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A label-free technique for accurate detection of nucleic acid–based self-avoiding molecular recognition systems supplemented multiple cross-displacement amplification and nanoparticles based biosensor

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ABSTRACT

Here, we devised a novel isothermal technique on the basis of standard multiple cross-displacement amplification (MCDA), which is assisted with self-avoiding molecular recognition system (SAMRS) components and antarctic thermal-sensitive uracil-DNA-glycosylase enzyme (AUDG), termed AUDG–SAMRS–MCDA. To enable product detection on the dipsticks, we firstly developed an analysis strategy, which did not require the labelled primers or probes, and thus, the analysis system avoids the false-positive results arising from undesired hybridization (between two labelled primers, or the labelled probe and primer). The SAMRS components are incorporated into MCDA primers for improve the assay's specificity, which can prevent the false-positive results yielding from off-target hybrids, undesired interactions between (hetero-dimer) or within (self-dimerization) primers. Two additional components (AUDG enzyme and dUTP) were added into the reaction mixtures, which were used for removing the false-positive results generating from carryover contamination, and thus, the genuine positives results were produced from the amplification of target templates. For the demonstration, the label-free AUDG–SAMRS–MCDA technique was successfully applied to detect *Pseudomonas aeruginosa* from pure culture and blood samples. As a proof-of-concept technique, the label-free AUDG–SAMRS–MCDA method can be reconfigured to detect different target sequences by redesigning the specific primers.

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P. aeruginosa; MCDA; SAMRS; AUDG; AUDG–SAMRS–MCDA; LFB

Introduction

Thus far, polymerase chain reaction (PCR)-based techniques are the most popular *in vitro* nucleic acid amplification methodologies and have been widely applied in clinical diagnosis, epidemiology, agriculture, basic research and many other fields [1]. However, PCR-based technologies require high-precision apparatus to perform thermal-cycling reactions, and these instruments are expensive, bulky and complex to operate, thus rendering them unsuitable for use in resource-poor settings. The growing use of *in vitro* nucleic acid amplification techniques has emphasized efficiency, simplicity, speed and low cost as key criteria for adoption in various fields. To this end, dozens of isothermal nucleic acid isothermal amplification techniques were devised and provided the solution to the shortcomings of PCR-based amplification counterparts [2].

Among the isothermal nucleic acid amplification techniques currently available, three assays, including multiple cross-displacement amplification (MCDA), loop-mediated isothermal amplification (LAMP) and cross-priming amplification (CPA), utilize primers' design to replace the role of PCR temperature cycles to denature target sequences and are conducted at a single temperature using only an enzyme with strong strand displacement activity, thus eliminating the use of complex protocols, special reagents and multiple enzymes [3]. Among them, MCDA assay has the advantages over LAMP and CPA techniques, namely quick results, high efficiency, sensitivity and specificity. Despite these advantages, the normal MCDA technique occasionally suffer from non-specific amplification, arising from primer dimers and off-target hybrids. This is since the higher ions, dNTPs and primers in isothermal amplification assays (such as MCDA, LAMP and CPA) compared with PCR-based techniques, as well as a relatively low amplification temperature, contribute to stabilization of non-specific primer dimers. In addition, the mixtures of primers in presence of polymerases and triphosphates are prone to "mischief", as isothermal amplification assays are not forced (at any point in the cycle) to follow Watson–Crick pairing rules by slowly annealing from high to low temperatures. Thus, high background (artefacts and primer dimers), low sensitivity, loss of signal, even false-positive results are generated. These problems posed by isothermal amplification methods cannot be mitigated by careful design of primer sequences and/or optimization of the reaction mixtures.

In order to address these shortcomings posed by MCDA technique, we utilized the MCDA primers that contained

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Supplemental data for this article can be accessed here.

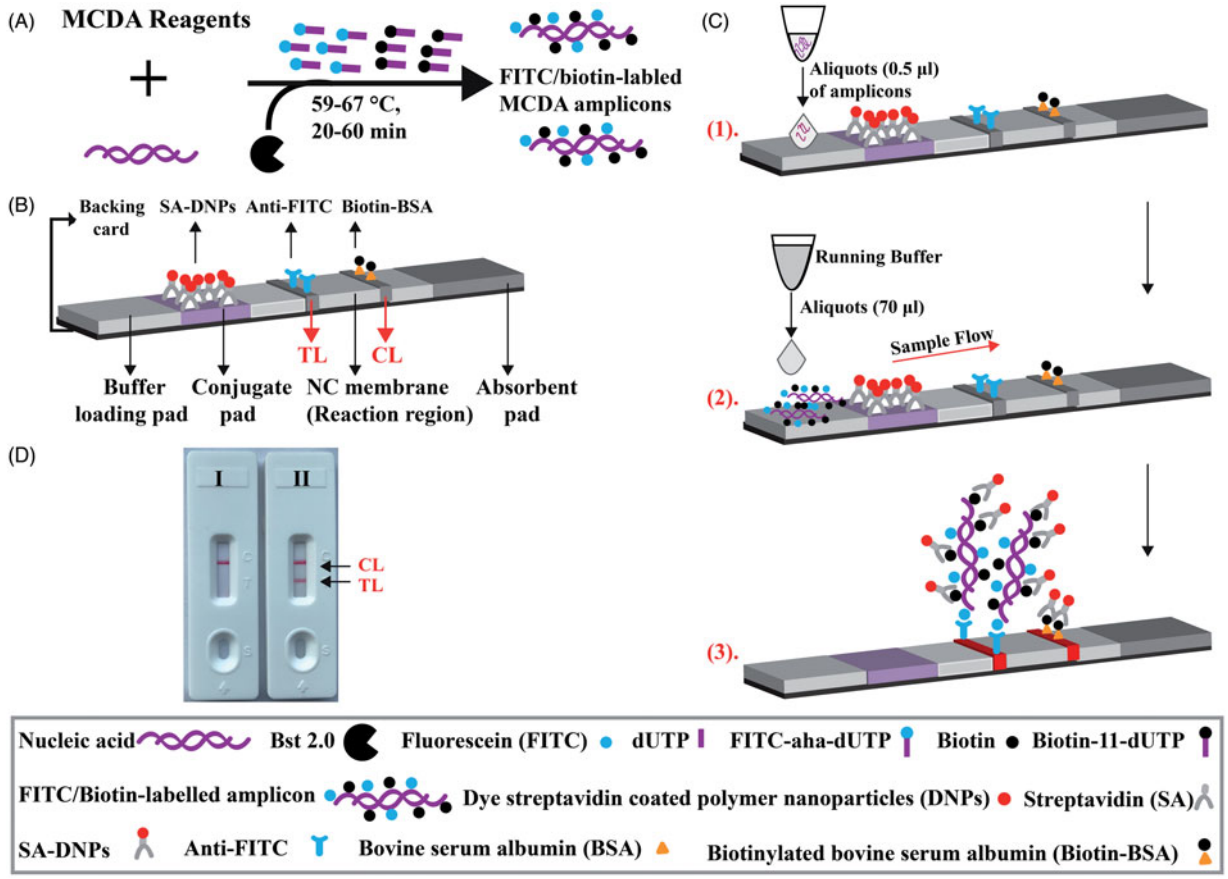

Figure 1. Outline of label-free MCDA coupled with lateral flow biosensor (LFB). (A) Outline of label-free MCDA with FITC-aha-dUTP and biotin-11-dUTP. (B) The detailed structure of LFB. (C) The schematic illustration of the LFB operation procedures: (1), 0.5 μl of amplification products was added to the buffer-loading pad; (2), 70 μl of diluent buffer was also added to the buffer-loading pad; (3), The capillary flow transferred MCDA amplicons and SA-DNPs from buffer-loading pad to the test line and control line. (D), Interpretation of the results: (I), negative (only the control line region showed a crimson red line); (II), positive (two crimson red bands, including test line and control line, appeared on the reaction region of the dipstick).

SAMRS (self-avoiding molecular recognition system) components (termed SAMRS-assisted MCDA, SAMRS–MCDA) to specifically amplify target sequences without non-target amplification. SAMRS molecules are these that are capable of binding to natural nucleotides but not to other SAMRS components. Hence, oligonucleotides built from SAMRS molecules do not interact with each other, even when target primers are poorly designed. The consideration is prompting research workers to construct SAMRS that is used in a variety of amplification methodologies. Thus far, SAMRS components only were applicable to multiplex PCR, RPA and HAD techniques [4–7]. Here, we reported that SAMRS components supported MCDA technique and were able to improve the performance of target assay. Then, the resultant MCDA products were analysed using an extremely simple but highly sensitive nanoparticles-based lateral flow biosensor (LFB). The LFB is a superior nucleic acid analysis tool owing to its simplicity, user-friendliness, high sensitivity and easy interpretation of results. Hence, a new nucleic acid lateral flow immuno-technique, which eliminates the use of the labeled primers and/or probes, is devised and applied to analyze amplification products (Figure 1(A)). However, the additional processing (such as reporting results by LFB) carries high risk of carry contamination, thus the effective strategies for removing the false-positive results yielding from carryover contaminations are extremely significant for accurate diagnostics. Towards a strategy to prevent carryover contamination, we utilized dUTP (deoxyruidine triphosphate) for MCDA reaction, such that all MCDA products incorporated uracil bases. Prior to conducting subsequent MCDA reactions, any uracil-containing MCDA products from previous MCDA reactions (such as carryover contaminants) were cleaved with AUDG (Antarctic thermal-sensitive uracil-DNA-glycosylase) enzyme, which specifically removes uracil bases in uracil-containing amplicons but has no effect on natural, thymine-containing template. Herein, AUDG digested uracil-containing MCDA products from previous MCDA reactions, hampering them from amplifying while leaving only the target template (natural DNA) intact for amplification. In sum, the conventional MCDA technique is assisted with SAMRS components, AUDG enzyme and LFB, which prevent the non-specific amplification arising from non-target amplification and carryover contamination and provide simple, rapid, sensitive, specific and user-friendliness detection of target sequences by merely observing a colorimetric signal with the naked eye.

Pseudomonas aeruginosa (*P. aeruginosa*), a rod-shaped Gram-negative obligatorily aerobic bacterium, is a ubiquitous environmental organism and belongs to the family of *Pseudomonadaceae* [8]. This bacterium is an opportunistic pathogen that infects animals and humans and is one of the

Table 1. Bacterial strains used in this study.

Bacteria	Strain no. (source of strains)[a]	No. of strains	MCDA Plus result[b]
P. aeruginosa			
P. aeruginosa	ZG-CDC-1417	1	P
P. aeruginosa	ZG-CDC-3165	1	P
P. aeruginosa	GZ-CDC-001	1	P
P. aeruginosa	GZ-CDC-002	1	P
P. aeruginosa	Isolated strains (ICDC)	14	P
Non-P.aeruginosa			
Staphylococcus aureus	Isolated strains (ICDC)	1	N
Staphylococcus epidemidis	Isolated strains (ICDC)	1	N
Plesiomonas shigelloides	Isolated strains (ICDC)	1	N
Enterohemorrhagic E. coli	EDL933 (ICDC)	1	N
Enteropathogenic E. coli	Isolated strains (ICDC)	1	N
Enterotoxigenic E. coli	Isolated strains (ICDC)	1	N
Enteroaggregative E. coli	Isolated strains (ICDC)	1	N
Enteroinvasive E. coli	Isolated strains (ICDC)	1	N
Aeromonas hydrophila	Isolated strains (ICDC)	1	N
Listeria monocytogenes	ATCC-EGD-e	1	N
Listeria ivanovii	ATCCBAA-678	1	N
Listeria grayi	ATCC25402	1	N
Listeria innocua	Isolated strains (ICDC)	1	N
Listeria welshimeri	Isolated strains (ICDC)	1	N
Listeria seeligeri	Isolated strains (ICDC)	1	N
Vibrio cholerae	ATCC14035	1	N
Vibrio alginolyticus	Isolated strains (ICDC)	1	N
Vibrio parahaemolyticus	Isolated strains (ICDC)	1	N
Shigella dysenteriae	Isolated strains (ICDC)	1	N
Shigella boydii	Isolated strains (ICDC)	1	N
Shigella flexneria	Isolated strains (ICDC)	1	N
Shigella sonneri	Isolated strains (ICDC)	1	N
Salmonella	Isolated strains (ICDC)	1	N
Enterococcus faecalis	ATCC35667	1	N
Enterococcus faecium	Isolated strains (ICDC)	1	N
Yersinia enterocolitica	ATCC23715	1	N
Enterobacter cloacae	Isolated strains (ICDC)	1	N
Bntorobater sakazakii	Isolated strains (ICDC)	1	N
Bacillus cereus	Isolated strains (ICDC)	1	N
Campylobacter jejuni	ATCC33291	1	N
Klebsiella pneumoniae	Isolated strains (ICDC)	1	N

[a]ATCC: American Type Culture Collection; GZ-CDC: GuiZhou Center for Disease Control and Prevention; ICDC: National Institute for Communicable Disease Control Disease Control and Prevention, Chinese Center for Disease Control and Prevention; ZG-CDC: ZiGong Center for Disease Control and Prevention.

[b]N: negative; P: positive. Only strains belonging to *P. aeruginosa* could be detected by the AUDG–SAMRS–MCDA technique, indicating the extremely high selectivity of the method.

top three causes of nosocomial infections, causing serious illnesses such as bacteraemia, septicaemia, otitis, pneumonia, keratitis, endocarditis and urinary tract infections [9]. Moreover, this pathogen is one of the most frequently identified bacteria in bottled water and tap water and is often monitored as an indicator of other bacterial contamination of faecal origin. As a major concern of nosocomial infection and with increased awareness in public health, establishment of a simple, cost-effective, rapid, sensitive, specific bacteriological *P. aeruginosa* detection assay is of the utmostly important and urgent necessity [10]. Therefore, *P. aeruginosa* is detected by the novel isothermal assay developed here to verify the feasibility of target analysis. The performance of target technique in diagnosing *P. aeruginosa* from pure culture and spiked blood samples is successfully demonstrated.

Materials and methods

Reagents and apparatus

Antarctic thermal-sensitive uracil-DNA-glycosylase (AUDG), dATP, dCTP and dGTP were obtained from New England Biolabs, INC. (Beijing, China). dUTP are obtained from Kuimo. Co., Ltd. (Beijing, China). Fluorescein (FITC)-aha-dUTP and biotin-11-dUTP were obtained from Thermo Scientific. Co., Ltd. (Shanghai, China). Biotinylated bovine serum albumin (biotin-BSA) and rabbit antifluorescein antibody (anti-FITC) and were purchased from Abcam. Co., Ltd. (Shanghai, China). Dye streptavidin-coated polymer nanoparticles (Crimson red, 129 nm) were purchased from Bangs Laboratories, INC. (IN). Visual detection reagent (Hydroxynaphthol blue, HNB) was obtained from Bei-Jing HaiTaiZhengYuan. Co., Ltd. (Beijing, China). Backing card, sample pad, conjugate pad, nitrocellulose membrane (NC) and absorbent pad were obtained from the Jie-Yi Biotechnology. Co., Ltd. (Shanghai, China). DNA extraction kits (QIAamp DNA minikits; Qiagen, Hilden, Germany) were obtained from Qiagen (Beijing, China).

Bacterial strains and DNA preparation

A total of 49 bacterial strains, including 18 strains of *P. aeruginosa* and 31 strains of non-*P. aeruginosa* bacteria, were used in this report (Table 1). DNA templates were extracted using DNA extraction kits (QIAamp DNA minikits, Hilden, Germany)

Table 2. The primers used in this study.

Primers name[a]	Sequences and modifications[b]	Length[c]
F1	5′-CCTCTCCCCTACCGCT-3′	16 nt
F2	5′-GCACCTTCACCGGAAGC-3′	17 nt
CP1	5′-GTTTGTAACCGTTGGCGACCGCGATGTGCGCGAGCCT-3′	37 mer
CP2	5′-TGAAATTCGGCAAATTTGCTGCGATCGCCGCCCTTGGAG-3′	39 mer
C1	5′-GTTTGTAACCGTTGGCGACCG-3′	21 nt
C2	5′-TGAAATTCGGCAAATTTGCTGCG-3′	23 nt
D1	5′-TCAGTTCAGGTAAGGGGA-3′	18 nt
D2	5′-CTCTGGCCATGGCTGTG-3′	17 nt
R1	5′-CCTAATGAACCCCAGTGT-3′	18 nt
R2	5′-AGTTACATGATGGAAATGC-3′	19 nt
SAMRS-F1	5′-CCTCTCCCCTA*C*C*G*CT-3′	16 nt
SAMRS-F2	5′-GCACCTTCACCG*G*A*A*GC-3′	17 nt
SAMRS-CP1	5′-GTTTGTAACCGTTGGCGACCGCGATGTGCGC*G*A*G*CCT-3′	37 mer
SAMRS-CP2	5′-TGAAATTCGGCAAATTTGCTGCGATCGCCGCCCT*T*G*G*AG-3′	39 mer
SAMRS-C1	5′-GTTTGTAACCGTTGGC*G*A*C*CG-3′	21 nt
SAMRS-C2	5′-TGAAATTCGGCAAATTTG*C*T*G*CG-3′	23 nt
SAMRS-D1	5′-TCAGTTCAGGTAA*G*G*G*GA-3′	18 nt
SAMRS-D2	5′-CTCTGGCCATGG*C*T*G*TG-3′	17 nt
SAMRS-R1	5′-CCTAATGAACCCC*A*G*T*GT-3′	18 nt
SAMRS-R2	5′-AGTTACATGATGGA*A*A*T*GC-3′	19 nt

[a]SAMRS, self-avoiding molecular recognition system.
[b]A*, 2-aminopurine; T*, 2-thiothymine; C*, N^4-ethylcytosine; G*, hypoxanthine.
[c]mer: monomeric; nt: nucleotide.

according to the manufacture's protocol and were determined with ultraviolet spectrophotometer (Nano drop ND-1000, Calibre, Beijing, China) at A260/280. Genomic templates were stored under at −20 °C before the templates were used. The strain of *P. aeruginosa* ZG-CDC-1417 was used for confirmation performance, optimal temperature and sensitivity analysis conducted in the report. The DNA templates of *P. aeruginosa* ZG-CDC-1417 was serially diluted (100 ng, 100 pg, 100 fg, 10 fg, 1 fg and 100 ag per microlitre) and a 1 μl aliquot of each dilution was used for the verification of assay's sensitivity.

Construction of lateral flow biosensor

Lateral flow biosensor (LFB; 4 mm ×60 mm) contains a buffer-loading pad, a conjugate pad, an NC membrane and an absorbent pad and the four components assembled on a backing card orderly by overlapping 2 mm among them (Figure 1(B)) [11]. Dye streptavidin-coated polymer nanoparticles (SA-DPNs) were gathered in the conjugate pad, and anti-FITC antibody and biotin-BSA were immobilized at the test line (TL) and control line (CL), respectively.

The schematic illustration of LFB operation procedures are shown in Figure 1(C). First, a volume of 0.5 μl of amplification products (FITC and biotin-labelled MCDA amplicons) was loaded into the buffer loading pad of the LFB. Next, a volume of 70 μl of diluent buffer was also added to the buffer-loading pad of the dipstick, and the LFB allowed to absorbing the whole running buffer. After 1 min, the MCDA amplicons analysis was indicated by the presence of crimson red lines on the NC membrane (Figure 1(D)).

Design of oligonucleotide primers

A specific primer set was designed using the *oprL* (outer membrane peptidoglycan-associated lipoprotein, GenBank, accession no. Z50191) gene of *P. aeruginosa*. The MCDA primer set consisting of two displacement primers (F1 and F2), two cross primers (CP1 and CP2) and six amplification primers (C1, C2, D1, D2, R1 and R2), which recognized 10 distinct regions on the target sequence. To verify the specificity of the MCDA primer set designed here, all primers are initially searched against these published sequences to screen possible cross reactivity with heterologous bacterial species not targeted for identification, using the Blastn software program (National Center for Biotechnology Information, U.S. National Library of Medicine, 8600 Rockville Pike, Bethesda MD). The details (such as primer design, sequences and modifications) are shown in Figure S1 and Table 2.

AUDG–SAMRS–MCDA reaction

AUDG–SARMS–MCDA is carried out in a one-step reaction in a 25 μl mixture containing 2.5 μl 10 X of the supplied buffer (New England Biolabs, Ipswich, MA), 1.6 μM each of cross primers SARMS-CP1 and SARMS-CP2, 0.8 μM each of amplification primers, 0.8 μM each of amplification primers SARMS-C1, SARMS-C2, SARMS-R1, SARMS-R2, SARMS-D1 and SARMS-D2, 0.4 μM each of displacement primers SARMS-F1 and SARMS-F2, 1.4 mM dATP, 1.4 mM dCTP, 1.4 mM dGTP, 0.5 mM dUTP, 1 μl of 0.1 mM biotin-11-dUTP, 0.5 μl of 0.1 mM FITC-aha-dUTP, 1.25 μl (10 U) of *Bst* 2.0 DNA polymerase (New England Biolabs), 0.8 M betaine (Sigma-Aldrich, St. Louis, MO), 0.3 μl (0.3 U) of AUDG and 1 μl DNA template.

A total of three monitoring methods, including colorimetric indicator (HNB), real-time turbidity (LA-320 C) and lateral flow biosensor detection, are applied to confirm and demonstrate the amplification of SAMRS–MCDA reaction. Then, we examine the optimal reaction temperature of SAMRS–MCDA, which are conducted at a fixed temperature ranging from 58 °C to 65 °C for 1 h and then incubated at 85 °C for 5 min to terminate the reaction. Reaction mixtures with 1 μl of double-distilled water (DW) were used as a blank control (BC), and reaction mixtures with 1 μl DNA template of *S. aureus* (isolated strain) and *K. pneumonia* (isolated strain) were used as negative controls (NC).

Sensitivity of SAMRS–MCDA assays

In order to evaluate the assay's sensitivity, limit of detection (LoD) of SAMRS-MCDA assay was performed using serial dilutions of purified DNA templates of *P. aeruginosa* ZG-CDC-1417. The initial purified genomic template concentration was 100 ngμl^{-1} and was used to make serial dilutions (100 ng, 100 pg, 100 fg, 10 fg, 1 fg and 100 ag per microliter). Then, a volume of 1 μl of each dilution is used as DNA template for SAMRS–MCDA assay, and the amplification products was analysed using HNB, real-time turbidity and LFB. The LoD was determined as the last dilution of each positive examination. All tests were conducted in triplicate.

Simulating carryover contamination

Products of SAMRS–MCDA reaction yielded from 100 pg/μl in the absence of AUDG enzyme, which is quantitated using ultraviolet spectrophotometer (NanoDrop ND-1000, Calibre, Beijing, China), was used to make serial dilution from 1×10^{-13} to 1×10^{-20} gμL^{-1}. The diluted amplicons served as the source of simulating carryover contaminants, and a volume of 1 μl of each dilution was used as DNA template in the SAMRS-MCDA assays.

Elimination of carryover contamination by AUDG–SAMRS–MCDA

In order to examine the ability of the established AUDG–SAMRS–MCDA assay to prevent the false-positive results due to carryover contaminants in detecting target sequences, we performed both AUDG–SAMRS–MCDA and SAMRS–MCDA reactions by adding 1 μl of simulated carryover contamination of 1×10^{-18} g/μl and 1 μl of diluted DNA templates (100 ng, 100 pg, 100 fg, 10 fg, 1 fg and 100 ag per microlitre) in the same amplification vessel. The total mass (1×10^{-18} g, approximately equivalent to a 0.2 μm diameter aerosol droplet) of the simulated carryover contamination for each reaction cannot be efficiently hampered by either fibrous pipette tip filters or high efficiency particulate air filters in the biosafety cabinets [12,13]. LoDs of the SAMRS–MCDA with and without AUDG digestion before reaction were compared to validate whether the devised AUDG–SAMRS–MCDA technique can efficiently obviate false-positive results.

Specificity of AUDG–SAMRS–MCDA assay

The assay's specificity is evaluated by performing the AUDG–SAMRS–MCDA assay on at least 10 ng of genomic templates extracted from a panel of *P. aeruginosa* and non-*P. aeruginosa* organisms (Table 1).

Practical application of AUDG–SAMRS–MCDA to P. Aeruginosa *in blood samples*

The human blood samples were acquired from a healthy donor with the written informed consent. Our study was reviewed and approved by the ethics committee of the National Institute for Communicable Disease Control and Prevention, China CDC, according to the medical research regulations of the Ministry of Health China (Approval No. ICDC2014003).

To evaluate the availability of AUDG–SAMRS–MCDA assay, the *P. aeruginosa* strain (ZG-CDC-1417) was used to examine the sensitivity of AUDG–SAMRS–MCDA in blood samples. Firstly, the bacteria suspension (with an optical density at 0.6), which contained *P. aeruginosa* ZG-CDC-1417, was prepared in 1 ml of sterile saline. The numbers of CFU (colony-forming units) in the suspension was calculated using a plate-counting technique according to previous study [14]. Briefly, serial 10-fold dilution (10^{-1}–10^{-8}) of the suspensions was carried out, and a volume of 100 μl appropriate dilution (10^{-6}) was spread onto brain–heart infusion agar (BHI) in triplicate. After 24 h at 37 °C, the numbers of CFU were counted. Next, a volume of 100 μl of each suspension with appropriate dilutions (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} and 10^{-7}) were added into the blood samples (900 μl), and the numbers of *P. aeruginosa* ZG-CDC-1417 were adjusted to approximate 380,000 CFU/ml, 38,000 CFU/ml, 3800 CFU/ml, 380 CFU/ml, 38 CFU/ml, 3.8 CFU/ml and 0.38 CFU/ml. A volume of 100 μl of the artificially contaminated blood samples was subjected to extract DNA template, and the genomic templates were eluted in 10 μl of Qiagen elution buffer. A volume of 2 μl of extracted DNA was used as templates for AUDG–SAMRS–MCDA assays. Non-contamination blood samples were selected as negative control (NC). This performance was independently conducted in triplicate.

Results

Development of label-free MCDA–LFB assay

A schematic illustration of the principle of label-free MCDA–LFB assay is showed in Figure 1. In the MCDA system, FITC-aha-dUTP and biotin-11-dUTP are added into the MCDA reaction mixtures (Figure 1(A)). During the reaction stage, MCDA primers anneal to target sequence, and are extended by *Bst* 2.0 polymerase. Thus, FITC-aha-dUTP and biotin-11-dUTP are simultaneously incorporated into the MCDA amplicons. As a result, FITC- and biotin-labelled MCDA amplicons, which are named as double-labelled detectable products, are successfully formed during the amplification stage.

A schematic illustration of the principle of the biosensor for the visualization of target amplicons is displayed in Figure 1(C). Firstly, a volume of 0.5 μl of MCDA reaction mixtures is deposited on the buffer-loading pad (Figure 1(C), step 1). Next, a volume of 70 μl of diluent buffer also is loaded into buffer-loading pad (Figure 1(C), step 2). The capillary flow transfers FITC- and biotin-labelled amplicons from buffer-loading pad to conjugate pad, so that the biotin-labelled products form a complex with SA-DNPs via streptavidin–biotin interactions. Finally, the capillary flow continuously transfers SA-DNPs and the SA-DNPs/amplicons complexes from conjugate pad to test line (TL) and control line (CL). The SA-DNPs/MCDA amplicons complexes are specifically captured at the TL by interaction between anti-FITC and FITC, whereas the

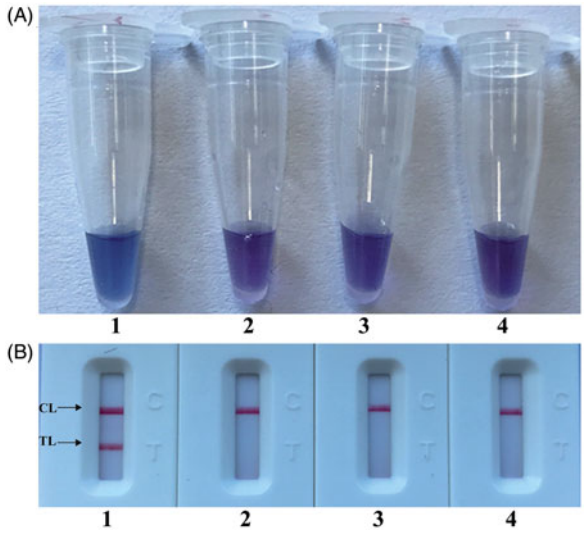

Figure 2. Validation and detection of SAMRS–MCDA products. (A) Reaction products of SAMRS–MCDA assay were visually detected by observation of the color change. (B) The dipsticks applied to for visual detection of SAMRS–MCDA products. Tube/strip 1, positive amplification of SAMRS–MCDA assay (*P. aeruginosa*, ZG-CDC-1417); Tube/strip 2, negative amplification of SAMRS–MCDA assay (*S. aureus*); Tube/strip 3, negative amplification of SAMRS–MCDA assay (*K. pneumonia*); Tube/strip 4, blank control (DW).

SA-DNPs that does not form complexes are immobilized at the CL by biotin-BSA via streptavidin–biotin interactions (Figure 1(C), step 3). Complexed and non-conplexed SA-DNPs are indicated by crimson red lines at the test region and control region, respectively. The colorimetric signal was easily visible to the naked eye within 2 min (Figure 1(D)).

Confirmation and validation of SAMRS–MCDA–LFB products

In order to verify the availability of SAMRS–MCDA primers, the SAMRS–MCDA reactions were performed in the presence or absence of target DNA templates within 60 min at a single temperature (60 °C). The positive SAMRS–MCDA tube was observed by naked eye as sky blue, whereas the negative and blank controls reminded violet (Figure 2(A)). By LFB, two crimson red lines, including TL and CL, were visualized in the positive SAMRS–MCDA reactions, only a crimson red line (CL) was seen in negative controls and blank control (Figure 2(B)). These results demonstrated that the SAMRS–MCDA primer set was a good candidate for the development of the AUDG–SAMRS–MCDA–LFB assay for target sequence detection.

The temperature optimization of the SAMRS–MCDA primer set

The SAMRS–MCDA assays were performed using the *P. aeruginosa* (ZG-CDC-1417) templates at the level of 100 pg per vessel to test the optimal reaction temperature during the amplification stage. Different reaction temperatures (from 58 °C to 65 °C at 1 °C interval) were compared for confirming the optimal assay temperature. Real-time measurement of turbidity was used for monitoring the SAMRS–MCDA reactions, and the typical kinetics graphs corresponding to eight reaction temperatures were obtained. As shown in Figure 3, the better results were generated from the reaction temperature of 58 °C to 62 °C. The assay temperature of 60 °C was used for performing the rests conducted in this study.

Performance of SAMRS–MCDA using different strains and double-distilled water

A total of 64 SAMRS–MCDA amplifications, including one positive control, 31 negative controls and 32 blank controls (DW), were carried out at the assay temperature (60 °C). As shown in Figure 4, only a positive result was produced from the positive control, and no non-specific amplification was obtained in negative control and blank controls. In contrast, MCDA assays with standard primer set yielded four false-positive results (Figure S2, signals 17, 20, 46 and 49). Two undesired positives (signals 17 and 20), termed non-specific amplification, were observed in non-*P. aeruginosa* strains and another two false positives (signals 46 and 49), named self-amplification, were obtained from blank controls. These results indicated that SAMRS–MCDA assay could effectively eliminate the undesired amplification, arising from off-target hybridization and self-amplification.

Analytical sensitivity of SAMRS–MCDA–LFB assay in pure culture

In order to determine the LoD of the SAMRS–MCDA–LFB assay, we performed SAMRS–MCDA reactions using serial dilutions (from 100 ng to 100 aq per microlitre) of pure *P. aeruginosa* (ZG-CDC-1417) DNA. As shown in Figure 5(A), the SAMRS–MCDA reaction mixtures were detected by LFB, and the analytical sensitivity of SAMRS–MCDA method for identifying *oprL* gene was 10 fg of DNA templates per vessel. Using the SAMRS–MCDA amplification by self-trail, the sensitivity of the assay was further examined by direct visual inspection of the SAMRS–MCDA products with HNB reagents and real-time turbidity analysis (Figure 5(B,C)). By HNB reagents and real-time turbidity, the LoD of SAMRS–MCDA assay was also 10 fg per tube, which was conformity with LFB analysis. Comparing with standard MCDA assay, the LoD of standard MCDA method for detecting *oprL* gene was also 10 fg per reaction tube, which was in complete accordance with that seen in the SAMRS–MCDA assay (Figure 5 and S3).

AUDG–SAMRS–MCDA reaction

The established AUDG–SAMRS–MCDA assay is a one-pot, closed-tube technique for specific detection of target sequences and prevention of carryover contamination (Figure 6). Comparing with SAMRS–MCDA, the developed AUDG–SAMRS–MCDA assay is assisted with AUDG enzyme and dUTP. During the amplification stage, dUTP can be incorporated into all SAMRS–MCDA products after amplifying the target sequences under certain conditions (shown in the section of "AUDG–SAMRS–MCDA reaction"). This processing was regarded as the first stage for carryover contamination

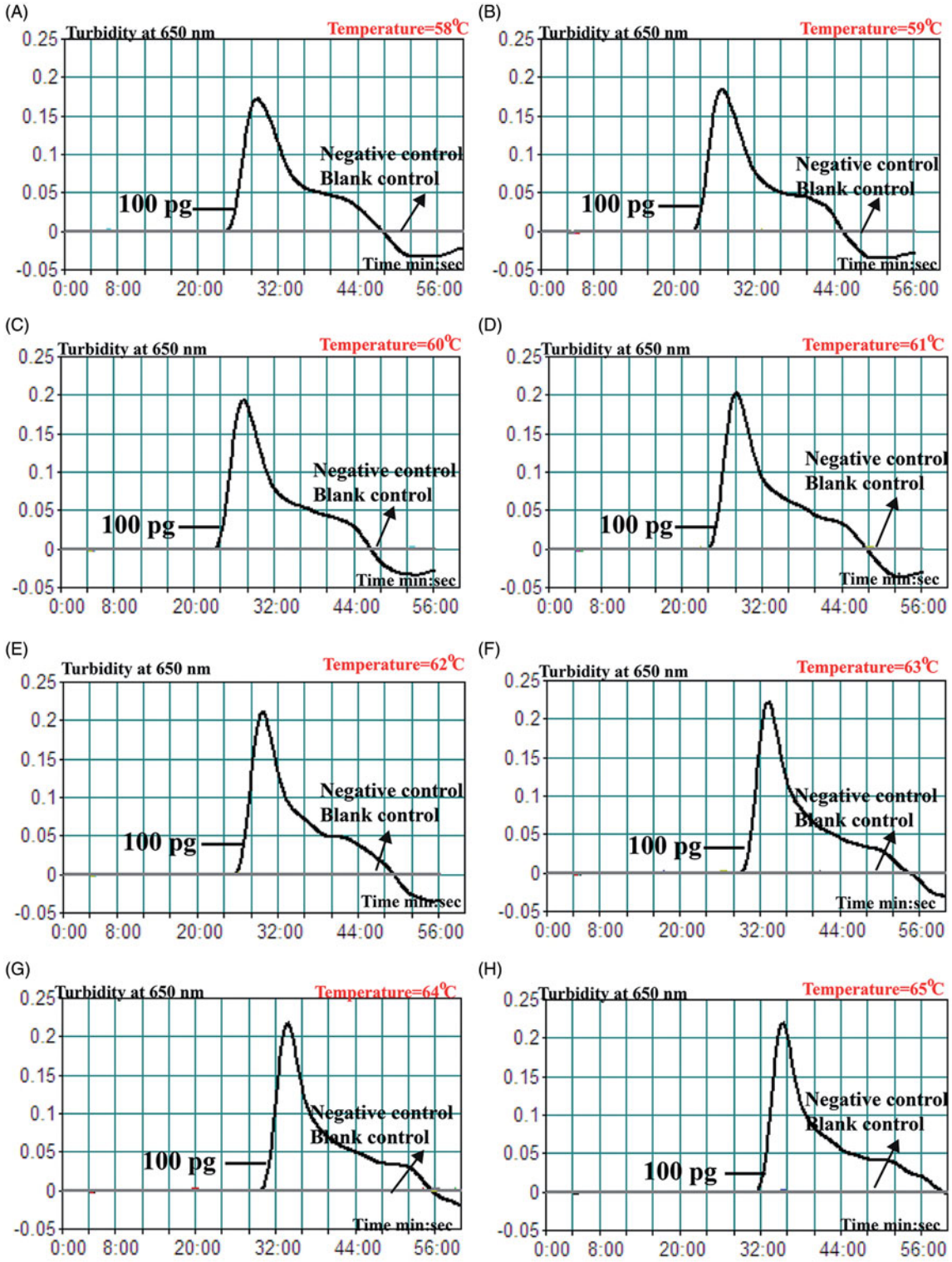

Figure 3. Optimal amplification temperature for SAMRS–MCDA primer set. The SAMRS–MCDA reactions for the detection of *P. aeruginosa* were monitored by real-time measurement of turbidity (LA-320c), and the corresponding curves of concentrations of genomic templates were marked in the figures. The threshold value was 0.1 and the turbidity of >0.1 was regarded as positive amplification. The graphs from (A) to (E) displayed robust amplification.

prevention. In order to cleave the dUTP-incorporated SAMRS–MCDA amplicons, AUDG treatment was conducted before SAMRS–MCDA amplification, and this processing was considered as the second stage for carryover contamination elimination. However, the natural templates cannot be cleaved by AUDG enzyme under this stage. Moreover, to construct the FITC-biotin double-labelled SAMRS–MCDA amplicons and enable them detection on the strip, two additional components (FITC-aha-dUTP and biotin-11-dUTP) are added into SAMRS–MCDA amplification mixtures (Figure 1, 6).

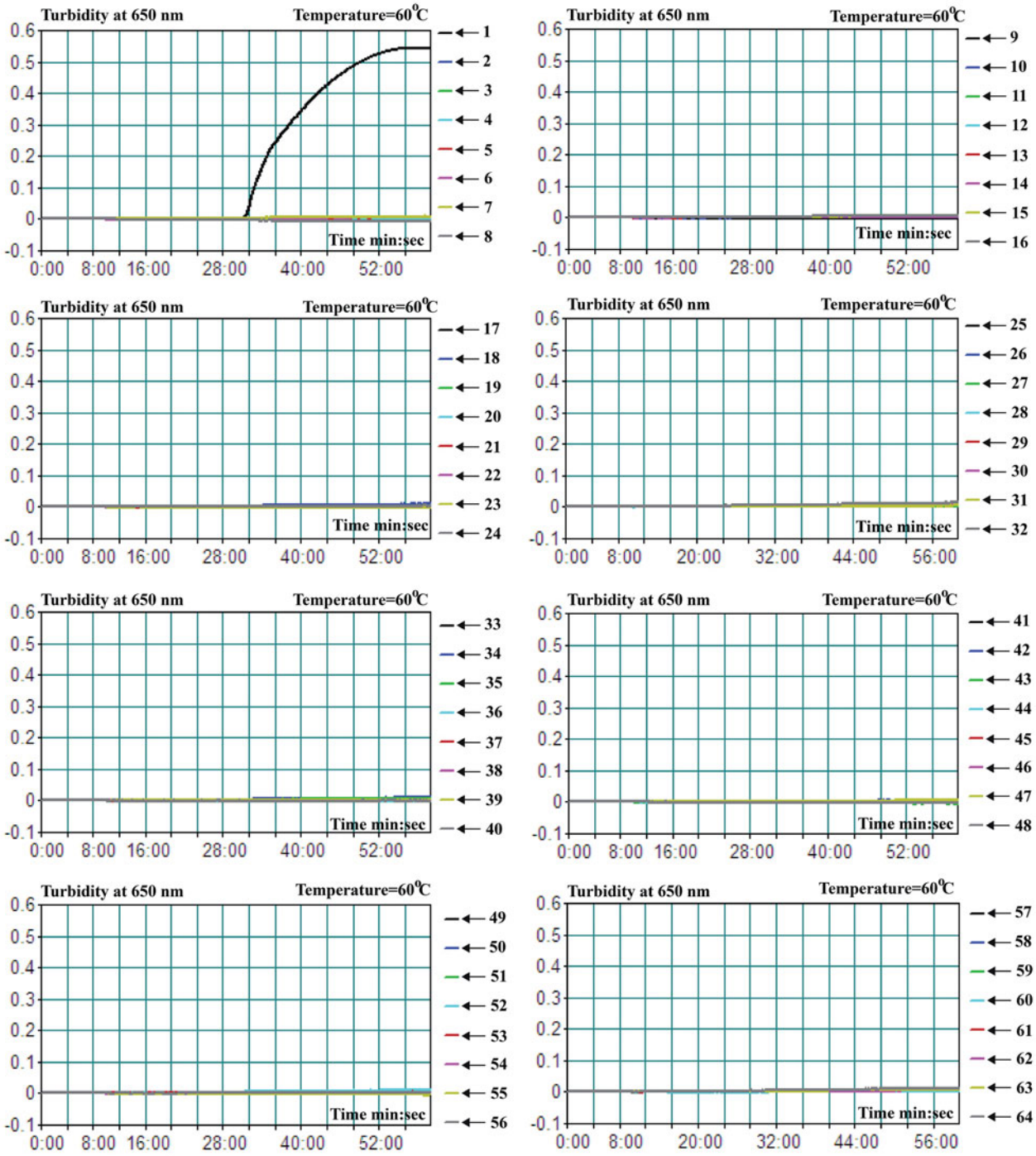

Figure 4. Performance of the SAMRS–MCDA using different genomic templates and double distilled water (DW). Signal 1, positive control (P. aeruginosa, ZG-CDC-1417); Signals 2-32, Staphylococcus aureus, Staphylococcus epidemidis, Plesiomonas shigelloides, Enterohemorrhagic E. coli, Enteropathogenic E. coli, Enterotoxigenic E. coli, Enteroaggregative E. coli, Enteroinvasive E. coli, Aeromonas hydrophila, Listeria monocytogenes, Listeria ivanovii, Listeria grayi, Listeria innocua, Listeria welshimeri, Listeria seeligeri, Vibrio cholera, Vibrio alginolyticus, Vibrio parahaemolyticus, Shigella dysenteriae, Shigella boydii, Shigella flexneria, Shigella sonneri, Salmonella, Enterococcus faecalis, Enterococcus faecium, Yersinia enterocolitica, Enterobacter cloacae, Bntorobater sakazakii, Bacillus cereus, Campylobacter jejuni, Klebsiella pneumonia; Signals 33-64, DW.

AUDG–SAMRS–MCDA detects simulated carryover contamination

In order to evaluate that simulated carryover contamination (dUTP-incorporated SAMRS–MCDA amplicons) from previous SAMRS–MCDA reactions is able to contaminate new SAMRS–MCDA amplifications. In this report, we compared the analytical sensitivity of the SAMRS–MCDA and AUDG–SAMRS–MCDA approaches using serial diluted SAMRS–MCDA amplicons with concentrations ranging from 1×10^{-13} to 1×10^{-20} g/μL. In the presence of AUDG treatment, the AUDG–SAMRS–MCDA assay only detected 1×10^{-14} g of simulated carryover contamination per tube (Figure 7(A)). In contrast, the SAMRS–MCDA without AUDG

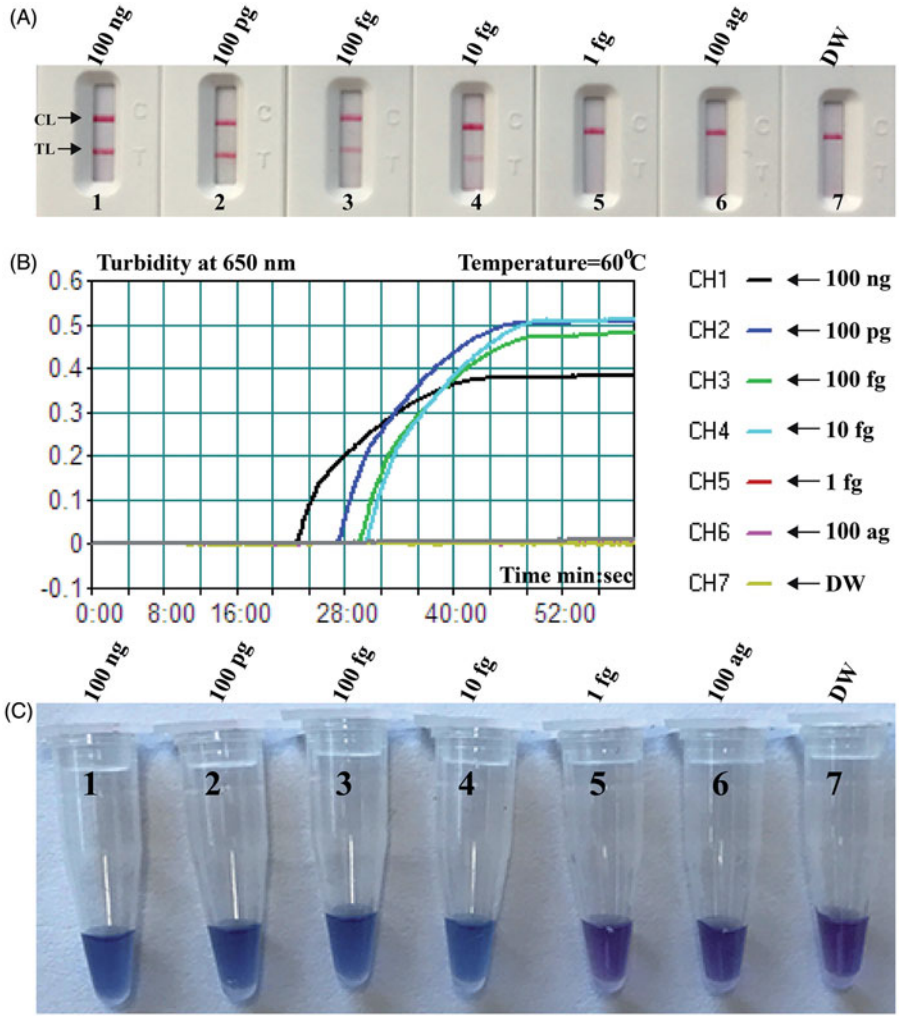

Figure 5. Analytical sensitivity of SAMRS–MCDA assay using serially diluted genomic DNA with *P. aeruginosa* (ZG-CDC-1417). A total of three monitoring techniques, including lateral flow biosensor (A) real time turbidity (B) colorimetric indicator (HNB, C) were applied for analysing the amplification products. The genomic DNA levels of 100 ng, 100 pg, 100 fg and 10 fg per reaction produced the positive reactions.

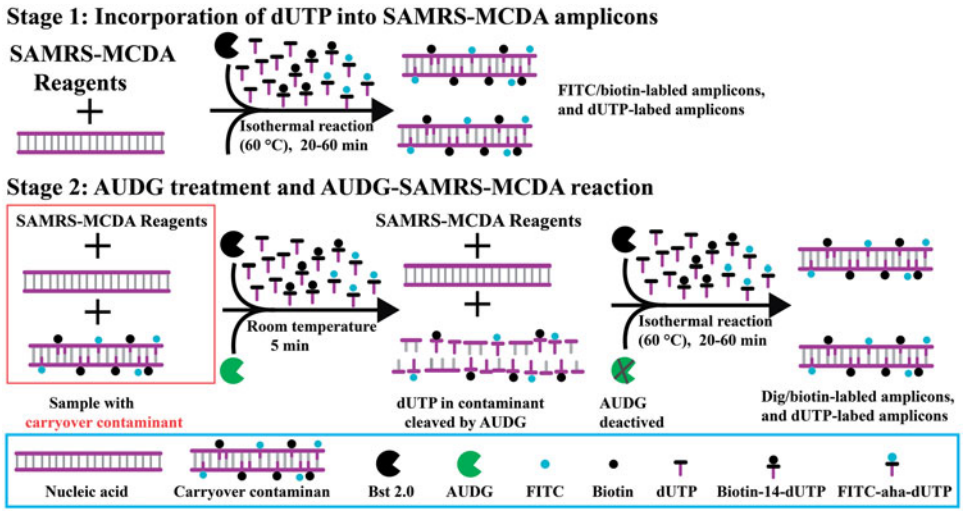

Figure 6. Schematic illustration of the principle of AUDG-SAMRS-MCDA technique for eliminating carryover contamination. AUDG–SAMRS–MCDA eliminates carryover contamination in two stages. During the first stage, all SAMRS–MCDA amplifications are conducted in the presence of dUTP and *Bst* 2.0 DNA polymerase, such that dUTP was incorporated into all SAMRS–MCDA amplicons. During the second stage, all subsequent SAMRS–MCDA reactions are treated with AUDG enzyme to digest carryover contaminants by removing uracil from amplicons from previous SAMRS–MCDA amplifications, but having no effect on natural DNA (target templates). During the SAMRS–MCDA amplification stage (60 °C), the AUDG enzyme is rapidly heat-inactivated and the cleaved contaminants are degraded, ensuring that only the target templates are amplified. In order to from the FITC- and biotin-attached duplex products and enable them detection on the dipsticks, two additional components, including FITC-aha-dUTP and biotin-11-dUTP, are added into SAMRS–MCDA reaction mixtures.

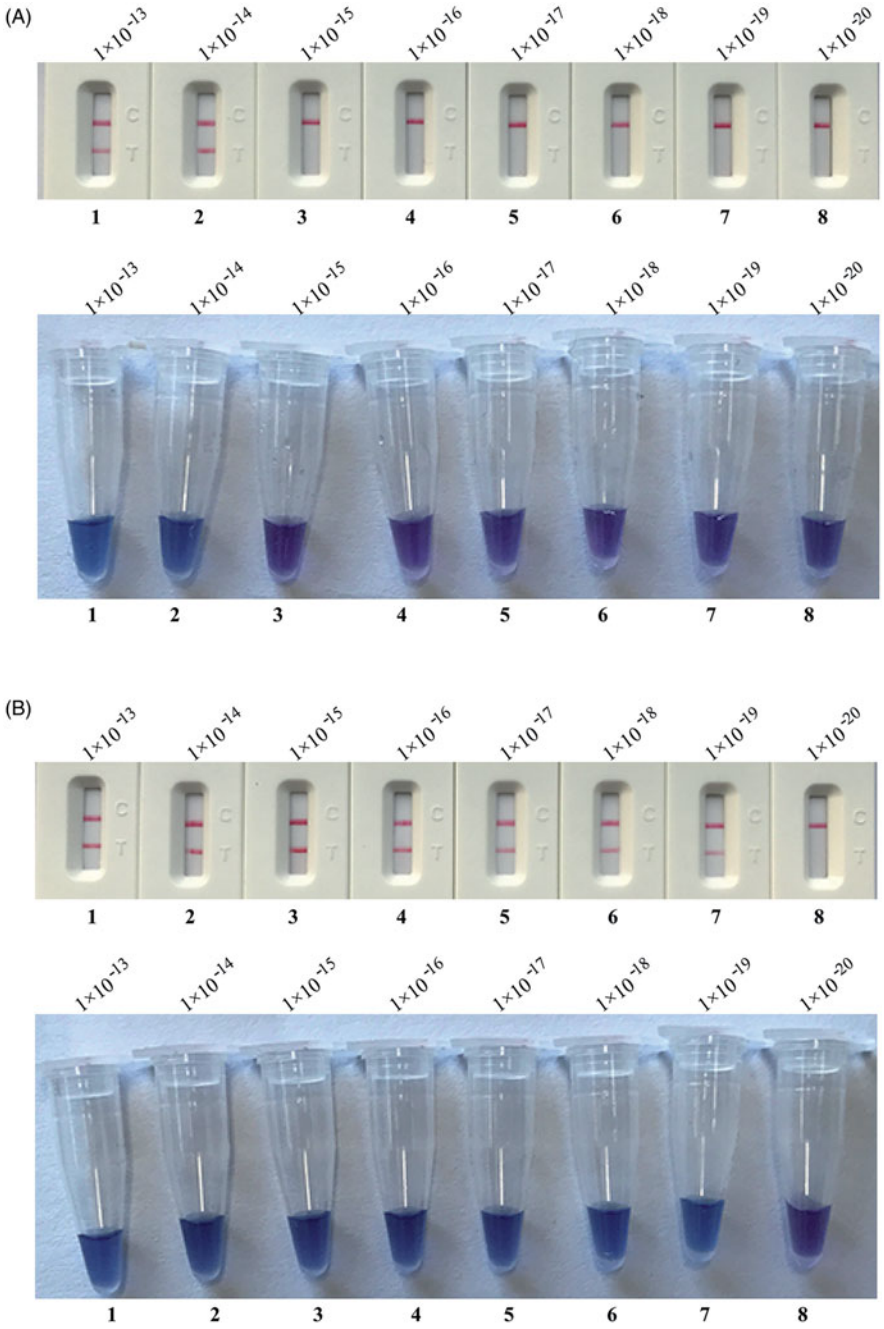

Figure 7. Control of carryover contamination in AUDG–SAMRS–MCDA assay. Sensitivity examination of AUDG–SAMRS–MCDA (A) and SAMRS–MCDA (B) using 10-fold serial dilutions of simulated carryover contamination (dUTP-incorporated amplicons DNA, concentration diluted from 1×10^{-13}, 1×10^{-14}, 1×10^{-15}, 1×10^{-16}, 1×10^{-17}, 1×10^{-18} 1×10^{-19} and 1×10^{-20}g μL^{-1}) as determined using LFB (top row) and HNB (bottom row).

treatment could detect as little as 1×10^{-19}g of simulated carryover contamination per vessel, which was equivalent to a 0.14 μm diameter aerosol droplet (Figure 7(B)). The result indicated that a very small amount of contaminants (1×10^{-18}g ~ 0.2 μm diameter aerosol droplet, even less), which could not be efficiently removed by fibrous pipette tip filters, was sufficient to contaminate new SAMRS–MCDA reactions and caused false-positive results. Thus, the AUDG enzyme could prevent the amplification of up to 100000-fold higher concentrations of carryover contaminant amplicons (1×10^{-18}g), which significantly reduced the likelihood of undesired results of SAMRS–MCDA detection.

AUDG–SAMRS–MCDA assay avoids the false-positive results

In order to further verify that AUDG–SAMRS–MCDA technique has the ability to reduce the likelihood of undesired results due to carryover contaminants, the sensitivity evaluation of AUDG–SAMRS–MCDA and SAMRS–MCDA reactions was carried out using serial dilution of the *P. aeruginosa* ZG-CDC-1417 templates, and simultaneously added 1×10^{-18}g of contaminants (dUTP-incorporated amplicons) into each reaction tube. In the presence of AUDG enzyme treatment, the LoD of AUDG–SAMRS–MCDA assay was consistent with the

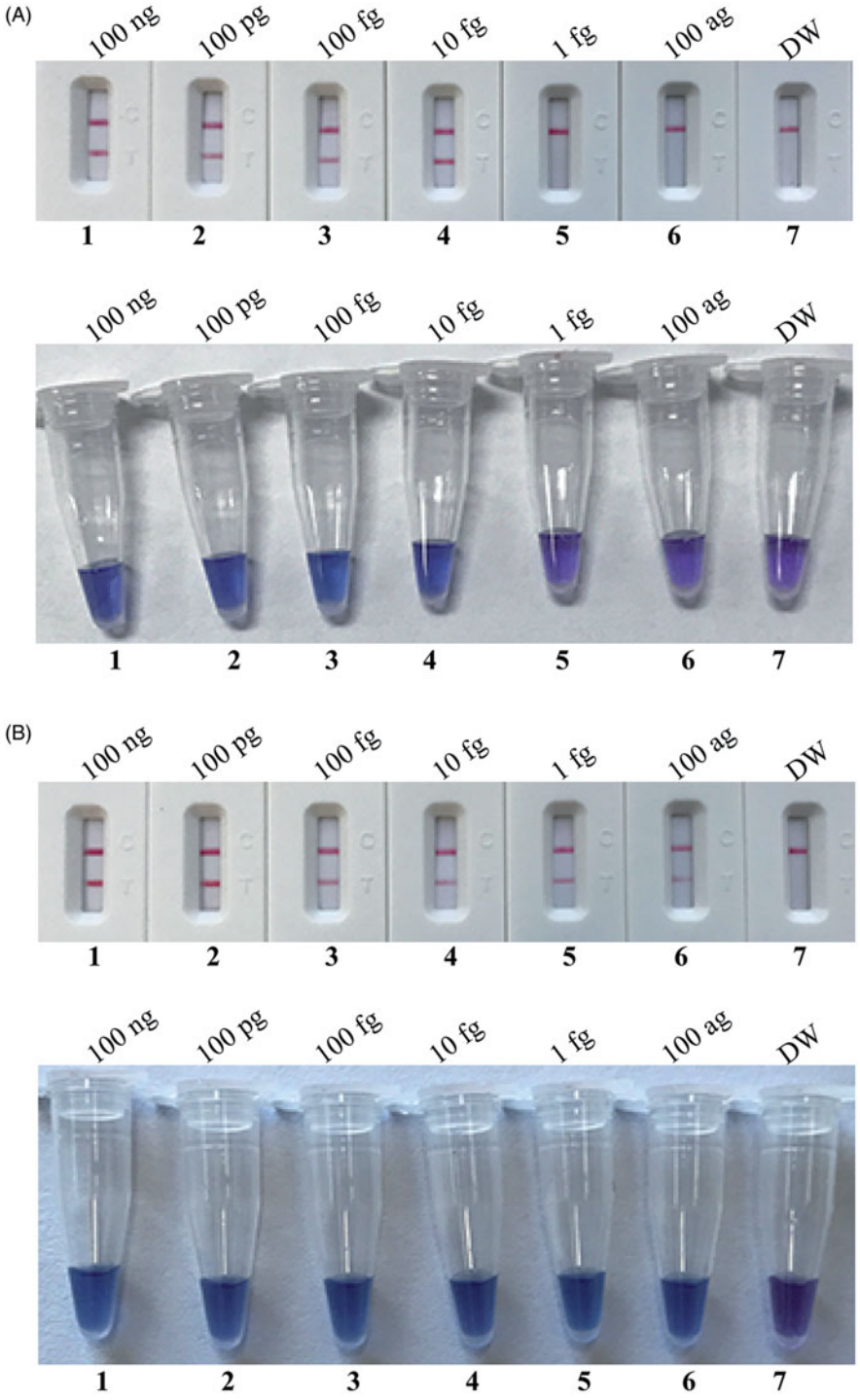

Figure 8. AUDG–SAMRS–MCDA technique eliminates false-positive detection due to carryover contamination. Sensitivity examination of AUDG–SAMRS–MCDA (A) and SAMRS–MCDA (B) assay using serial dilutions (100 ng μl^{-1}, 100 pg μl^{-1}, 100 fg μl^{-1}, 10 fg μl^{-1}, 1 fg μl^{-1} and 100 ag μl^{-1}) of ZG-CDC-1417 and 1×10^{-18} g μL^{-1} of simulated carryover contamination (dUTP-incorporated amplicons DNA) as determined using HNB (**bottom row**) and LFB (**top row**).

aforementioned sensitivity testing (Figure 8(A) and 5). In the SAMRS–MCDA reactions without AUDG treatment, all tested samples exhibited positive results, even including these samples with undetectable level of *P. aeruginosa* ZG-CDC-1417 templates (less than 10 fg per reaction), which were regarded as false-positive amplifications (Figure 8(B)). As such, we did not correctly confirm the LoD of SAMRS–MCDA assay without AUDG treatment. These results indicated that the AUDG–SAMRS–MCDA assay devised here could successfully remove false-positive results arising from carryover contamination.

Analytical specificity of AUDG–SAMRS–MCDA assay

To examine the specificity of AUDG–SAMRS–MCDA technique, 18 *P. aeruginosa* strains and 31 non-*P. aeruginosa* strains are determined. All positive results was observed after 60 min of

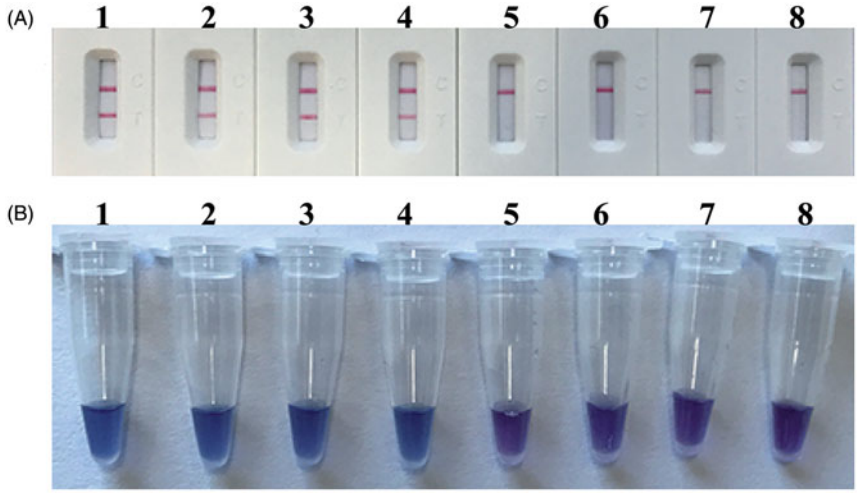

Figure 9. Analytical sensitivity of AUDG–SAMRS–MCDA for detecting target pathogen in blood samples. Two monitoring techniques, including later flow biosensor (A), and colorimetric indicator (HNB, B), were applied for analysing the amplification products. The serial dilutions of target templates were subjected to AUDG–SAMRS–MCDA reactions. Strips (A)/Tubes (B) 1–8 represented the DNA levels of 7600 CFU, 760 CFU, 76 CFU, 7.6 CFU, 0.76 CFU, 0.076, 0.0076 CFU per reaction, negative control (non-contaminated blood samples). The genomic DNA levels of 7600 CFU, 760 CFU, 76 CFU and 7.6 CFU per reaction produced the positive reactions.

incubation in *P. aeruginosa* strains tested, whereas other non-*P. aeruginosa* strains displayed negative results (Table 1). These data demonstrate that our AUDG–SAMRS–MCDA assay was very specific to *P. aeruginosa*.

Practical application of AUDG–SAMRS–MCDA to P. Aeruginosa *in blood samples*

To demonstrate the feasibility of AUDG–SAMRS–MCDA assay as a nucleic acid detection tool, the artificially contaminated blood samples with target pathogen were examined using AUDG-SAMRS-MCDA technique. As shown in Figure 9, AUDG–SAMRS–MCDA assay produced positive results when the contaminated numbers of *P. aeruginosa* were 380 CFU per millilitre (~7.6 CFU per reaction). No positive results were generated from negative control (non-contaminated blood samples).

Discussion

Multiple cross-displacement amplification (MCDA) is a sensitive strand displacement technique and amplifies target sequences from trace levels to detectable levels less than 60 min at a constant temperature (58–67 °C) [3]. MCDA technique, similar to LAMP and CPA assays, utilizes primers' design to replace the role of PCR temperature cycles to denature target sequences. Thus, the shortcomings (false-positive results arising from off-target hybridization and primer-dimers) posed by MCDA are shared with LAMP and CPA approaches (Figure S2) [15]. Normally, isothermal amplification methods (MCDA, LAMP and CPA) did not produce non-specific amplification within 30 min. However, the amplification of very small amounts of target templates (less than 10 copies per vessel) for more than 30 min yielded positive signals that were similar with those seen in the non-specific amplifications (Figure S2). As a result, there is always the possibility that a sample with extremely low concentration of target sequences may be somewhat ambiguous to the researcher. In order to address these problems, some researchers have explored use of monitoring methods (such as melting curves and agarose gel electrophoresis) to differentiate specific amplification from non-specific amplification. Unfortunately, these strategies required complicated apparatus, additional post-procedure and trained personnel [15]. Most importantly, these monitoring techniques, including melting curves and agarose gel electrophoresis, which cannot eliminate the non-specific results, are used for only distinguishing non-specific amplifications from specific amplifications.

In this report, we introduce a "self-avoiding molecular recognition system" (SAMRS) as a class of DNA analogues employed to support MCDA assay [4,5]. With the MCDA primers containing SAMRS components, no undesired results were obtained in blank controls and negative controls, and only a positive signal was seen in positive control (Figure 4). In SAMRS–MCDA assay, the SAMRS components, including A* (2-aminopurine), T* (2-thiotymine), C* (N^4-ethylcytosine) and G* (hypoxanthine), are used for replacing the natural A, T, C and G nucleobases at some sites (near the 3′-end of primers) of MCDA primers (Table 2) [4]. SAMRS T* pairs with natural A; SAMRS A* pairs with natural T; SAMRS G* pairs with natural C and SAMRS C* pairs with natural G. However, SAMRS A*: SAMRS T* and SAMRS C*: SAMRS G* base pairs cannot contribute the stability of the helix due to they either form 1 or 1.5 bonds [5,16,17]. The special pairing rules indicate that SAMRS components cannot interact with other SAMRS components (even when these molecules exhibit 100% complementarity), thus MCDA primers containing SAMRS molecules bind solely to their intended natural complements (target sequence) and prevent primer-dimers in SAMRS–MCDA assay. Accordingly, SAMRS–MCDA method successfully removes the false-positive results, arising from self-amplification (false-positive results generated from interactions between or

within primers) and non-specific amplification (false-positive results generated from non-target hybrids) (Figure 4).

The MCDA results can be determined by colorimetric indicators (such as HNB), agarose gel electrophoresis, real-time turbidity and lateral flow biosensors [18]. In particular, lateral flow biosensors have been used as an analysis tool to achieve simple, rapid and user-friendliness detection of various amplicons by merely observing a colorimetric signal with the naked eye. Traditionally, to incorporate nucleic acid amplification techniques and LFB detection, two primers involved in amplification were labelled, one with hapten and one with biotin [18–22]. In this way, a double-labelled detectable amplicon was formed during the amplification stage, which derived from two labelled primers. However, the hybridization between two labelled primers can construct a double-labelled detectable product, which generates a false-positive signal without amplification. To overcome the disadvantages posed by conventional methodologies, we devised a label-free strategy to construct a double-labelled detectable amplicon, which eliminate the use of label-primer or label-probe (Figure 1). The label-free strategy, which is a universal technique, can be applied to a wide of amplification approaches (such as MCDA, LAMP, CPA, PCR) and completely avoids the false positive results yielding from interaction between labelled primers. In this report, the label-free MCDA coupled with LFB (MCDA–LFB) was devised and applied to validate the feasibility of the label-free strategy. In the MCDA assay, two additional components, including FITC-aha-dUTP and biotin-11-dUTP, were added into MCDA reaction mixtures. FITC-aha-dUTP and biotin-11-dUTP were incorporated into all MCDA amplicons when MCDA primers extended by Bst 2.0 polymerase, thus all amplification products were simultaneous labelled (Figure 1(A)). Then, the detectable amplicons could be directly analysed by the LFB, alleviating the use of any post-hybridization procedure (Figure 1(C)). Moreover, label-free MCDA-LFB assay also obviated the use of label-probe for this biosensor, and thus, there is no incubation step prior to LFB detection.

Further processing, such as reporting SAMRS–MCDA result by LFB and agarose gel electrophoresis, requires opening of the reaction vessel, which easily yielded aerosol droplets of different sizes that contain high concentrations of amplicons. Due to its high sensitivity, the MCDA assay is extremely vulnerable to aerosol droplets (carryover contamination) (Figure 7). Particularly, repeated detection of certain target sequences is performed in the same laboratory, and carryover contamination is produced through pipettes, work space surfaces, reagents or the skin of researcher. Thus, the carryover contamination of amplified DNA from previous reactions is one of very tricky problems affecting nucleic acid amplifications because it can generated false-positive results. In this report, two changes were successfully introduced into SAMRS–MCDA (an updated version of standard MCDA) procedures to eliminate carryover contamination: (1) incorporation of dUTP into all SAMRS–MCDA amplicons and (2) applying Antarctic thermal-sensitive uracil-DNA-glycosylase (AUDG) to digest contaminants before the next SAMRS–MCDA procedure (Figure 6). AUDG is an enzyme that is capable of catalysing the removal of uracil bases from uracil-containing single- and double-stranded DNA by digesting the N-glycosidic bond between sugar-phosphate backbone and the uracil base at relative low temperature (i.e. room temperature). If dTTP are replaced by dUTP in SAMRS–MCDA reaction mixture, SAMRS–MCDA amplicons containing uracil instead of thymine residues are synthesized in the amplification mixtures. The SAMRS–MCDA products containing uracil are targets for AUDG enzyme, while the uracil-free DNAs (natural DNA template) is not. Thus, carryover contamination caused by amplicons from previous reactions can be eliminated by treatment with AUDG enzyme.

Importantly, the AUDG enzyme can be rapidly and completely inactivated at an elevated temperature (above 50 °C) because it is extremely sensitive to heat. As a result, the use of AUDG enzyme enables AUDG–SAMRS–MCDA assay to be performed in a single closed tube because the contaminants from previous reaction are designedly degraded within 5 min at room temperature prior to amplification, then AUDG enzyme is rapidly, automatically and completely inactivated at assay temperature (60 °C). In this way, genuine amplification products subsequently produced from the target DNAs during the reaction remain completely unaffected and cannot be degraded, permitting SAMRS–MCDA amplification to normally proceed (Figure 6). Once AUDG–SAMRS–MCDA reaction is finished, the results can be determined using various monitoring methods (such as colorimetric indicator, gel electrophoresis and turbidimeters and LFB).

As a proof of concept, *P. aeruginosa*, an important human opportunistic pathogen, is responsible for serious infections including pneumonia, endocarditis, septicaemia, keratitis and otitis, was selected as model for validating the feasibility of AUDG–SAMRS–MCDA assay [9]. AUDG–SAMRS–MCDA's LoD for *P. aeruginose* detection is 10 fg of genomic DNA per tube in pure culture (Figures 5 and 8). The results obtained using LFB were in conformity with HNB reagent, real-time turbidity and agarose gel electrophoresis detection (Figures 5, 8 and 9). Although the AUDG–SAMRS–MCDA amplicons could be analysed equally with other techniques employed in this report, the dipstick is likely the preferred monitoring method as indicating the results is less subjective and does not require apparatus. The practical application of AUDG–SAMRS–MCDA method was successfully evaluated by detecting *P. aeruginose* in blood sample with high specificity and sensitivity (Figure 9). For the analytical specificity test, the desired results were obtained for target pathogens, and no positive signals were seen in the assay of non-*P. aeruginose* strains (Table 2).

Conclusions

In conclusion, we devised a novel technique on the basis of standard MCDA assay, termed AUDG–SAMRS–MCDA. In order to enable product detection on the biosensor, we firstly developed a new analysis strategy, which did not require the labelled primers or probe, and thus, the analysis system avoids the false-positive result arising from undesired hybridization (between two labelled primers, or the labelled probe and primer). The SAMRS components are incorporated into MCDA primers for improve the assay's specificity, which can

prevent the false-positive result yielding from off-target hybrids, undesired interactions between (hetero-dimer) or within (self-dimerization) primers. Moreover, two additional components (AUDG enzyme and dUTP) were added into the reaction mixtures, which were used for removing the false-positive result generating from carryover contamination. The assay developed in this report was assisted with new detection system, SAMRS components and AUDG enzyme, and thus, the genuine positives results were produced from the amplification of target templates. For demonstration purpose, *P. aeruginosa*, which is an important human opportunistic pathogen, was detected by the AUDG–SAMRS–MCDA technique to validate the feasibility for target analysis. The performance of AUDG–SAMRS–MCDA method in detecting target pathogen from pure culture and blood samples was successfully determined. Importantly, the AUDG–SAMRS–MCDA assay, as the proof of concept technique, can be reconfigured to detect different target sequences by redesigning the specific primers.

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Disclosure statement

The authors report no conflicts of interest.

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