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Development of a thermostabilised triplex LAMP assay with dry-reagent four target lateral flow dipstick for detection of *Entamoeba histolytica* and non-pathogenic *Entamoeba* spp.

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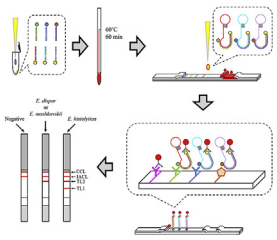
HIGHLIGHTS

- The developed triplex LAMP-NALFIA assay is ready-to-use and cold-chain-free.
- The triplex LAMP reagent mix is thermostabilized and lyophilised.
- The LAMP assay is designed to simultaneously amplified three specific DNA targets.
- The biosensor is developed to facilitate interpretation of post-LAMP analysis.
- The triplex LAMP-NALFIA assay could detect as low as 10 *Entamoeba* trophozoites.

GRAPHICAL ABSTRACT



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ABSTRACT

This study highlighted the development of a four target nitrocellulose-based nucleic acid lateral flow immunoassay biosensor in a dry-reagent strip format for interpretation of double-labelled double-stranded amplicons from thermostabilised triplex loop-mediated isothermal amplification assay. The DNA biosensor contained two test lines which captured biotin and texas red labelled amplicons; a LAMP internal amplification control line that captured digoxigenin labelled amplicon; and a chromatography control line that validated the functionality of the conjugated gold nanoparticles and membrane. The red lines on detection pad were generated when the gold nanoparticles conjugated antibody bound to the fluorescein labelled amplicons, and the capture agents bound to their specific hapten on the other 5′ end of the double-stranded amplicon. The applicability of this DNA biosensor was demonstrated using amoebiasis-causing *Entamoeba histolytica* simultaneously with the non-pathogenic but morphologically identical *Entamoeba dispar* and *Entamoeba moshkovskii*. The biosensor detection limit was 10 *E. histolytica* trophozoites, and revealed 100% specificity when it was evaluated against 3 medically important *Entamoeba* species and 75 other pathogenic microorganisms. Heat stability test showed that the biosensor was stable for at least 181 days at ambient temperature. This ready-to-use and cold-chain-free

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biosensor facilitated the post-LAMP analysis based on visualisation of lines on strip instead of observation of amplicon patterns in agarose gel.

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1. Introduction

The introduction of nucleic acid amplification technology had revolutionised disease diagnostics. The ability of polymerase chain reaction (PCR) to amplify as little as a single gene copy had established it as a highly sensitive diagnostic assay [1]. However, PCR relied on repetition of several temperatures in a continuous chain reaction which made it dependent on thermocycler. The advent of several isothermal amplification methods as alternatives to PCR had facilitated molecular diagnostics. In particular, loop-mediated isothermal amplification (LAMP) introduced by Notomi, Okayama, Masubuchi, Yonekawa, Watanabe, Amino and Hase [2] had innovated the DNA amplification platform by circumventing thermal cycling to amplify its target. LAMP is basically an amplification method that uses either two or three sets of primers and a high strand-displacement activity DNA polymerase to amplify DNA target at a constant temperature ranging from 60 °C to 65 °C. It also provides highly efficient DNA amplification of up to 10^9-10^{10} times in 15–60 min, and the concentration of the LAMP product is much higher than that generated by conventional PCR [3]. Since then, LAMP had been widely applied in various studies for detection of medically important pathogens such as *Salmonella* Typhi, *Leptospira* spp. and *Burkholderia pseudomallei* [4–6].

Despite its potential, LAMP had not been well studied for multi-target detection due to its ladder-like products pattern that restricted the differentiation among the targets on agarose gel after electrophoresis. Attempts to use signal quantification method in detecting the multiplex LAMP products had been reported to be promising [7–9]. However, requirement of trained personnel and expensive real-time PCR thermal cycler were deterrents to its application in resource-limited laboratories. Therefore, innovation on improving the visualisation and interpretation of amplified LAMP products was still pertinent when it involved two or more targets in a single reaction. Lateral flow platform technology had been reported to be promising in enhancing the visualisation and analysis of amplified LAMP products [10,11]. Preference of lateral flow platform to colorimetric agents, fluorescent agents, lab-on-a-chip devices and turbidimeter was due to its rapid display, disposable format and ease-of-use as well as its potential to be developed into point-of care biosensor [12].

Molecular detection of *Entamoeba histolytica* was important for diagnosis of amoebiasis as it was morphologically indistinguishable from two other non-pathogenic *Entamoeba* species, namely *Entamoeba dispar* and *Entamoeba moshkovskii* [13–15]. Several highly sensitive and specific LAMP assays for detection of *E. histolytica* had been successfully developed [16–18]. To date, all these assays were cold-chain dependent and the post-amplification analysis required agarose gel electrophoresis and UV trans-illuminator for result visualisation. Besides, these reported LAMP assays were unable to verify the validity of negative results as internal amplification control (IAC) was not incorporated. Validation of amplification process was pertinent in this context, as diagnosis of enteric pathogens such as *E. histolytica* involved extraction of DNA from stool samples which may contain amplification inhibitors; hence inclusion of IAC was important to verify false negative results [19,20].

Nurul Najian et al. [21] had previously described a duplex LAMP assay with label-based lateral flow dipstick for detection of pathogenic *Leptospira*. However, application of the previous study was not convenient at remote areas because the LAMP reagent mix and the lateral flow were dependent on cold-chain storage. Moreover, in addition to IAC, the system had only one test line which was not applicable for detection that required two test lines. The objective of this study was to develop a thermostabilised triplex LAMP assay coupled with a dry-reagent nucleic acid lateral flow immunoassay (NALFIA) for simultaneous detection of *E. histolytica* and *Entamoeba* spp. with incorporation of IAC. A nitrocellulose membrane-based NALFIA was developed prior to the triplex LAMP assay development in order to facilitate the development and interpretation of the LAMP double-labelled double-stranded amplicons. Then, a thermostabilised triplex LAMP assay was developed for detection and differentiation of *E. histolytica* from *E. dispar* and *E. moshkovskii* as well as simultaneous validation of the assay using IAC.

2. Experimental

2.1. Reagents and apparatus

Rabbit polyclonal IgG texas red antibody was purchased from Invitrogen, Thermo Fisher Scientific (Massachusetts, USA); while streptavidin, sheep polyclonal IgG digoxigenin antibody, goat anti-mouse IgG secondary antibody and fluorescein isothiocyanate (FITC) IgG1 monoclonal antibody were purchased from Pierce Thermo Fisher Scientific (Massachusetts, USA). The colloidal gold solution (40 nm) was purchased from Kestrel Bio Sciences (Thailand). Bovine serum albumin (BSA) was purchased from Amresco (Solon, USA) and western blocking reagent (WBR) was purchased from Roche (Indianapolis, USA). Mineral oil, betaine, sodium azide (NaN_3), polyvinylpyrrolidone (PVP), polyvinyl alcohol (PVA), Tween-20, Triton X-100, sucrose, and other common chemicals were from Sigma (St. Louis, USA). All reagents and chemicals in this study were prepared using ultrapure water (>18MΩ) from a Millipore Milli-Q water purification system (Billerica, USA). Nitrocellulose membrane card HF135, cellulose fiber pads, glass fiber pads and Amicon Ultra 50 K were also Millipore products.

All labelled and non-labelled oligonucleotides were synthesised by Integrated DNA Technologies (Singapore). The LAMP isothermal amplification *Bst* DNA polymerase was purchased from New England Biolabs (Massachusetts, USA) and amplification reactions were performed using Cole-Parmer chilling heating block (Illinois, USA). The LAMP amplicons were analysed using Alpha Innotech ChemiImager 5500 UV illuminator and image capturing unit (California, USA). The NALFIA was lined with streptavidin and antibodies using BioDot XYZ3050 dispensing platform (Irvine, USA) and were cut into strips using Matrix 2360 programmable strip cutter from Kinematic Automation (Twain Harte, USA).

2.2. Entamoeba species and other microorganisms

Microorganism isolates used in this study were listed in Table 1 and were acquired from the Departamento de Medicina Experimental, Facultad de Medicina, Universidad Nacional Autónoma de México, Mexico; the London School of Hygiene and Tropical

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Table 1
List of reference organisms and LAMP-NALFIA analytical specificity evaluation results.

Strains	Serogroup or species	No. of isolates	Analytical specificity of LAMP-NALFIA assay		
			TL1	TL2	IACL
Entamoeba spp.	*Entamoeba histolytica HM-1*:IMSS	1	+	+	+
	Entamoeba dispar SAW760	1	−	+	+
	Entamoeba moshkovskii Laredo	1	−	+	+
Other enteric pathogens	*Acinetobacter baumannii*	1	−	−	+
($n = 75$)	*Aeromonas hydrophila*	2	−	−	+
	Bacillus subtilis	1	−	−	+
	Burkholderia cepacia	1	−	−	+
	Burkholderia pseudomallei	1	−	−	+
	Burkholderia thailandensis	1	−	−	+
	Citrobacter freundii	1	−	−	+
	Enterococcus faecalis	1	−	−	+
	Enterococcus faecium	1	−	−	+
	Enterococcus gallinarum	1	−	−	+
	Enterohemorrhagic *Escherichia coli*	1	−	−	+
	Enteroinvasive *Escherichia coli*	1	−	−	+
	Enteropathogenic *Escherichia coli*	1	−	−	+
	Enterotoxigenic *Escherichia coli*	1	−	−	+
	Escherichia coli	1	−	−	+
	group A *Streptococcus*	1	−	−	+
	group B *Streptococcus*	1	−	−	+
	group F *Streptococcus*	1	−	−	+
	group G *Streptococcus*	1	−	−	+
	Haemophilus influenza	1	−	−	+
	Klebsiella pneumoniae	2	−	−	+
	Leptospira biflexa ser. Patoc	1	−	−	+
	Leptospira interrogans ser. Canicola	1	−	−	+
	Leptospira interrogans ser. Hebdomadis	1	−	−	+
	Leptospira interrogans ser. Pomona	1	−	−	+
	Leptospira licerasiae ser. Varillal	1	−	−	+
	Listeria monocytogenes	1	−	−	+
	Salmonella enterica ser. Agona	1	−	−	+
	Salmonella enterica ser. Albany	1	−	−	+
	Salmonella enterica ser. Bardo	1	−	−	+
	Salmonella enterica ser. Bordeaux	1	−	−	+
	Salmonella enterica ser. Braenderup	1	−	−	+
	Salmonella enterica ser. Emek	1	−	−	+
	Salmonella enterica ser. Enteritidis	1	−	−	+
	Salmonella enterica ser. Hadar	1	−	−	+
	Salmonella enterica ser. Heidelberg	1	−	−	+
	Salmonella enterica ser. Java	1	−	−	+
	Salmonella enterica ser. Kibi	1	−	−	+
	Salmonella enterica ser. Kissi	1	−	−	+
	Salmonella enterica ser. Newport	1	−	−	+
	Salmonella enterica ser. Oslo	1	−	−	+
	Salmonella enterica ser. Paratyphi A	2	−	−	+
	Salmonella enterica ser. Paratyphi B	2	−	−	+
	Salmonella enterica ser. Paratyphi C	1	−	−	+
	Salmonella enterica ser. Poona	1	−	−	+
	Salmonella enterica ser. Regent	1	−	−	+
	Salmonella enterica ser. Richmond	1	−	−	+
	Salmonella enterica ser. Tshiongwe	1	−	−	+
	Salmonella enterica ser. Typhi	2	−	−	+
	Salmonella enterica ser. Typhimurium	1	−	−	+
	Salmonella enterica ser. Uppsala	1	−	−	+
	Salmonella enterica ser. Virchow	1	−	−	+
	Salmonella enterica ser. Weltevreden	1	−	−	+
	Shigella boydii	2	−	−	+
	Shigella dysenteriae	2	−	−	+
	Shigella flexneri	2	−	−	+
	Shigella sonnei	2	−	−	+
	Staphylococcus aureus	1	−	−	+
	Staphylococcus epidermidis	1	−	−	+
	Vibrio cincinnatiensis	1	−	−	+
	Vibrio fluvialis	1	−	−	+
	Vibrio mimicus	1	−	−	+
	Vibrio parahaemolyticus	1	−	−	+
	Vibrio vulnificus	1	−	−	+
	Yersinia enterocolitica	2	−	−	+
Total		78			

TL1, first test line; TL2, second test line; IACL, internal amplification control line; +, positive result; -, negative result.

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Medicine, London, UK; the Institute for Medical Research, Malaysia; and the Department of Medical Microbiology and Parasitology, School of Medical Sciences, Universiti Sains Malaysia, Malaysia. All the isolates were listed in Table 1. DNAs isolated from axenically grown *E. histolytica HM-1*:IMSS and lyophilised *E. dispar* SAW760 were used as positive controls in this study. Both organisms were a gift from Alfonso Olivos-Garcia (Departamento de Medicina Experimental, Facultad de Medicina, Universidad Nacional Autónoma de México, Mexico). DNA extraction was performed using Qiagen QIAamp DNA Stool extraction kit (Germany). DNA of *E. moshkovskii* Laredo was a kind gift from Professor Graham C. Clark (London School of Hygiene and Tropical Medicine, London, UK) and it was used to validate the designed primer set that targeted *Entamoeba* spp., as well as to determine the specificity of the developed assay in this study.

2.3. Establishment of dry-reagent triplex LAMP

2.3.1. LAMP primers design

E. histolytica species-specific primers were designed based on the reported serine-rich *E. histolytica* protein (*SREHP*) gene (GenBank accession no. M80910.1) while *Entamoeba* spp. specific primers were derived from *E. histolytica* large subunit ribosomal RNA (LSU-rRNA) gene (GenBank accession no. Z29969.1). LAMP IAC primers were designed based on the *Plasmodium falciparum* 3D7 Thrombospondin-related anonymous protein (*TRAP*) gene (GenBank accession no. XM_001350052.1). Primer sets for all the three targets were designed using Primer Explorer V4 software (http://primerexplorer.jp/elamp4.0.0/index.html). All primer sequences were submitted to BLAST search for *in silico* evaluation of its specificities. In order to allow the amplicons to be capturable by the NALFIA, the primers were modified and labelled as showed in Table 2. The first target (T1), second target (T2) and IAC loop primers were labelled respectively with biotin, texas red and digoxigenin while their forward or backward inner primers were commonly labelled with fluorescein at 5′ end. The sensitivity and specificity of these primers were verified before the formulation of triplex LAMP assay.

2.3.2. Formulation of triplex LAMP assay

The outer primer to inner primer ratio for each of the 3 targets (T1, T2 and IAC) was optimised separately in monoplex LAMP reactions. These 3 primer sets were pooled in a ratio of 1:1:1, and were further optimised based on primer set ratio. The primer sets ratio was introduced and optimised according to targets priority before establishment of triplex LAMP assay. The final concentrations of primers for T1 were optimal with 2 μM of each forward inner primer (FIP) and backward inner primer (BIP), 0.167 μM of each forward outer primer (F3) and backward outer primer (B3) and 0.67 μM of backward loop primer (LB) while primer set T2 was optimal with 0.5 μM of each FIP and BIP, 0.167 μM of each F3 and B3 and 0.67 μM of LB. The IAC primer set was optimised at 0.67 μM of each FIP and BIP, 0.083 μM of each F3 and B3 and 0.33 μM of forward loop primer (LF). Apart from these optimal concentration of primers, the triplex reaction was carried out with a total of 30 μL reaction mixture containing 1 × isothermal amplification buffer [20 mM Tris-HCl (pH 8.8), 50 mM KCl, 10 mM $(NH4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Tween® 20] (New England Biolabs, Massachusetts, USA), 0.8 M betaine (Sigma, Missouri, USA), 1.2 mM of each dNTP (Thermo Fisher Scientific, USA), supplementary 6 mM $MgSO_4$ (New England Biolabs, Massachusetts, USA), 44 U of *Bst* 2.0 WarmStart DNA polymerase (New England Biolabs, Massachusetts, USA) and 1 fg of IAC DNA plasmid.

2.3.3. Preparation of thermostablised triplex LAMP mix

Deglycerolisation of *Bst* DNA polymerase by centrifugal filtration was performed using Amicon Ultra 50 K. *Bst* 2.0 WarmStart DNA polymerase which was stored in a suspension buffer [10 mM Tris—HCl, (pH 7.5), 50 mM KCl, 1 mM DTT, 0.1 mM EDTA, 0.1% Triton X-100 and 50% glycerol] was added into a centrifugal filter unit to a final volume of 500 μL. The centrifugal device was centrifuged at 14,000 × *g* for 10 min at 4 °C and the flow-through was discarded. The retentate was washed with 10 mM Tris—HCl (pH 7.5) followed by inverting the centrifugal filter unit. The retentate was retrieved by centrifugation at 1000 × *g* for 2 min at 4 °C. Deglycerolised *Bst* DNA polymerase was immediately placed into triplex LAMP mix prior to lyophilisation process. To convert the triplex LAMP mix into a dry-reagent format different combinations of bulking agents with stabilising sugars were tested. The optimised dry-reagent triplex LAMP mix (30 μL) contained 2 μM of each Eh-FIP-SER and Eh-BIP-SER-FITC, 0.167 μM of each Eh-F3-SER and Eh-B3-SER, 0.67 μM of Eh-LB-SER-Biotin, 0.5 μM of each Eh-FIP-HLY and Eh-BIP-HLY-FITC, 0.167 μM of each Eh-F3-HLY and Eh-B3-HLY, 0.67 μM of Enta-LB-HLY-Tex, 0.67 μM of each IAC-FIP-SSP2-FITC and IAC-BIP-SSP2, 0.083 μM of each IAC-F3-SSP2 and IAC-B3-SSP2, 0.33 μM of IAC-LB-SSP2-Dig, 1.2 mM of each dNTP, 44 U of *Bst* 2.0 WarmStart DNA

Table 2
List of oligonucleotide sequences.

Primers	Sequences (5′-3′)	Length (mer)	5′ Label
Targeting *Entamoeba histolytica* (*SREHP* gene) [T1]			
Eh-FIP-SER	GCTTCGTTCTTTAAAAATACACCGTCATTCTTGATTTGGATCAAGAAGT	49	–
Eh-BIP-SER-FITC	AGTAGCTCAGCAAAACCAGAATCACTTGCTTTTTCATCTTCATCA	45	Fluorescein
Eh-F3-SER	TGCATTCACTAGTGCAACT	19	–
Eh-B3-SER	GCTTGATTCTGAGTTATCACTTG	23	–
Eh-LB-SER-Biotin	AAGTTCAAATGAAGATAATGAA	22	Biotin
Targeting *Entamoeba* spp. (LSU-rRNA gene) [T2]			
Eh-FIP-HLY	TACGCCATTTCGTTTCCTTACTCGATTTCTTAACTGATACTCGACCG	47	–
Eh-BIP-HLY-FITC	AGATTGAAACTGTCCTTAGTGCAGCAGTTCTAAGATGTTTTTTTCCTC	48	Fluorescein
Eh-F3-HLY	CCTGAAAATGGATGGCATTA	20	–
Eh-B3-HLY	CCCTAATCCAAGTAATGTTGTT	22	–
Enta-LB-HLY-Tex	CTTGGTGGTAGTAGCAAATACTAAG	25	Texas red
Internal amplification control (*TRAP* gene) [IAC]			
IAC-FIP-SSP2-FITC	TGCAGAATCAGCATACAAGTTACATGCTTTCAACAGATTTCTTGTAGG	48	Fluorescein
IAC-BIP-SSP2	GTTATCGGACCCTTTATGAAGGCCGTCCCAAACACCACAAC	41	–
IAC-F3-SSP2	GTTTTTGGTATTGGACAAGGTA	22	–
IAC-B3-SSP2	CCTTTACCACAAGTTACACTAC	22	–
IAC-LF-SSP2-Dig	TACCATCTGATGGATGACAA	20	Digoxigenin

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polymerase, 7.5% (w/v) raffinose, 1 fg of IAC DNA plasmid template and ultrapure water. The aqueous triplex LAMP mix was subjected to a drying process for 2.5 h at 1.8^{-2} mBar pressure using a Heto vacuum concentrator (Thermo Scientific Heto) connected to a LyoLab 3000 freeze-dryer (Thermo Scientific Heto) as described in a previous study with some modifications until an amorphous glassy matrix was formed [22]. The thermostabilised triplex LAMP mixes were sealed in aluminium pouches containing silica gel and stored in a desiccator cabinet until further experiments.

2.4. Construction of NALFIA strip

2.4.1. Bio-conjugation of antibody to gold nanoparticles (GNPs)

Bio-conjugation of GNPs with FITC antibody was conducted to functionalise the GNPs as signal generator for the NALFIA. The conjugation process was essentially performed as described by Ang, Yu and Yean [23] with slight modifications to maximise the yield of functionalised GNPs. To produce 1 mL of functionalised GNPs, 10 mL of colloidal gold solution with optical density at 522 nm (OD_{522}) value of 1 were adjusted to pH 8.0 followed by conjugation of 40 μg FITC antibody on GNPs. The mixture was shaken gently at ambient temperature for 1 h followed by supplemented with 1% (w/v) BSA and allowed to mix for another 1 h at room temperature. The GNPs-FITC antibody conjugates mixture were then centrifuged at 10,000 × *g* for 30 min at 4 °C. The pellet was suspended in 10 mM phosphate buffer (PB) containing 1% (w/v) BSA and 0.05% (w/v) NaN_3.

2.4.2. Preparation of dry-reagent NALFIA strip

The dry-reagent NALFIA strip (5 mm × 77 mm) composed of a buffer application pad, a gold conjugate pad, a 25 mm in length laminated nitrocellulose membrane and an absorbent pad as shown in Fig. 1. Prior to affixing the three amplicons capturing proteins, Millipore HF135 nitrocellulose membrane card was assembled with cellulose fiber as the device absorbent pad. The absorbent pad was overlaid 2 mm on the membrane card to ensure continuous capillary action. The dipsticks were then affixed with three different double-labelled double-stranded amplicons-capturing-proteins onto nitrocellulose membrane using BioDot FrontLine dispenser (Irvine, USA) at a rate of 1.5 μL cm^{-1} followed by drying overnight in desiccator cabinet. A mixture of 0.2% WBR with 0.05% triton X-100 which dissolved into 2 mM PB (pH 7.4) buffer was used to block the uncoated surface of nitrocellulose membrane at the rate of 32 μL cm^{-1}. A conjugated GNPs suspension with OD_{522} value of 5 in 2 mM PB supplemented with 0.01% (v/v) PVA, 0.01% (v/v) Tween-20 and 20% (w/v) sucrose was dispensed onto a glass fiber conjugate pad and dried overnight in a desiccator cabinet. The absorbent pad, membrane card, conjugate pad and buffer application pad were then assembled together with 2 mm overlapping between each component, then cut into 5 mm width strips with Matrix 2360 Programmable Shear. The completed dry-reagent NALFIA strips were sealed in aluminium pouches containing silica gel and stored in a desiccator cabinet until further experiments.

2.4.3. Dry-reagent triplex LAMP-NALFIA assay protocol

Detection of the multiplex LAMP amplicons was performed by applying a mixture of 4 μL LAMP product and 16 μL of running buffer [1 × PBS and 1% (v/v) Tween-20] onto the detection region (nitrocellulose membrane) which adjacent to the conjugate pad. The NALFIA strip was then lifted up vertically and the buffer application pad was dipped into 250 μL of running buffer. Red lines formed on the nitrocellulose membrane indicated the reaction of the NALFIA towards the LAMP amplicons, which were visualised with unaided eyes within 10–15 min.

2.5. Evaluation of dry-reagent triplex LAMP-NALFIA assay

2.5.1. Shelf-life of dry-reagent LAMP mix and NALFIA strip

Q_{10} accelerated ageing technique described by Clark [24] was performed to estimate the shelf-life of the dry-reagent triplex LAMP mix and NALFIA strip. Both dry-reagent triplex LAMP mix and dry-reagent NALFIA strip were stored at 4 different temperatures and tested periodically for up to 60 days. The recorded duration was then used as data to estimate its stability at ambient temperature based on the recommended formula.

2.5.2. Analytical sensitivity and specificity

The analytical sensitivity of the dry-reagent triplex LAMP-NALFIA assay was determined using DNA extracted from spiked stool samples. First, the number of *E. histolytica* trophozoites was estimated based on Trypan blue exclusion method using a Neubauer chamber. A known amount of trophozoites was then diluted 10-fold to produce a range of dilutions from 10^6 to 10^{-3}. Then, each dilution of trophozoites was spiked into a 200 mg of human faecal sample and left for 1 h at ambient temperature prior to DNA isolation. The analytical specificity was determined using DNA isolated from *E. histolytica*, *E. dispar*, *E. moshkovskii* Laredo and 75 other pathogens as shown in Table 1.

3. Results and discussion

3.1. Principle of triplex LAMP-NALFIA assay

The dry-reagent triplex LAMP-NALFIA assay was developed to facilitate visualisation and interpretation of the generated double-labelled double-stranded amplicons without delay. Fig. 1 illustrated a schematic principle of the amplified amplicons from triplex LAMP captured by the NALFIA. The specific recognition of the NALFIA was formulated through the binding of the specific hapten labelled on the 5′ end of amplicons to the hapten capture proteins on NALFIA strip. The double-labelled amplicon for T1 was biotin and fluorescein labelled, T2 amplicon was texas red and fluorescein labelled whereas the IAC amplicon was digoxigenin and fluorescein labelled. A pTOPO 2.1 plasmid DNA containing *P. falciparum TRAP* gene, a gene unrelated to any *Entamoeba* species was used as IAC DNA template in this study.

The lyophilised triplex LAMP mix containing hapten labelled primers was first reconstituted with 10.2 μL of LAMP amplification buffer containing isothermal amplification buffer, betaine and $MgSO_4$; and 20 μL of sample DNA. The reconstituted mixture was then run at 63 °C for 60 min followed by 5 min of termination at 80 °C. The presence of target DNA allowed the formation of double-stranded amplicons generated by labelled primers with specific hapten (either biotin, texas red or digoxigenin) and common fluorescein on respective 5′ ends. After the amplification process, 4 μL of LAMP product was mixed with 16 μL of running buffer to dilute and distance the non-label double-stranded amplicons from labelled amplicons during capillary migration process. A total mixture of 20 μL was then placed onto the sample application region which is also detection region and incubated for 30 s before the running buffer was introduced at the proximal end of the strip. Rehydration of the NALFIA strip induced by running buffer through capillary migration released the dried gold conjugates from the conjugate pad to the detection region. Proteins affixed on the strip (detection region) then immobilised the double-labelled double-stranded amplicons by forming specific affinity interaction with their ligand labelled on the amplicons. The other 5′ end of double-stranded amplicon that was labelled with fluorescein then captured the mobile goat anti-mouse antibody that was conjugated on GNPs. Visualisation of the protein-ligand interaction was

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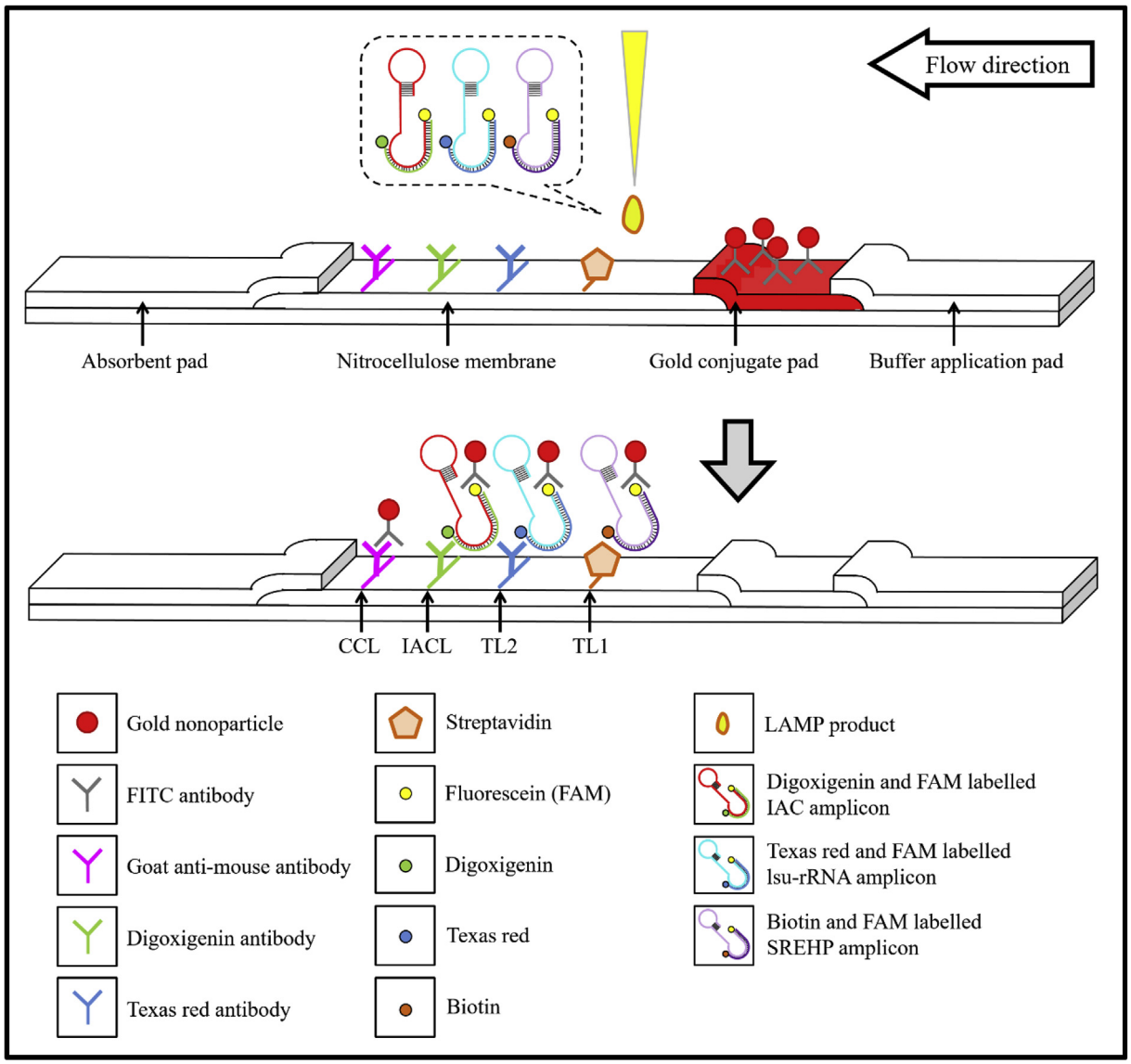

Fig. 1. Schematic illustration of the principle of the NALFIA. Schematic illustration of the principle of the DNA biosensor for visualisation of triplex LAMP amplicons using capture proteins affixed onto nitrocellulose membrane. The T1 LAMP amplicon was biotin and fluorescein labelled which captured on TL1, T2 LAMP amplicon was texas red and fluorescein labelled which captured on TL2 while IAC amplicon was digoxigenin and fluorescein labelled which captured on the IACL. Gold conjugate immobilised through specific recognition of the fluorescein produced visual signal. Working condition of the biosensor is validated by excess gold conjugate captured on the CCL. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

indicated by gold conjugates aggregation in the form of a pinkish red line, in which intensity was directly proportional to the amount of analyte.

The detection region of NALFIA strip consisted of a chromatography control line (CCL), an IAC line (IACL) and two test lines [first test line (TL1) and second test line (TL2)]. Among the four lines, TL1, TL2 and IACL required double-labelled double-stranded amplicons as the connection bridge to immobilise signal generator while the CCL affixed with goat anti-mouse antibody could directly capture FITC antibody conjugated on GNPs. The validity of the NALFIA strip was verified by the formation of the CCL signal. A lateral flow result was considered invalid if the CCL signal was not visible. IACL immobilised with digoxigenin antibody was designed to capture amplicons labelled with digoxigenin. IACL was incorporated to ascertain the validity of the amplification process which could be disrupted by inhibitors from the DNA sample matrix. The absence of the IACL signal implied the occurrence of disruption during amplification process which could justify false negative test result [25]. TL1 that was affixed with streptavidin was designed to immobilise the amplicon labelled with biotin while TL2 that was affixed with texas red antibody was designed to capture the amplicon labelled with texas red. This design enabled the TL1 and TL2 to produce signals when both *E. histolytica SREHP* and LSU-rRNA genes were present while TL1 was idle when only LSU-rRNA gene (non-pathogenic *Entamoeba* spp.) was detected. Since LAMP relied on auto strand-displacement activity, its amplification was extensive and beyond exponential. This caused the amplification of IAC to be unpredictable as the concentration of the IAC primers and target DNA was not directly proportional to the amount of the amplicons produced. In order to avoid amplification competition dominated by IAC, the triplex LAMP assay was fine-tuned to ensure the amplification of the IAC took place at its minimal point. Therefore, the appearance of IACL signal during interpretation of positive TL1 and TL2 might not be necessary as

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presence of large amounts of DNA template for the T1 and T2 could deter the amplification of IAC.

3.2. Optimisation of dry-reagent triplex LAMP

Combination of the 3 sets of primer in a single reaction mix was performed to ascertain the amplification efficacies of the target genes without altering their yields. The 3 primer sets were first inspected for their specificity (BLAST), hetero-dimer formation and self-dimerisation (OligoAnalyzer) prior to optimisation. Incorporation of loop primers not only function as the hapten carrier for formation of double-labelled double-stranded amplicon, investigation showed that the inclusion of loop primer into LAMP does accelerate and increase the amplification yield. However, modification was made where only one loop primer was incorporated into each target primer set as it is sufficient to only enhance the amplification of the side of inner primer that carried fluorescein.

Primer set for T1 was designed based on *SREHP* gene which demonstrated only amplified DNA isolated from *E. histolytica* while primer set for T2 was designed based on LSU-rRNA gene which demonstrated to be able to amplify DNA isolated from *E. histolytica*, *E. dispar* and *E. moshkovskii*. The performance of IAC amplification was minimised by reducing its DNA plasmid target in order to diminish the risks of amplification competition with T1 and T2 primers. To discover the detection limit of IAC primers, 5-fold dilution on its DNA plasmid target was conducted and used for amplification. The lowest concentration of the DNA plasmid template (1 fg) was then selected as optimal concentration for IAC amplification [Fig. S1]. After optimising the primers ratio of the 3 different monoplex LAMP, the primers were mixed together for development of triplex LAMP. The outer primers to inner primers ratio of 1:12 for T1 and 1:8 for both T2 and IAC primers were retained throughout the primer sets optimisation. T1 primer set was prioritised in the optimisation process instead of T2 primer set due to the higher copy number of T2 target in *Entamoeba* spp. [26]. IAC primer set concentration was set at the lowest as this target was meant to be only essentially available when the sample DNA was absent with T1 and T2 target gene. The optimum primers ratio (Set T1: set T2: set IAC) was recorded to be 12:3:4 where the amplification of T1 was superior than the other targets without hindering the amplification efficiency of T2 and IAC [Fig. 2]. Although ratio D in Fig. 2 showed CCL signal was presented in low intensity, it was later enhanced with additional amount of gold conjugate. Other LAMP components such as betaine, $MgSO_4$, dNTP, *Bst* DNA polymerase and enzyme stabiliser concentrations were determined empirically and the optimised conditions were recorded as described above.

3.3. Optimisation of the NALFIA

The concentration of gold conjugate, which functioned as NALFIA signal generator was optimised to generate the best line intensity. GNPs were suited for lateral flow device as they were inert, non-toxic and remain red under both dry and liquid condition [27]. The optimal concentration of FITC antibody coated onto GNPs was optimised in a concentration range of 0.625–60 μg mL^{-1} and the optimal FITC antibody was found to be 40 μg mL^{-1} [Fig. S2(a)]. The amount of GNPs conjugates was also optimised in a OD_{522} value range (1–6) to enhance the intensity of the detection lines. OD_{522} value of 5 was recorded to be the lowest OD_{522} required to generate the best intensity for all the four lines at the detection region [Fig. S2(b)]. Sucrose was added into the gold conjugate suspension to stabilise the dried GNPs together with Tween-20 and PVA that facilitated the release of the gold conjugate during rehydration.

It was not possible to distinguish the different LAMP products using agarose gel electrophoresis. However, development of a specific detection multiple target NALFIA strip could overcome the problem. The four detection lines on NALFIA strip were optimised based on their antibody or protein concentrations. The optimal concentrations of capture agents were essential to generate similar signal intensities on the detection pad. Excessive capture agents were avoided to prevent disruption of the detection lines. Streptavidin was positioned nearest to the gold conjugate pad as it was the prioritised detection target, followed by texas red antibody, digoxigenin antibody and goat anti-mouse antibody. Goat anti-mouse antibody was located furthest from the conjugate pad as it was permitted to only produce signal when there were sufficient conjugate GNPs for the other three capturing agents. Streptavidin concentration was optimised at 2.0 mg mL^{-1}, texas red antibody at 0.6 mg mL^{-1}, digoxigenin antibody at 0.7 mg mL^{-1} and goat anti-mouse antibody at 1.0 mg mL^{-1} [Fig. S3].

Despite having the four capture proteins immobilised on the lateral flow detection region, the uncoated open surface of membrane would risk the attachment of unexpected protein or amplicon. Blocking of strip detection region was essential in preventing any non-specific binding that could lead to formation of false positive result. However, excessive blocking agent would cause reduction of signal and slowed down capillary migration on the nitrocellulose membrane. BSA, WBR, PVA and PVP were selected as blocking agent candidates and were optimised in range of 0.1–1% (v/v). The best blocking agent was found to be 0.2% (w/v) WBR [Fig. S4(a)]. In order to retain the capillary performance of the nitrocellulose membrane, Triton X-100 (v/v), Tween-20 (v/v) and SDS (w/v) were selected as surfactant candidates and were optimised in range of 0.001–0.1%. Triton X-100 with 0.01% concentration was recorded as the best surfactant to be added into blocking

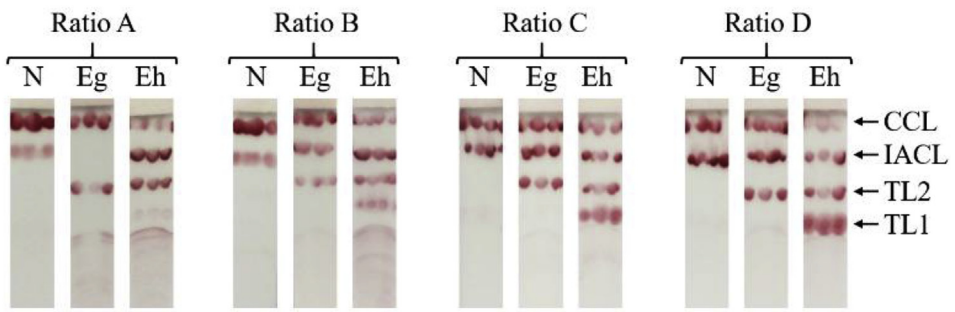

Fig. 2. Optimisation of primer sets ratio for 3 targets during triplex LAMP development. Ratio of primer sets, T1 primer set: T2 primer set: IAC primer set; Ratio A, 12:12:4; Ratio B, 12:9:4; Ratio C, 12:6:4; Ratio D, 12:3:4. Ratio D showed the optimal ratio distribution of primers as TL1, TL2 and IACL presented in similar intensity with only excess gold conjugate attached on CCL.

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reagent to facilitate rewetting of the detection region after loading of sample [Fig. S4(b)]. Between PB and PBS, PBS was found to be a better rehydration buffer for gold conjugate and allowed protein-ligand interaction to take place without additional hybridisation buffer. PB was not replaced by PBS as the solvent for blocking buffer. This was because the presence of large amount PBS could reduce the migration flow rate on the nitrocellulose membrane.

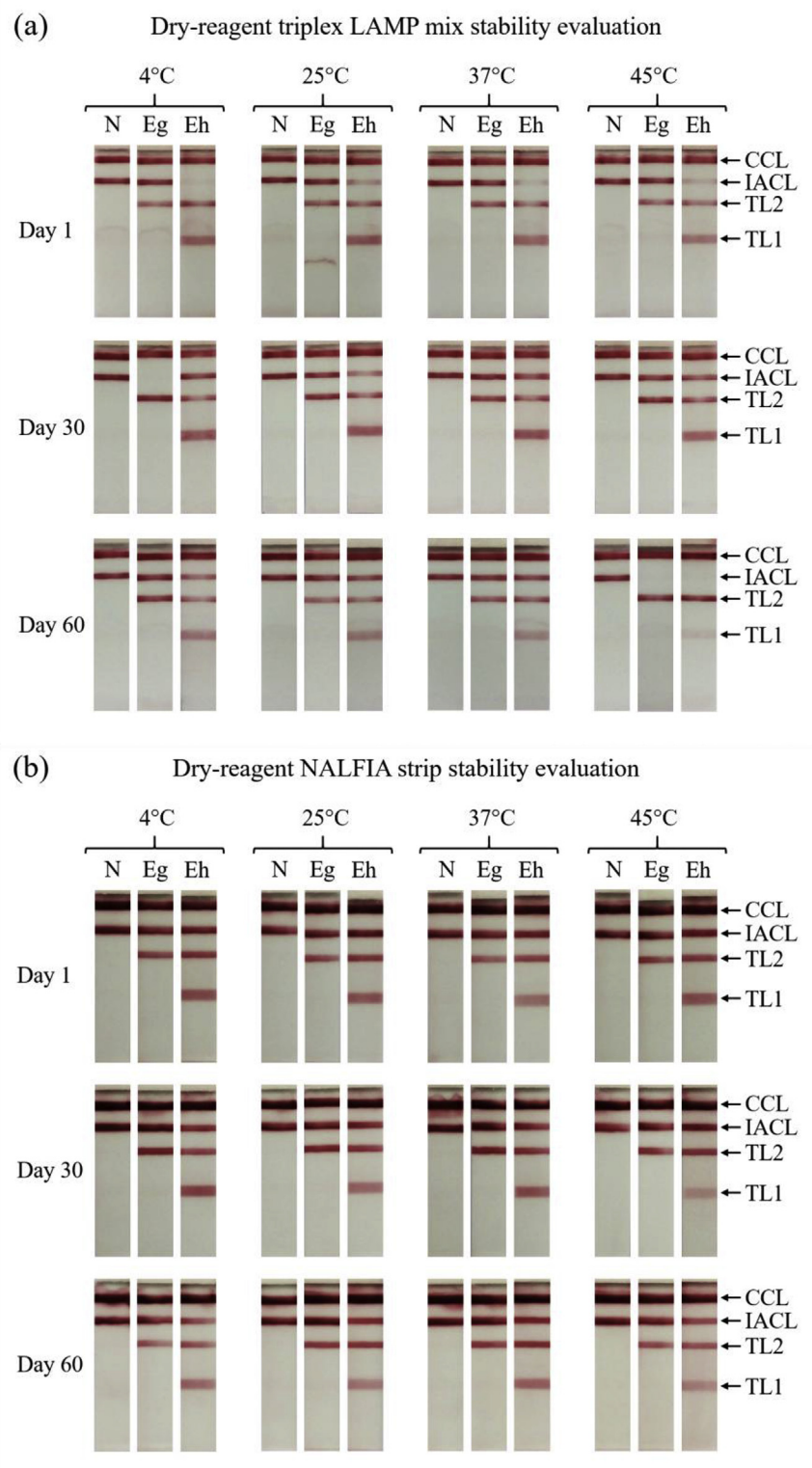

Fig. 3. Stability evaluation on dry-reagent triplex LAMP mix and NALFIA strip. N, negative control with IAC; Eg, *E. dispar* or *E. moshkovskii*; Eh, *E. histolytica*. The recorded 60 days at 37 °C was used as the duration and temperature for (a) dry-reagent triplex LAMP mix shelf-life estimation while 60 days at 45 °C used for (b) dry-reagent NALFIA strip shelf-life estimation. Based on accelerated aging method, the estimated shelf-life at room temperature for thermostabilised triplex LAMP mix was 181 days and dry-reagent NALFIA strip was 254 days.

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3.4. Performances of the dry-reagent triplex LAMP-NALFIA assay

The shelf-life of the developed dry-reagent triplex LAMP mix and NALFIA strip were estimated using accelerated aging technique (Q_{10} method) [24]. Q_{10} method was used to measure and study the storage stability of degradable medical devices in shorter duration at elevated temperatures, and mathematically correlate the temperatures into real time [28]. The stability of the dry-reagent triplex LAMP-NALFIA assay was studied for 60 days at various temperatures (4 °C, 25 °C, 37 °C and 45 °C). Both the dry-reagent triplex LAMP mix and NALFIA strips were stored in separate aluminium pouches containing silica gel and only minimal loss of signal was recorded after 60 days of storage. Lyophilised triplex LAMP mix was recorded to be stable up to 60 days at 37 °C [Fig. 3(a)] while dry-reagent NALFIA strip remain stable for at least 60 days at 45 °C [Fig. 3(b)]. Based on the formula of accelerated aging method, the dry-reagent triplex LAMP mix was estimated to remain stable for at least 181.5 days at ambient temperature while NALFIA strip was estimated to have a shelf-life of at least 254 days. The dry-reagent triplex LAMP mix had shorter stability duration compared to the NALFIA strip because the LAMP mix contained *Bst* DNA polymerase enzyme that previously needed −20 °C cold-chain storage while the strip was affixed with affinity-binding-proteins (non-enzymes) that only required 4 °C of storage prior thermostabilisation.

The detection limits of the dry-reagent triplex LAMP-NALFIA were investigated periodically up to 60 days. Both dry-reagent triplex LAMP mix and NALFIA strip were kept at 37 °C in accordance with the temperature used for triplex LAMP mix stability estimation to mimic the prolonged real time duration. To mimic clinical samples, the 10-fold diluted trophozoites were spiked into stool samples and left for 1 h prior to DNA isolation. The detection limit of the dry-reagent triplex LAMP-NALFIA assay after 181 days of storage at room temperature (estimated from 60 days at 37 °C) was recorded as 10 *E. histolytica* trophozoites [Fig. 4].

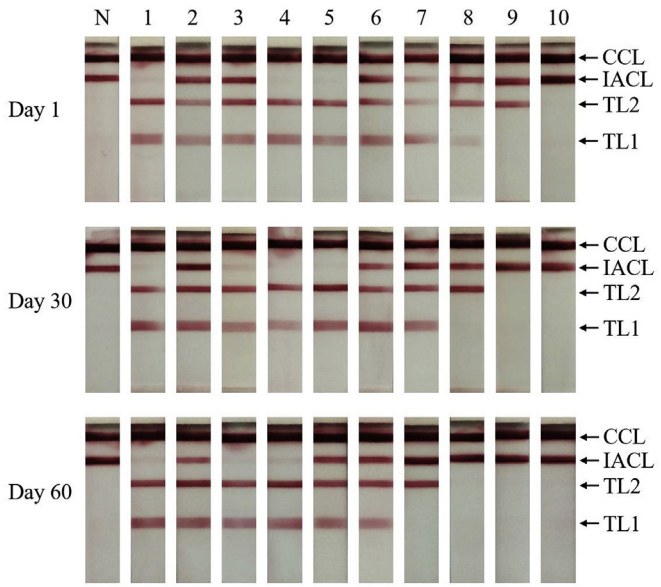

Fig. 4. Spiked stool analytical sensitivity using 10-fold dilutions on *Entamoeba histolytica* trophozoites on dry-reagent triplex LAMP-NALFIA assay under 37 °C for 60 days. N, negative control with IAC; 1–10, 10-fold dilution of trophozoites: strip 1, 10^6; strip 2, 10^5; strip 3, 10^4; strip 4, 10^3; strip 5, 10^2; strip 6, 10; strip 7, 1; strip 8, 0.1; strip 9, 10^{-2}; strip 10, 10^{-3}. Ten trophozoites were detected at day 60 under 37 °C and was interpreted as detection limit of dry-reagent triplex LAMP-NALFIA assay at 25 °C on day 181.

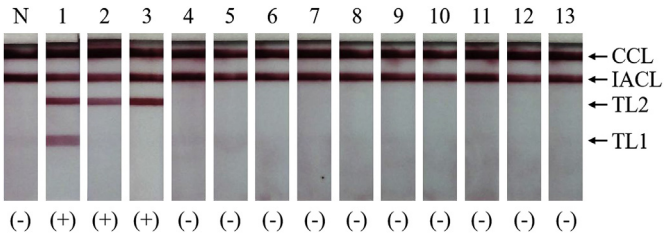

Fig. 5. Representative results of analytical specificity evaluation using other known microorganisms. N, LAMP negative control; 1, *Entamoeba histolytica*; 2, *Entamoeba dispar*; 3, *Entamoeba moshkovskii*; 4, *Salmonella enterica* ser. Typhi; 5, *Salmonella enterica* ser. Enteritidis; 6, *Leptospira interrogans* ser. Canicola; 7, *Shigella flexneri*; 8, *Burkholderia pseudomallei*; 9, *Enterococcus gallinarum*; 10, *Staphylococcus aureus*; 11, *Escherichia coli*; 12, *Aeromonas hydrophila*; 13, *Vibrio fluvialis*. +, positive result; -, negative result.

3.5. Analytical specificity of triplex LAMP-NALFIA assay

Analytical specificity of the dry-reagent triplex LAMP-NALFIA assay was performed using DNA isolated from *E. histolytica*, *E. dispar*, *E. moshkovskii* and 75 other microorganisms. Test sample with *E. histolytica* DNA produced 4 lines signal on the NALFIA detection region, whereas sample with *E. dispar* and *E. moshkovskii* produced only 3 lines signal. Meanwhile, DNA isolated from other microorganisms only amplified the IAC target [Fig. 5] which indicated true negative results with no inhibition. The results revealed that the dry-reagent triplex LAMP-NALFIA assay was 100% specific, in which all non-*Entamoeba* DNA gave true negative results. The summary of the analytical specificity test results was presented in Table 1.

4. Conclusion

Dry-reagent triplex LAMP-NALFIA platform assay could rapidly amplify multiple DNA targets under a single temperature and the results could be rapidly interpreted without aid of equipment such as agarose gel electrophoresis system. Thermostabilisation of LAMP reagents and gold conjugate enhanced the biosensor shelf-life and made it cold-chain free. Hence, this molecular biosensor is potentially useable in field settings and deliverable to resource-limited areas.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://

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