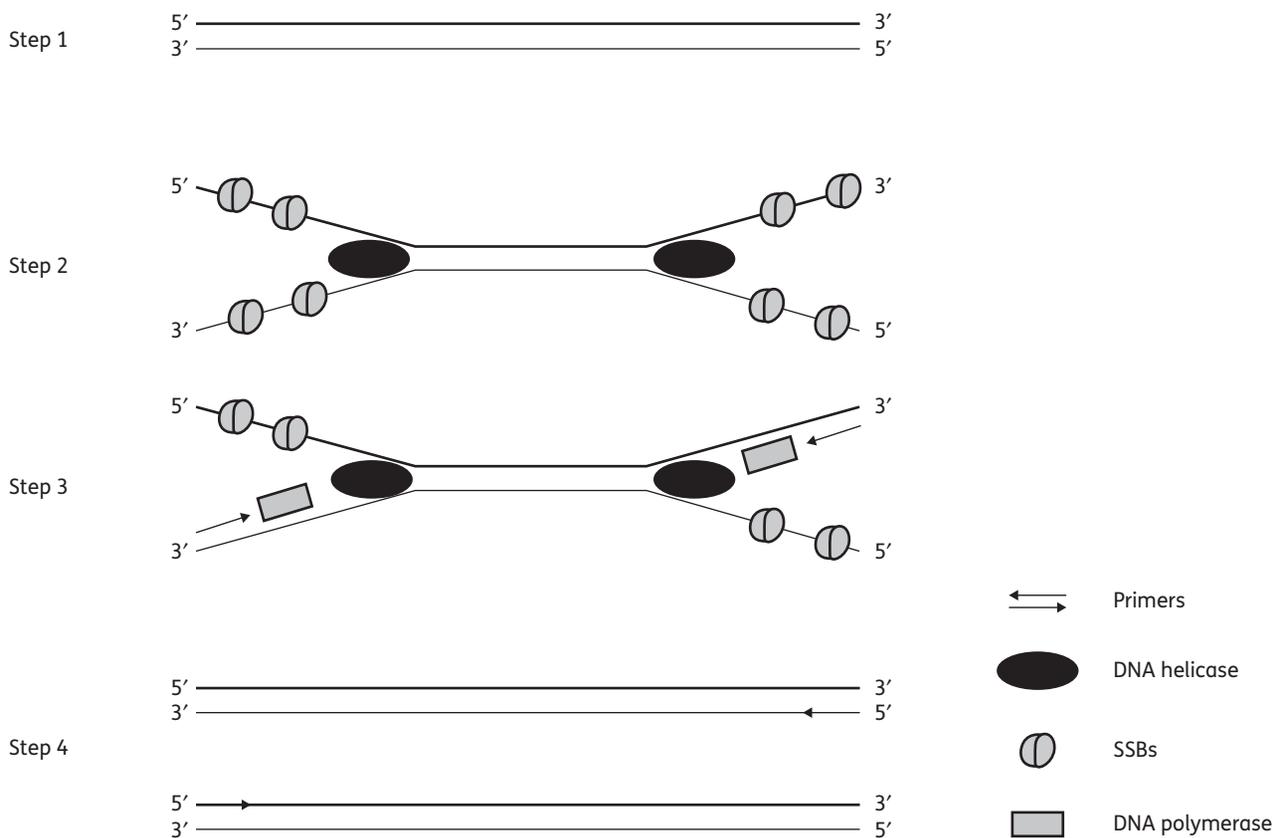


Figure 1. Schematic representation of tHDA. Steps 1 and 2: DNA helicase binds to double-stranded DNA and begins unwinding while SSBs attach to stabilize the single strands. Step 3: sequence-specific primers bind and DNA polymerase synthesizes new DNA strands in the 5'→3' direction. Step 4: duplex DNA strands formed serve as a template for another round of amplification.

which is performed at 37°C.⁷² However, because both methods are isothermal and do not require thermocycling, they present a relatively cheaper platform than PCR.

The tHDA platform has been successfully used for the detection of several pathogens.⁷³ When compared with culture methods for detection of *Helicobacter pylori*, tHDA-ELISA had a sensitivity and specificity of 90% and 96%, respectively, and a higher sensitivity (97%) and specificity (97%) when compared with histology.⁷⁴ However, when using colorimetric detection with gold nanoparticle probes, the sensitivity was higher (93% compared with culture methods and 100% with histology) and specificity similar or higher (95% compared with culture methods and 99% with histology detection).⁷⁵ The sensitivity and specificity for the detection of different pathogens was higher when amplified DNA was applied onto a vertical-flow strip embedded in a disposable cassette (Table 1).^{76–80} Recently, clinical and analytical performance of tHDA for *Plasmodium* detection and species-level identification in blood samples was reported, with overall sensitivity of 97% (95% CI, 87%–99%) and specificity of 100% (95% CI, 85%–100%).⁸¹

Adaptation of the tHDA on a microarray platform can be carried out directly on the surface of a glass slide by immobilizing one primer on the glass substrate and leaving the corresponding primer in solution. With the helicase unwinding the DNA template, the resulting DNA single strand is able to anneal at the immobilized primer and will be subsequently elongated by the DNA

polymerase. Labelling the corresponding primer with a reporter allows successful detection of amplified targets from the microarray platform by laser scanning or total internal reflection fluorescence technologies.⁸²

Other isothermal amplification assays with potential for use in malaria diagnosis

In recent years, a vast array of isothermal amplification methods targeting DNA, RNA or both have been developed or used for the diagnosis of pathogens. These are T7 promoter-driven amplifications: transcription-mediated amplification (TMA),⁸³ single primer isothermal amplification,⁸⁴ strand displacement methods such as strand displacement amplification (SDA) and smart amplification (SmartAmp),^{85,86} recombinase polymerase amplification (RPA),^{87,88} isothermal and chimeric primer-initiated amplification of nucleic acids,⁸⁹ self-sustained sequence replication reaction,⁹⁰ exponential amplification reaction,⁹¹ cross-priming amplification,^{92,93} rolling circle amplification⁹⁴ and the genome exponential amplification reaction technique.⁹⁵ Table 2 summarizes the comparison of some important features between PCR and the isothermal amplification methods described in this review. Most of these assays require multiple enzymes (two or more), rigorous optimization, a heat source and post-amplification analysis.

Based on the WHO ASSURED criteria for diagnostic assays for resource-limited settings, two of the isothermal amplification methods, which are deemed more advanced towards field implementation, namely RPA and SDA, were selected for further discussion. Their suitability for deployment as field-based molecular tests for malaria parasite detection was based on assay chemistry, simplicity of design and operation, cost-efficiency and robustness.

RPA

In RPA, the isothermal amplification of specific DNA fragments is achieved by the binding of opposing oligonucleotide primers to template DNA and their extension by a DNA polymerase. Global melting of the template is not required for the primers to be directed to their complementary target sequences. Instead, RPA employs recombinase–primer complexes to scan double-stranded DNA and facilitate strand exchange at cognate sites.^{96–98} The resulting structures are stabilized by SSBs interacting with the displaced template strand, thus preventing the ejection of the primer by branch migration (Figure 2).⁹⁹ Recombinase

disassembly leaves the 3'-end of the oligonucleotide accessible to a strand-displacing DNA polymerase, in this case the large fragment of *Bacillus subtilis* PolI (Bsu),¹⁰⁰ and primer extension ensues. Exponential amplification is accomplished by the cyclic repetition of this process.

Key to RPA is the establishment of a dynamic reaction environment that balances the formation and disassembly of recombinase–primer filaments.⁸⁷ The reaction system is provided in a stabilized dried format, which may permit transportation and limited storage without refrigeration even though long-term storage under refrigeration is still recommended.¹⁰¹ RPA has also been recently reported in a SlipChip microfluidic platform that enables multistep manipulation in parallel with large numbers of small volumes, consisting of two plates containing wells and ducts that can be brought into contact and moved relatively to one another to manipulate fluids by creating and breaking fluidic paths.⁸⁸ Recently, a highly sensitive isothermal RPA assay for the detection of Rift Valley fever virus (RVFV) RNA on a mobile device was published, though the assay is yet to be validated on clinical samples.¹⁰²

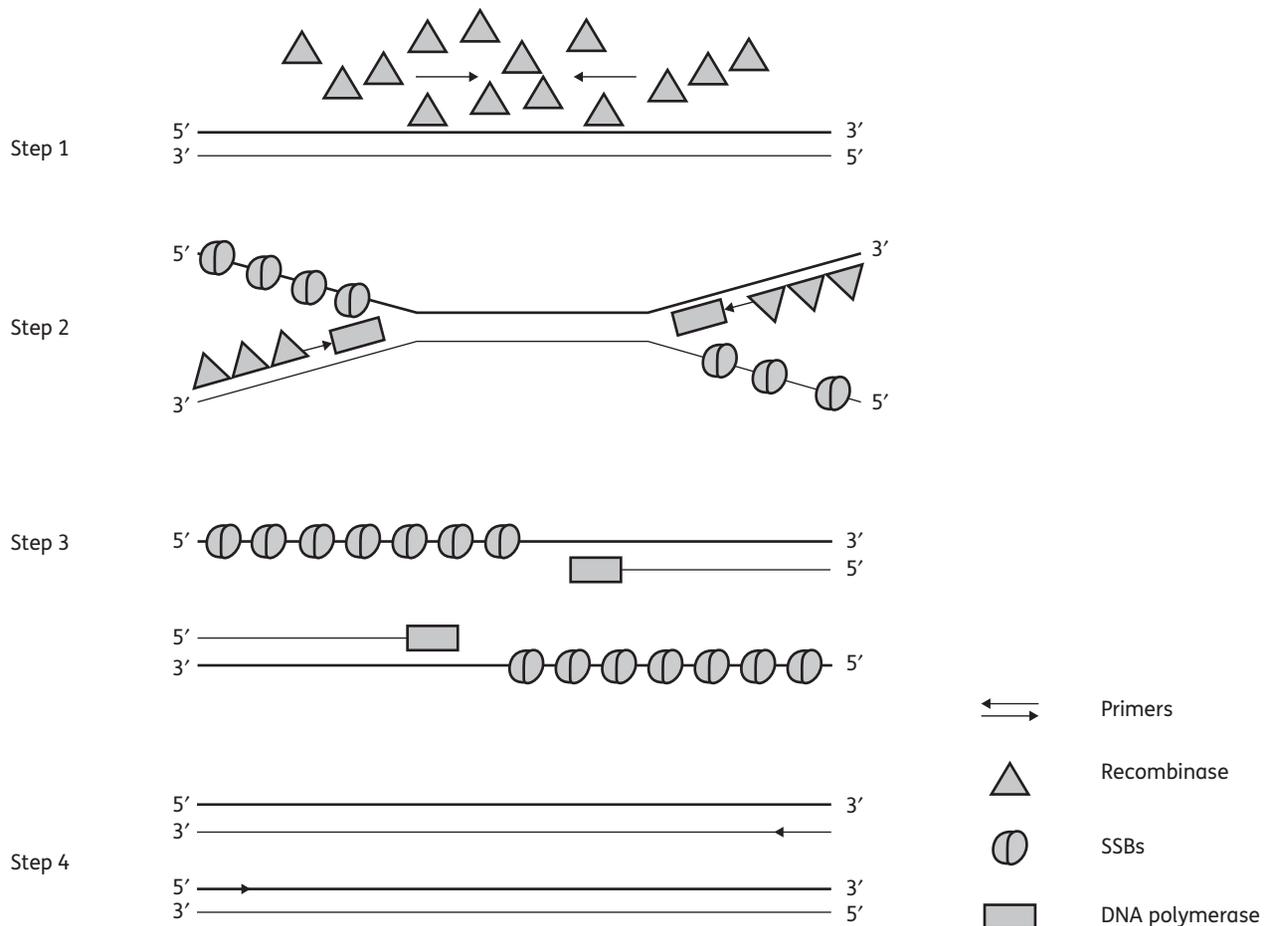


Figure 2. Schematic representation of RPA. Step 1: primers and recombinases form a complex that targets a homologous DNA sequence. Step 2: DNA polymerase synthesizes a new strand by displacing the complementary strand (strand exchange). SSBs help stabilize the displaced single strands. Step 3: the parent DNA strand separates and synthesis continues to form two new duplex DNA strands. Step 4: duplex DNA strands formed serve as a template for another round of amplification.

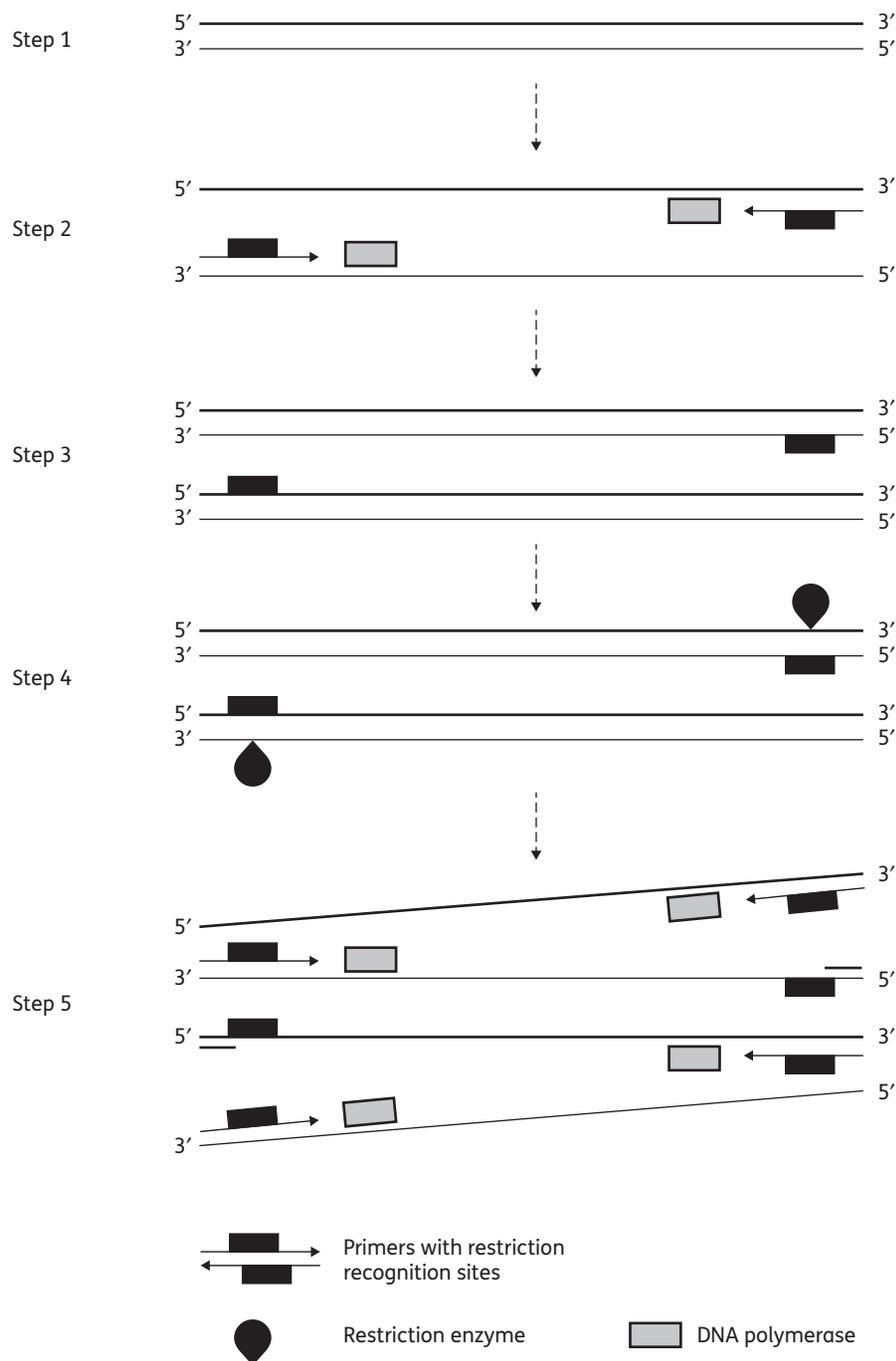


Figure 3. Schematic representation of SDA. Step 1: denaturation of double-stranded DNA. Steps 2 and 3: primers bind on each strand while DNA polymerase extends to produce double-stranded DNA with a modified (hemiphosphorothioate) recognition site. Step 4: a restriction enzyme cleaves the unmodified strand of the newly synthesized double-stranded DNA, displacing it. Step 5: DNA polymerase synthesizes a new strand from the cleaved DNA strand for another round of restriction digest and polymerization.

SDA

SDA is based on the ability of a restriction enzyme to nick or cut the unmodified strand of a hemiphosphorothioate form of its recognition site as well as the ability of a DNA polymerase to initiate replication at the 'cut site' and displace the downstream non-template strand. Primers containing recognition sites for the

nicking restriction enzyme bind to opposite strands of target DNA at positions flanking the sequence to be amplified. The target fragment is exponentially amplified by coupling sense and antisense reactions in which strands displaced from the sense reaction serve as a target for the antisense reaction and vice versa.¹⁰³ The method consists of two parts (Figure 3): (i) a target

generation process that makes copies of the target sequence flanked by enzyme restriction sites; and (ii) the exponential amplification of these modified target sequences by repeated nicking, strand displacement and priming of displaced strands.¹⁰⁴

Despite the seemingly complicated sequence of events, SDA operates under a very simple protocol. Target DNA is heat denatured in the presence of all reagents except the restriction enzyme and polymerase; amplification then proceeds at 40°C after cooling and addition of the enzymes.⁸⁵ Amplified products may then be detected by a variety of methods.¹⁰⁵ The original SDA process was not very efficient and it has been improved by incorporating a thermostable polymerase and a different exonuclease to increase the yield and rate of amplification. These new conditions allow a 10¹⁰-fold amplification of target after 15 min at 60°C.^{106,107} SDA can also be used to detect RNA by incorporating a reverse transcription step.¹⁰⁸ Varying sensitivities and specificities have been reported for diagnosis of *Mycobacterium tuberculosis*, *Neisseria gonorrhoeae*, *Chlamydia trachomatis* and enterohaemorrhagic *Escherichia coli* (Table 1).^{109–111}

Discussion and conclusions

Isothermal amplification techniques have potential for the field diagnosis of malaria infection. They eliminate the need for a costly and power-intensive thermocycler, produce results in a short time (from 30 min to 1 h), can be used to process large numbers of samples required for active surveillance and are capable of detecting infections of <1 parasite/μL of blood, of both sexual and asexual stages.^{25,46,69,70,112} The sensitivity and specificity of these techniques are comparable to those of PCR-based diagnostics.^{5,37} However, it should be noted that isothermal amplification techniques, though relatively cheaper than PCR, are not totally equipment- or infrastructure-free but rather have a better potential for field deployment due to their simplified amplification conditions; thus, alternative heat sources are being explored.²⁰

One major limitation for the field deployment of these isothermal amplification assays is the endpoint detection of amplified products. Real-time fluorescence or turbidity measurement is the most reliable method of post-amplification detection.¹⁸ With LAMP assays, positive samples can be visualized and identified with the naked eye as a result of the white precipitate of magnesium pyrophosphate formed during the reaction.⁴¹ Adaptation of the vertical-flow strip embedded in a disposable cassette or lab-on-chip portable devices for endpoint detection of amplified product with the isothermal amplification assays would be more user-friendly and field deployable.^{77,81,87}

The Foundation for Innovative New Diagnostics has been working with the Hospital for Tropical Diseases in London and Eiken Chemical Company (Japan) in the development of a simplified LAMP assay for the diagnosis of malaria. Prototypes of this test have been compared with PCR using samples from febrile patients in two clinical trials, one in London (travellers) and the other in an endemic setting in Uganda.^{59,60,113} This places LAMP at the forefront of all the isothermal amplification assays with the potential to replace PCR in the nearest future. Commercial LAMP reaction kits have also been developed recently for numerous viral, bacterial and protozoan pathogens.³⁷ The combination of LAMP into a 'lab on a chip' with diagnosis performed on a single-use device would offer a sensitive alternative to microscopy and RDTs.^{25,114}

As the global malaria map continues to shrink and elimination is seriously being considered in certain territories, active detection of asymptomatic carriers may be scaled up in these regions. Therefore, a robust field-deployable, molecular-based assay that is able to give results comparable to laboratory-based assays, using crude sample sources and simple end product detection, would be the best approach to handle the large number of samples that would be generated. The development of isothermal amplification techniques has paved the way for this.

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Transparency declarations

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