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Ambient temperature detection of PCR amplicons with a novel sequence-specific nucleic acid lateral flow biosensor

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ABSTRACT

In the field of diagnostics, molecular amplification targeting unique genetic signature sequences has been widely used for rapid identification of infectious agents, which significantly aids physicians in determining the choice of treatment as well as providing important epidemiological data for surveillance and disease control assessment. We report the development of a rapid nucleic acid lateral flow biosensor (NALFB) in a dry-reagent strip format for the sequence-specific detection of single-stranded polymerase chain reaction (PCR) amplicons at ambient temperature (22–25 °C). The NALFB was developed in combination with a linear-after-the-exponential PCR assay and the applicability of this biosensor was demonstrated through detection of the cholera toxin gene from diarrheal-causing toxigenic *Vibrio cholerae*. Amplification using the advanced asymmetric PCR boosts the production of fluorescein-labeled single-stranded amplicons, allowing capture probes immobilized on the NALFB to hybridize specifically with complementary targets *in situ* on the strip. Subsequent visual formation of red lines is achieved through the binding of conjugated gold nanoparticles to the fluorescein label of the captured amplicons. The visual detection limit observed with synthetic target DNA was 0.3 ng and 1 pg with pure genomic DNA. Evaluation of the NALFB with 164 strains of *V. cholerae* and non-*V. cholerae* bacteria recorded 100% for both sensitivity and specificity. The whole procedure of the low-cost NALFB, which is performed at ambient temperature, eliminates the need for preheated buffers or additional equipment, greatly simplifying the protocol for sequence-specific PCR amplicon analysis.

1. Introduction

The development of nucleic acid biosensors is currently at the forefront of diagnostics research, which attests to the importance of detecting unique genetic sequences in various fields. Nucleic acid amplification using the polymerase chain reaction (PCR) is a well-established technique and continues to be widely used, as evidenced by the various lateral flow devices that have been developed for the detection of molecularly amplified nucleic acid products in the last decade alone. The lateral flow device, referred to occasionally as a "dipstick", is of particular interest as a rapid, user-friendly alternative to the conventional PCR analysis, which is heavily instrument-dependent and time-consuming because it requires agarose gel preparation, an electrophoresis step, and nucleic acid staining with carcinogenic ethidium bromide (EtBr). One format of such devices is the nucleic acid lateral flow immunoassay (NALFI), which detects unique haptens labeled at the termini of double-stranded DNA products (Blazkova et al., 2011; Blazkova et al., 2009; Chua et al., 2011; Rigano et al., 2010; Soo et al., 2006). The major drawback of the NALFI is that signal generation is independent of the nucleic acid sequence of amplicons and, thus, the device is vulnerable to false positive results from non-specific amplifications.

The need for sequence-specific detection led to the incorporation of a hybridization step in the detection scheme, such as in the primer extension (PEXT) reaction, which generates labeled amplicons following a standard PCR assay (Kalogianni et al., 2009; Litos et al., 2009; Vlachou et al., 2010). The oligonucleotide probe that is included in the PEXT reaction mixture will hybridize to the extended segment of the labeled PEXT primer in the presence of the target sequence. The PEXT reaction allows each target sequence to be tagged with a unique label and recognized with specific probes but the method involves addition of various reagents and extra thermal cycling steps after completion of the PCR assay for the PEXT reaction to take place.

A method that does not involve the PEXT reaction entailed the denaturation of double-stranded biotinylated amplicons before hybridization in solution with specific probes at temperatures ranging from 37 °C to 63 °C for 5–7 min followed by affinity-capture with streptavidin on a dipstick (Glynou et al., 2003; Kalogianni et al., 2007a, 2007b, 2006; Konstantou et al., 2010; Nimitphak et al., 2010;

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Srisala et al., 2008). However, manual addition of detection probes and elevated temperature are still required for hybridization with the target sequence. Recently, several studies have reported on the detection method of unlabeled amplicons (Deborggraeve et al., 2008; Mugasa et al., 2009) as well as biotin and/or digoxigenin-labeled amplicons (Akinwale et al., 2008; Laurent et al., 2009) in which probes complementary to the target sequence were conjugated to gold nanoparticles. Despite the apparent advantages of circumventing manual addition of probes and an incubation step, these assays were performed with buffer preheated at 55 °C and, notably, the composition of the migration buffer was not disclosed.

We report on a nucleic acid lateral flow biosensor (NALFB) that is a dry-reagent device for the detection of sequence-specific PCR-amplified target DNA at ambient temperature (22–25 °C) without multiple incubation or washing steps. We demonstrate the simplicity and feasibility of this novel biosensor through detection of the cholera toxin gene (*ctxA*), which implicates the presence of toxigenic *Vibrio cholerae*, a food- and water-borne bacterium responsible for an acute diarrheal disease called cholera. Cholera toxin (CT) is the principal virulent factor in the manifestation of massive body water reflux that can lead rapidly to severe dehydration and death (Sack et al., 2004). Therefore, it is imperative to detect the CT gene as, CT production is recognized as one of the two important determinants in assessing the public health significance of a *V. cholerae* isolate (Kaper et al., 1995). The proposed NALFB has potential application in rapid screening and identification of infectious disease and biological weapons especially the etiological agent of cholera, which can be introduced easily into new geographical areas to cause explosive outbreaks.

2. Experimental

2.1. Reagents and apparatus

NeutrAvidin biotin-binding protein (neutravidin), mouse anti-FITC antibody (Ab) and goat anti-mouse IgG Ab were purchased from Pierce Biotechnology (Rockford, USA) and colloidal gold solution (40 nm) was from Heron Diagnostics (Pathumthani, Thailand). Methanol and bovine serum albumin (BSA) were purchased from Amresco (Solon, USA) and western blocking reagent was from Roche (Indianapolis, USA). Magnesium chloride ($MgCl_2$), polyethyleneglycol 3350 (PEG 3350), sucrose, Tween-20, Triton X-100, polyvinyl alcohol (PVA), polyvinylpyrrolidone (PVP), sodium azide (NaN_3) and all other common chemicals were from Sigma (St. Louis, USA). All reagents used in this study were of analytical reagent grade and all solutions were prepared using ultrapure water ($> 18\ M\Omega$) from a Millipore Milli-Q water purification system (Billerica, USA).

Laminated nitrocellulose membrane card (ref. HF135MC100), cellulose fiber pads (ref. CFSP173000), glass fiber pads (ref. GFCP083000) and Amicon Ultra 30K were Millipore products. PCR primers, capture probes and synthetic targets were synthesized by Integrated DNA Technologies (Coralville, USA) and the nucleotide sequences are given in Table 1. All PCR reagents were obtained from Fermentas (Vilniuas, Lithuania) and amplifications were performed in an Eppendorf Mastercycler (Hamburg, Germany). A XYZ dispensing platform from BioDot (Irvine, USA) and Matrix 2360 programmable shear from Kinematic Automation (Twain Harte, USA) were used in this study.

2.2. LATE-PCR for cholera toxin gene

Linear-after-the-exponential (LATE)-PCR assay targeting the *ctxA* gene was carried out in a 20 μl reaction mixture containing 1X PCR buffer, 320 μM dNTPs, 2.5 mM $MgCl_2$, 0.75 U of *Taq* DNA polymerase, 10 pg of internal amplification control (IAC) plasmid, 2 μl of target DNA template, and deionized water. The target gene was amplified with 0.1 μM forward primer (ctxA_FL) and 0.5 μM reverse primer (ctxA_RX). The IAC sequence was amplified with 0.5 μM forward primer (IAC_FX) and 0.05 μM reverse primer (IAC_RL). Optimization was performed using genomic DNA extracted from *V. cholerae* strain J3321 using a Nucleospin Tissue Kit from Macherey–Nagel (Düren, Germany). The thermal cycling conditions were as follows: an initial denaturation step at 95 °C for 5 min, followed by 35 cycles at 95 °C for 30 s, 61 °C for 30 s, 72 °C for 30 s, a final extension step at 72 °C for 5 min and a denaturation step at 95 °C for 5 min was added to increase the yield of single-stranded PCR amplicons. The amplification products were snap-cooled on ice before proceeding with the NALFB assay.

2.3. Construction of NALFB

2.3.1. Functionalization of gold nanoparticles

The signal generator of the biosensor consisted of gold nanoparticles conjugated with mouse anti-FITC Ab and the conjugation was performed essentially as described by Yu et al. (2011) but with slight modification in the incubation and centrifugation time to increase the final yield of gold conjugates (data not shown). Briefly, 10 ml of colloidal gold nanoparticles adjusted to pH 8.0 was added with 22 μg of anti-FITC Ab followed by gentle shaking at room temperature for 1 h. The colloidal gold nanoparticles were supplemented with 1%(w/v) BSA and allowed to

Table 1
Oligonucleotide sequences used in this study.

Oligonucleotide	Sequence (5′→3′)	Length (mer)	5′ Label	Amplicon size (bp)
PCR primers				
ctxA_FL	GGGAATGCTCCAAGATCATCGA	22	–	91
ctxA_RX	CTT TAGATTGGTATTCGTCAAGGA	24	Fluorescein	
IAC_FX	TGTAGGTTGTCATCCATCAG	20	Fluorescein	112
IAC_RL	TTCTACTTCAACACAAACAGCCTTC	25	–	
Capture probes				
CP_ctxA	AAAAAACCCAAAGTCTAGGTGTAAA	25	Biotin	NA
CP_IAC	GGTCCGATAACATTTTTTACATTTT	25	Biotin	NA
Synthetic targets				
ST_ctxA	CTTTAGATTGGTATTCGTCAAGGAATTTTACACCTAGACTTTGGGT TTTTTCATCGCAAGTATTACTCATCGATGATCTTGGAGCATTCCC	91	Fluorescein	NA
ST_IAC	TCCATCAGATGGTAAATGTAACTTGTATGCTGATTCTGCATGGGAAAATGT AAAAAATGTTATCGGACCCTTTATGAAGGCTGTTTGTGTTGAAGTAGAA	100	Fluorescein	NA

NA, not applicable.

mix for another 1 h. The colloidal gold-anti-FITC Ab conjugates were collected by centrifugation at 10,000*g* for 30 min at 4 °C. The soft pellet was suspended in 10 mM phosphate buffer (PB) containing 1%(w/v) BSA and 0.05%(w/v) NaN_3.

2.3.2. Preparation of capture reagents

Biotinylated capture probes (CP_ctxA and CP_IAC) were conjugated to neutravidin in order to maintain entrapment within a designated area of the detection region. A total of 1.53 nmol of each capture probe was allowed to react with 2.04 mg of neutravidin for 1 h at room temperature. The reaction mixture was transferred to a centrifugal filter device and centrifuged at 14,000*g* for 15 min. The retentate was washed twice with 10 mM PB and reconstituted with 10 mM PB (pH 7.4) containing 1%(w/v) BSA and 1%(v/v) methanol after retrieval from the filter device.

2.3.3. Preparation of NALFB

The dry-reagent NALFB (4 mm × 77 mm) composed of a buffer application pad, a conjugate pad, a laminated nitrocellulose membrane and an absorbent pad as shown in Fig. 1. Capture reagents for the test line, the IAC line and the chromatography control line were applied separately onto the nitrocellulose membrane using a Front Line dispenser at a rate of 1 μl/cm. The nitrocellulose membrane was dried overnight in a desiccator. The remaining protein-binding sites of the membrane were blocked with 1%(v/v) western blocking reagent and 0.1%(v/v) Triton X-100 in 2 mM PB (pH 7.4). A suspension of gold conjugates at an optical density at 525 nm (OD_{525}) of 4 in 2 mM PB supplemented with 0.01%(v/v) Tween-20, 0.01%(v/v) PVA and 20%(w/v) sucrose was dispensed onto a glass fiber conjugate pad and dried overnight in a desiccator. The buffer application pad, conjugate pad, nitrocellulose membrane and absorbent pad were assembled with 2 mm overlapping between each component and the assembled master card was cut into 4 mm wide strips with a programmable shear. The strips were stored in a desiccator or sealed in aluminum pouches containing silica gel.

2.4. NALFB assay protocol

The test sample for the NALFB was prepared by adding an equal volume of hybridization buffer [10%(w/v) PEG 3350 in 1.5 M $MgCl_2$] to the PCR product and mixed by simple pipetting. A 10 μl sample of the mixture was applied to the sample application region adjacent to the conjugate pad and the buffer application pad of the NALFB was placed into contact with 250 μl of running buffer [2 mM PB, 0.01%(v/v) Triton X-100]. The results can be visualized in the form of red lines by unaided eyes within 20–25 min.

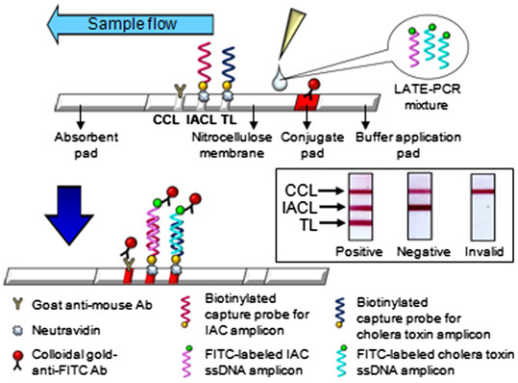

Fig. 1. An illustration of the principle of the NALFB. The inset photo shows the interpretation of the NALFB for positive, negative and invalid test results. CCL, chromatography control line; IACL, internal amplification control line; TL, test line; ssDNA, single-stranded DNA.

2.5. Application to clinical specimens

Evaluation of the NALFB was performed with spiked stool samples and the panel of 164 archived clinical strains included 94 toxigenic *V. cholerae* strains, 7 non-toxigenic *V. cholerae* strains, 35 other Gram-negative and 28 Gram-positive bacteria (Table 2). The spiked stool samples were prepared from all the bacterial strains as described by McOrist et al. (2002) before inoculation into 5 ml of alkaline peptone water (APW). After incubation at 37 °C for 6 h, 2 μl of boiled cell lysate was used as template in the LATE-PCR assay followed by detection using the NALFB.

3. Results and discussion

3.1. Principle of the NALFB

The dry-reagent NALFB was designed for the visual detection of single-stranded PCR amplicons in an easy-to-perform two-step assay that generates sequence-specific results in minutes. End-users are required only to add the PCR mixture containing hybridization buffer to the biosensor before the running buffer is introduced at the proximal end of the strip. The running buffer serves to rehydrate and release the dried gold conjugates from the conjugate pad to the detection region by capillary migration. At the detection region, capture probes immobilized on the strip will hybridize with complementary target DNA allowing gold conjugates to bind to the label at the 5′ termini of the captured strand. Aggregation of the gold conjugates enables visualization of the hybridization reaction in the form of a red/pink line with intensity proportional to the amount of target analyte.

The detection region of the NALFB consists of a test line, an IAC line and a chromatography control line. The test line provides a visual response to the presence/absence of the target DNA and in the present study we selected the CT gene that encodes for a potent enterotoxin. The portion of the target DNA that is recognized by the capture probes was deliberately designed without overlapping with the primer binding regions to avoid detection of non-specific amplification products from mispriming. In view of the fact that the biosensor was designed to detect a molecularly amplified target, multifactorial events ranging from a faulty thermal cycler, incorrect preparation of the PCR mix, poor DNA polymerase activity or the presence of PCR inhibitory substances from the sample matrix can lead to a false negative result simply because the amplification process is inhibited (Hoorfar et al., 2004). Therefore, this limitation was addressed by the inclusion of an IAC line, which provides a validation for the amplification process because a biological sample, especially stool that can contain inhibitory substances, is involved in the PCR assay.

Interpretation of a positive result on the test line might not necessarily be accompanied by the appearance of a positive result on the IAC line because the presence of very large amounts of template from the target sample could hinder the amplification of IAC. However, interpretation of a negative result on the test line must be validated by a positive test result on the IAC line. The third line, which is the chromatography control line, completes the foolproof design of the NALFB by assessing the functionality of each individual strip. The chromatography control line comprised of goat anti-mouse Ab, which binds to excess labeled conjugate as an indication of proper reagent flow in each device from proximal to distal end. Any test result obtained when the chromatography control line is negative is an invalid result.

Table 2
Summary of test results with spiked stool specimens.

Organism	No. of strain	No. of positive test	
		NALFB	GM_1-ELISA
Toxigenic *V. cholerae* strains			
Vibrio cholerae O1	79	79	79
Vibrio cholerae O139	15	15	15
Non-toxigenic *V. cholerae* strains			
Vibrio cholerae non-O1/non-O139	7	0	0
Other *Vibrio* sp.			
Vibrio fluvialis	1	0	0
Vibrio furnisii	1	0	0
Vibrio metschnikovii	1	0	0
Vibrio mimicus	1	0	0
Vibrio parahaemolyticus	1	0	0
Vibrio vulnificus	1	0	0
Other bacteria strains			
Acinetobacter baumanii	1	0	0
Aeromonas hydrophilia	1	0	0
Bacillus cereus	2	0	0
Bacillus subtilis	1	0	0
Citrobacter fruendii	1	0	0
Corynebacterium diphtheriae	1	0	0
Enterobacter cloacea	1	0	0
Enterococcus faecalis	3	0	0
Enterococcus faecium	1	0	0
Enterococcus gallinarum	2	0	0
Enterococcus sp.	2	0	0
Enteroinvasive *Escherichia coli* (EIEC)	1	0	0
Enteropathogenic *Escherichia coli* (EPEC)	1	0	0
Enterotoxigenic *Escherichia coli* (ETEC)	2	0	0
Escherichia coli O157:H7	1	0	0
Gardnerella vaginallis	1	0	0
Goup A *Streptococcus*	1	0	0
Group B *Streptococcus*	1	0	0
Group F *Streptococcus*	2	0	0
Group G *Streptococcus*	1	0	0
Klebsiella oxytoca	1	0	0
Klebsiella pneumoniae	1	0	0
Listeria monocytogenes	2	0	0
Morganella morganii	1	0	0
Pleisiomonas shigelloides	1	0	0
Proteus mirabilis	1	0	0
Proteus vulgaris	1	0	0
Providencia stuartii	1	0	0
Pseudomonas aeruginosa	1	0	0
Salmonella braenderup	1	0	0
Salmonella enteriditis	1	0	0
Salmonella flexneri	1	0	0
Salmonella paratyphi A	1	0	0
Salmonella paratyphi B	1	0	0
Salmonella typhi	1	0	0
Serratia marcescens	1	0	0
Shigella boydii	1	0	0
Shigella dysentery	1	0	0
Shigella sonnei	1	0	0
Staphylococcus aureus	4	0	0
Staphylococcus epidermidis	1	0	0
Staphylococcus haemolyticus	2	0	0
Streptococcus agalactiae	1	0	0
Streptococcus pyrogenes	1	0	0
Yersinia enterolitica	1	0	0
Total	164	94	94

3.2. LATE-PCR for cholera toxin gene

Combination of LATE-PCR and the NALFB was aimed at maximizing the yield of single-stranded DNA target in a single reaction for hybridization and, therefore, increasing the sensitivity of the overall detection platform. Conventional asymmetric PCR is inefficient, requires extensive optimization and tends to promote non-specific amplification. LATE-PCR overcomes these limitations with primer pairs designed for use at unequal concentrations (Pierce et al., 2005). LATE-PCR initiates with an exponential phase in which double-stranded DNA products are amplified until the limiting primer is depleted and the reaction switches to linear amplification of single-stranded product from the excess primer strands (Sanchez et al., 2004).

A pair of LATE-PCR primers was designed for the target gene. These primers spanned a 91 bp region of the *ctxA* gene and a second primer pair that served as IAC was designed to flank a 112 bp region of the IAC plasmid template. At the initial stage, the LATE-PCR assay was optimized with different concentrations of fluorescein-labeled excess primer (0.1–1.5 μM) at a primer ratio of 1:10. Signal intensity on the NALFB was the lowest when excess primer concentration was 0.1 μM and, although the band intensity increased when excess primer was present at 0.5 μM and 1.0 μM, the signal began to decrease when 1.5 μM excess primer was used. A large amount of excess primers was detrimental as they compete with the target amplicons for binding to the colloidal gold conjugates.

With 0.5 μM as the selected optimal concentration for the excess primer, primer ratios of 1:5, 1:10, 1:20, 1:30, 1:50 and 1:100 were used to study the efficiency of the amplification. We found that when the primer ratio was increased beyond 1:20, signal intensity on the NALFB began to reduce and a primer ratio of 1:5 was selected as the optimum. Likewise, optimization of the IAC primer pair was performed and the excess primer concentration of 0.5 μM with a primer ratio of 1:10 was selected as optimum. In addition to the primer concentration and primer ratio, other components of the PCR reaction such as dNTP, Mg^{2+} and *Taq* DNA polymerase concentrations were determined empirically and the optimized conditions were as reported above.

3.3. Optimization of the NALFB parameters

Colloidal gold nanoparticles were selected as the signal generator because their surface plasmon resonance gives them a characteristic red color in dried and liquid form, making the particles visible to the naked eye. Furthermore, the gold nanoparticles are inert and non-toxic, and the ability to retain their optical properties over time makes them highly suited for lateral flow devices (Thobhani et al., 2010). Conjugation of the colloidal gold nanoparticles was optimized by a flocculation assay in which ionic salt was used to challenge the stability of the conjugates. High concentrations of ionic salt reduce the electrostatic repulsion charges between the colloidal nanoparticles, resulting in aggregation of the particles accompanied by a color shift from red to bluish-gray (Chun et al., 2009). The optimal coating of Ab on the colloidal gold nanoparticles prevents flocculation and can be determined experimentally as the concentration that produces a minimal decrease in absorbance at 525 nm after the addition of sodium chloride.

We investigated the effect of pH in the range 6–10 by coating colloidal gold nanoparticles with 40 μg/ml of Ab and we found that the conjugates were most stable at pH 8 [Fig. S1(a)]. In order to determine the minimal concentration of Ab required to stabilize 1 ml of colloidal gold at pH 8, concentrations of Ab in the range 1.25–40 μg/ml were studied and 20 μg/ml of Ab was found to be sufficient [Fig. S1(b)]. We added an excess of 10% to the selected Ab concentration to ensure that the colloidal gold nanoparticles were optimally coated during the conjugation process.

In addition to the stability of the conjugates, the performance of the NALFB was dependent on the amount of colloidal gold conjugates available for binding to the label of the target DNA. The intensity of the line increased with increasing amounts of conjugates but we found that greater amounts of gold conjugates

were associated with a longer release time from the conjugate pad, which prolonged the assay time. The amount of gold conjugates was selected at an OD_{525} of 4 for the higher signal intensity generation although additional clearing time is required when compared to OD_{525} of 3. The suspension buffer contained sucrose to stabilize the gold conjugate in dried form whilst Tween-20 and PVA were added to facilitate release of the gold conjugates from the conjugate pad during rehydration.

Other parameters of the NALFB, such as the nitrocellulose region of the strip, were optimized with various blocking agents [BSA (1–10%, w/v), western blocking reagent (0.1–5%, v/v), PVA (0.1–5%, v/v) and PVP (0.1–5%, v/v)] in order to prevent background signal due to non-specific binding of the test sample or signal generators on the membrane and the best blocking agent was 1%(w/v) western blocking reagent. Different concentrations of detergents [SDS (0.001–0.1%, w/v), Tween-20 (0.001–0.1%, v/v) and Triton X-100 (0.001–0.1%, v/v)] were added to the blocking reagent to promote rewetting of the nitrocellulose membrane. Triton X-100 (0.1%, v/v) was selected as the optimal rewetting agent to facilitate sample loading in the sample application region.

The hybridization reaction, which is crucial to the performance of the NALFB, was optimized to ensure the selected ionic strength of the buffer enables the capture probes to hybridize specifically with its complementary target DNA at ambient temperature. The effect of $MgCl_2$ (0.50–1.25 M), NaCl (0.5–2.0 M) and sodium chloride/sodium citrate (1–10X) solution on hybridization of the target DNA to capture probes were investigated. Overall, we found that $MgCl_2$ had the best performance, which could be attributed to its property of stabilizing nucleic acid duplexes (Owczarzy et al., 2008). The signal intensity was found to be enhanced significantly in the presence of 5%(w/v) PEG 3350 after further tests with a series of hybridization accelerators (See details of parameters that were optimized in supplementary Fig. S2).

3.4. Performance of the NALFB

The analytical performance of the NALFB was assessed by using synthetic targets ranging from 0 ng to 10 ng. The results of the NALFB with different amounts of synthetic target for *ctxA* (ST_ctxA) and IAC (ST_IAC) are presented in Fig. 2(a). The limit of detection (LoD), which is defined as the lowest concentration of target that produces a visible signal, was found to be 0.3 ng. We have shown that the LoD of individual synthetic target was not affected when ST_ctxA and ST_IAC were present simultaneously. In combination with the LATE-PCR assay, the LoD of the NALFB was 1 pg of genomic DNA [Fig. 2(b)] and 10 colony-forming units (CFU)/ml of cultured *V. cholerae* [Fig. 2(c)]. Reproducibility of the LATE-PCR assay in generating detectable amplicons from 1 pg of genomic DNA and 10 CFU/ml of cultured *V. cholerae* was assessed ($n=9$) and found to be excellent (Fig. S3). The LoD of the proposed

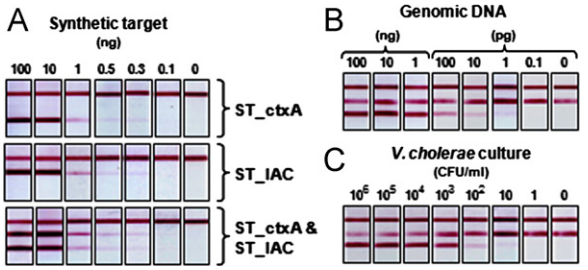

Fig. 2. Limit of detection of the NALFB. Photographic results of the NALFB at various amounts of (a) synthetic target ST_ctxA (top), ST_IAC (middle) or equal amount of both synthetic targets (bottom), (b) genomic DNA and (c) at various concentrations of *V. cholerae* culture.

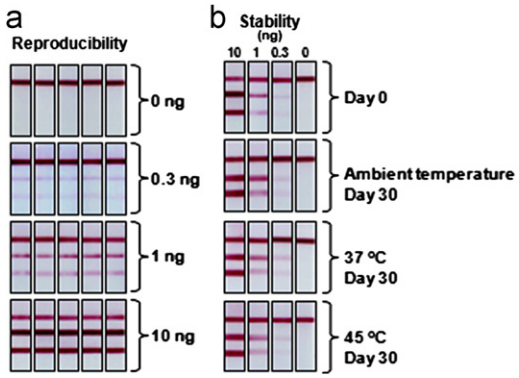

Fig. 3. (a) Stability of NALFB before and after storage at ambient temperature, 37 °C and 45 °C for 1 month. (b) Reproducibility of NALF biosensor at 0 ng, 0.3 ng, 1 ng and 10 ng of synthetic targets ($n=5$).

NALFB is lower than that reported by Chua et al. (2011) (5 ng) and Blazkova et al. (2011) (8 ng).

The reproducibility of the NALFB was demonstrated by testing four different amounts (0 ng, 0.3 ng, 1 ng and 10 ng) of synthetic targets. In Fig. 3(a), the NALFB is shown to be highly reproducible; similar responses were generated when tested repeatedly ($n=5$) for each amount of synthetic targets. The stability of the NALFB was assessed also by storing the strips in individual aluminum pouches containing silica gel for 30 day at ambient temperature (25 °C), 37 °C and 45 °C. The stability of the capture reagents and dried colloidal gold conjugates were evidenced by the minimal loss of signal after storage for 1 month, even at elevated temperature [Fig. 3(b)]. The accelerated stability test result of the developed NALFB at 37 °C for 30 day provides an estimated shelf life of at least 3 months when stored at ambient room temperature (Chua et al., 2011).

3.5. Application to clinical specimens

The NALFB evaluation was performed with spiked stool specimens to mimic clinical samples and the result was 100% for both sensitivity and specificity (Table 2). Concurrent with the evaluation of NALFB, a GM_1-ELISA that is used to detect the production of cholera toxin in bacteria culture was carried out as described by Ang et al. (2010) on overnight yeast extract peptone culture for all the bacterial strains. Full concordance between the results of both assays indicated that the NALFB produces accurate results with only a fraction of the time, effort and instruments involved in performing an ELISA for the detection of toxigenic *V. cholerae*.

4. Conclusions

The proposed sequence-specific NALFB that operates at ambient temperature offers significant advantages over the traditional method in molecularly-amplified nucleic acid analysis. The user-friendly strip format of the biosensor eliminates the need for skilled personnel and generates rapid visual results in minutes without multiple washing or incubation at elevated temperature. The disposable biosensor is cost-effective, generates less waste and poses no health risk to the end-user when compared to conventional agarose gel analysis, which involves the use of carcinogenic EtBr dye and UV light. The potential application of the NALFB may extend from the detection of cholera toxin to other applications, such as screening and/or identification of other infectious disease agents, genetic disorders and biowarfare agents. The cold-chain-free biosensor when combined with thermostabilized PCR mixes and portable, battery-operated PCR

machines will substantially facilitate the transfer of diagnostics from the confinement of the laboratory to the point-of-care and in field settings.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2012.05.019.

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