Accepted Manuscript

Loop-mediated isothermal amplification using self-avoiding molecular recognition systems and antarctic thermal sensitive uracil-DNA-glycosylase for detection of nucleic acid with prevention of carryover contamination

Yi Wang, Dongxin Liu, Jianping Deng, Yan Wang, Jianguo Xu, Changyun Ye

PII: S0003-2670(17)31181-9

DOI: 10.1016/j.aca.2017.10.022

Reference: ACA 235496

To appear in: *Analytica Chimica Acta*

Received Date: 29 July 2017

Revised Date: 11 October 2017

Accepted Date: 21 October 2017

Please cite this article as: Y. Wang, D. Liu, J. Deng, Y. Wang, J. Xu, C. Ye, Loop-mediated isothermal amplification using self-avoiding molecular recognition systems and antarctic thermal sensitive uracil-DNA-glycosylase for detection of nucleic acid with prevention of carryover contamination, *Analytica Chimica Acta* (2017), doi: 10.1016/j.aca.2017.10.022.

Title page:

Loop-mediated isothermal amplification using self-avoiding molecular recognition systems and antarctic thermal sensitive uracil-DNA-glycosylase for detection of nucleic acid with prevention of carryover contamination

Yi Wang [a], Dongxin Liu [b], Jianping Deng [c], Yan Wang [a], Jianguo Xu [a], Changyun Ye [*, a]

[a] State Key Laboratory of Infectious Disease Prevention and Control, National Institute for Communicable Disease Control and Prevention, Collaborative Innovation Center for Diagnosis and Treatment of Infectious Diseases, Chinese Center for Disease Control and Prevention, Changping, Beijing 102206, PR, China.

[b] National Institute for Communicable Disease Control and Prevention, Chinese Center for Disease Control and Prevention, Changping, Beijing 102206, PR, China.

[c] Zigong Center for Disease Control and Prevention, Zigong 643000, Sichuan Province, PR China.

* Corresponding author. Prof. Changyun Ye; E-mail: yechangyun@icdc.cn; Tel: +86 10 58900747; Fax: +86 10 58900748

ABSTRCT

Loop-mediated isothermal amplification (LAMP) is the most popular technique to amplify nucleic acid sequence without the use of temperature cycling. However, LAMP is often confounded by false-positive results, arising from interactions between (hetero-dimer) or within (self-dimerization) primers, off-target hybrids and carryover contaminants. Here, we devised a new LAMP technique that is self-avoiding molecular recognition system (SAMRS) components and antarctic thermal sensitive uracil-DNA-glycosylase (AUDG) enzyme-assisted, termed AUDG-SAMRS-LAMP. Incorporating SAMRS components into 3'-ends of LAMP primers can improve assay's specificity, which completely prevents the non-specific amplification yielding from off-target hybrids and undesired interactions between or within primers. Adding AUDG into reaction mixtures can effectively eliminate the false-positive results arising from carryover contamination, thus the genuine positive reactions are generated from the amplification of target templates. Furthermore, AUDG-SAMRS-LAMP results are confirmed using a new analysis strategy, which is developed for detecting LAMP amplicons by lateral flow biosensor (LFB). Only a single labeled primer is required in the analysis system, thus the false positive results arising from hybridization (the labeled primer and probe, or between two labeled primers) are avoided. Hence, the SAMRS components, AUDG and LFB convert traditional LAMP from a technique suited for the research laboratory into one that has practical value in the field of diagnosis. Human Tuberculosis (TB) is caused by infection with members of *Mycobacterium tuberculosis*

complex (MTC), which are detected by the AUDG-SAMRS-LAMP technique to demonstrate the availability of target analysis. The proof-of-concept method can be reconfigured to detect various nucleic acids by redesigning the specific primers.

Keywords:

Loop-mediated isothermal amplification; Biosensor; Self-avoiding molecular recognition systems; Antarctic thermal sensitive uracil-DNA-glycosylase; *Mycobacterium tuberculosis* complex

1. Introduction

Polymerase chain reaction (PCR) was the first and remains the most popular methodology for amplifying and analyzing miniscule amounts of nucleic acids. However, PCR-based techniques are restricted by the necessary laboratory equipment and are also time-consuming. In recent years, isothermal amplification of nucleic acids was invented as a promising alternative in which efficient and rapid amplification was achieved at a fixed temperature without cycling thermally the system required in PCR-based methods [1]. Among the isothermal amplification methodologies devised for specific analysis and detection of nucleic acids, one of the most widely cited isothermal assays is named loop-mediated isothermal amplification (LAMP) that operates at a constant temperature, and that displays interesting performances, in terms of stability, sensibility and amplification rates [2, 3]. Since the inception and development of LAMP in 2000, the technique has been established for widespread use, including genetically modified ingredients, pathogenic microorganisms, tumor detection and embryo sex identification [2].

In practice, LAMP has shortcomings, some of which are shared with other isothermal techniques, and others are specific to LAMP. Especially, mixtures of LAMP primers at high concentrations in the presence of triphosphates and polymerases are prone to “mischief”. This is a common disadvantage with isothermal reaction procedures, because isothermal amplification system is not forced at each cycle reform Watson-Crick pairing rules by annealing from high to low temperatures. Hence, LAMP system easily

generates artifacts, including off-target hybrids, primer-dimers and non-canonical folds (wobble mismatches or G-quartets). The consequences include poor amplification efficiency, low sensitivity, loss of signal, even as false positive and negative. These issues cannot be completely mitigated in LAMP by optimization of the reaction mixtures and/or careful design of LAMP primer set. In order to solve these problems posed by the LAMP system, we developed a new strategy by using LAMP primers that contain components of a self-avoiding molecular recognition system (SAMRS), named SAMRS-assisted LAMP (SAMRS-LAMP).

The analysis of LAMP products has become a pivotal concern because the simplifying analysis tool is a key concern. To date, various LAMP detection techniques, including fluorescent agents, colorimetric agents, turbidimeters, lab-on-a-chip devices and lateral flow biosensors (LFB), have been established [4-6]. Particularly, LFB has been applied to achieve field, 'on-site' and point-of-care diagnostics [5-7]. Currently, affordable, simple, reliable and rapid diagnostic tests are especially required by people in resource-challenged settings that do not have access to laboratory services. Thus, we devise a new strategy for the analysis of LAMP products, which integrates LAMP assay with label-based lateral flow biosensor. Furthermore, the further processing (such as reporting LAMP results by LFB) carries high risk of carryover contamination, and the effective techniques for removing the false positive results yielding from contamination are extremely significant for reliable diagnostics. Herein, we also developed a one-pot, closed-vessel enzymatic technique that can prevent carryover contamination while

preserving robust amplification in LAMP, offering accurate identification of target sequences in contaminated samples. In the current study, a novel LAMP assay (termed AUDG-SAMRS-LAMP), which merged LAMP chemistry with SAMRS components and antarctic thermal sensitive uracil-DNA-glycosylase (AUDG) digestion of carryover contaminants, was devised and combined with LFB for simple, rapid, sensitive and reliable detection of target nucleic acids.

Tuberculosis (TB) is a communicable disease caused by a group of closely related bacterial species named *Mycobacterium tuberculosis* complex (MTC), which remains globally a major health threat. Thus, efficient, rapid and reliable diagnosis of infectious TB is one of the crucial concerns in the global fight to prevent and control the infectious disease. Smear microscopy is the routine test used for diagnosis of TB in resource-poor endemic areas due to its simplicity and low cost. However, smear microscopy is a relatively insensitive test and often requires more than a single visit of a patient to be finished. Due to the high specificity and sensitivity, mycobacterial culture is still the gold standard test for detection of MTC, while it is a technically demanding and time-consuming technique, and limited to reference centers. Although the PCR-based approaches are the promising and attractive techniques for diagnosis of MTC, sophisticated technical skill and expensive laboratory apparatuses needed to perform these examinations make them inaccessible in resource-poor regions where the majority of TB cases occur. In order to achieve the simple, rapid, sensitive, specific and reliable diagnosis of TB, AUDG-SAMRS-LAMP assay developed in this study is applied to

detect MTC, based on the amplification of the insertion sequence 6110 (IS6110). Here, we provide the details on the AUDG-SAMRS-LAMP technique and evaluate its feasibility by detecting the target pathogens using pure cultures and clinical samples.

2 Experimental Section

2.1 Reagents and apparatus

Backing card, sample pad, conjugate pad, nitrocellulose membrane (NC) and absorbent pad were purchased from the Jie-Yi Biotechnology. Co., Ltd. (Shanghai, China). The sheep anti-digoxigenin antibody (anti-dig) and biotinylated bovine serum albumin (biotin-BSA) were purchased from Abcam. Co., Ltd. (Shanghai, China). Dye streptavidin coated polymer nanoparticles (Crimson red, 129 nm) were purchased from Bangs Laboratories, INC. (Indiana, USA). *Bst* 2.0 DNA polymerase, dATP，dTTP, dCTP, dGTP and antarctic thermal sensitive uracil-DNA-glycosylase (AUDG) were obtained from New England Biolabs, INC. (Beijing, China). dUTP are obtained from Kuimo. Co., Ltd. (Beijing, China). Biotin-14-dCTP was obtained from Thermo Scientific. Co., Ltd. (Shanghai, China). Visual detection reagent (Hydroxynaphthol blue, HNB) was purchased from Bei-Jing HaiTaiZhengYuan. Co., Ltd. (Beijing, China). DNA extraction kits (QIAamp DNA minikits; Qiagen, Hilden, Germany) were obtained from Qiagen (Beijing, China).

2.2 Bacterial strains and genomic DNA extraction

Bacterial strains used in this study, including 32 non-mycobacterial species and 21 mycobacterial species, were shown in **Table 1**. DNA templates from the mycobacterial strains was extracted following the method of Tomotada Iwamoto and coworkers [8]. DNA concentrations were quantified using ultraviolet spectrophotometer (NanoDrop ND-1000, Calibre, Beijing, China) at A260/280. DNA templates from non-mycobacterial species strains were prepared using DNA extraction kits (QIAamp DNA minikits, Hilden, Germany) according to the manufacturer's instructions. The extracted genomic DNAs were stored under at -20°C until use.

2.3 Preparation of the lateral flow biosensor

As shown in **Figure 1**, lateral flow biosensor consists of four components assembled together on a plastic adhesive backing card: an immersion pad, a conjugate pad, a NC membrane and an absorbent pad [5]. The anti-dig (0.25 mg mL^{-1}) and biotin-BSA (4 mg mL^{-1}) in 0.01M phosphate-buffered saline (PBS, pH 7.4) were sprayed onto the NC membranes to form test line (T line) and control line (C line) at an interval of 5 mm. The detector reagents (dye streptavidin coated polymer nanoparticles, SA-DNPs) in 0.01M PBS (pH 7.4) were sprayed onto the conjugate area of LFB. After the LFB were dried at room temperature for 2 h, they were cut to 4 mm×60 mm with a strip cutter (CM4000) and stored in a desiccator.

2.4 Visual analysis of amplicons using the biosensor

A volume of 0.5 μL of reaction mixtures was added to the sample application zone of biosensor. Then, a 80 μL aliquot of running buffer (10 mM PBS, pH 7.4 with 1% Tween 20) was deposited on the sample application zone of biosensor, and the biosensor allowed to absorbing the whole running buffer. After 1 minute, the detection results were indicated by the presence of crimson red lines on the NC membrane.

2.5 Design of LAMP assay primers

Based on the insertion sequence 6110 (IS6110; Genbank no. X17348), a set of LAMP primers is designed by PrimerExplorer V4 (Eiken Chemical, Japan). IS6110 is specific for the members of MTC, which are obligate organisms with more than 99% genetic homology [9]. The details (sequences and modifications) are shown in **Table 2**.

2.6 LAMP, SAMRS-LAMP and AUDG-SAMRS-LAMP reactions

LAMP is performed in a one-step reaction in a 25-μL mixture containing 0.4 μM each of F3 and B3, 0.8 μM each of LF and LB, 1.6 μM each of FIP and BIP, 2.5 μL 10 × of the supplied LAMP buffer (New England Biolabs, Ipswich, MA, USA, Cat. #M0537L), 0.8 M betaine (Sigma-Aldrich, St. Louis, MO, USA), 1.4 mM dTTP, 1.3 mM dCTP, 0.1 mM biotin-14-dCTP, 1.4 mM dATP, 1.4 mM dGTP, 1.25 μL (10 U) of *Bst* 2.0 DNA polymerase (New England Biolabs, Ipswich, MA, USA, Cat. #M0537L) and 1μL DNA template.

SAMRS-LAMP is performed in a one-step reaction in a 25-μL mixture containing

0.4 μM each of SAMRS-F3 and SAMRS-B3, 0.8 μM each of SAMRS-LF and SAMRS-LB, 1.6 μM each of SAMRS-FIP and SAMRS-BIP, 2.5 μL 10 × of the supplied LAMP buffer (New England Biolabs, Ipswich, MA, USA, Cat. #M0537L), 0.8 M betaine (Sigma-Aldrich, St. Louis, MO, USA), 1.4 mM dTTP, 1.3 mM dCTP, 0.1 mM biotin-14-dCTP, 1.4 mM dATP, 1.4 mM dGTP, 1.25 μL (10 U) of *Bst* 2.0 DNA polymerase (New England Biolabs, Ipswich, MA, USA, Cat. #M0537L) and 1μL DNA template.

AUDG-SAMRS-LAMP is performed in a one-step reaction in a 25-μL mixture containing 0.4 μM each of SAMRS-F3 and SAMRS-B3, 0.8 μM each of SAMRS-LF and SAMRS-LB, 1.6 μM each of SAMRS-FIP and SAMRS-BIP, 2.5 μL 10 × of the supplied LAMP buffer (New England Biolabs, Ipswich, MA, USA, Cat. #M0537L), 0.8 M betaine (Sigma-Aldrich, St. Louis, MO, USA), 0.7 mM dTTP, 0.7 mM dUTP, 1.3 mM dCTP, 0.1 mM biotin-14-dCTP, 1.4 mM dATP, 1.4 mM dGTP, 1.25 μL (10 U) of *Bst* 2.0 DNA polymerase (New England Biolabs, Ipswich, MA, USA, Cat. #M0537L), 0.2 μL (0.2 U) of AUDG and 1μL DNA template.

Reaction mixtures with 1 μL of templates of *M. kansasii* (ATCC 12478), *S. aureus* (Isolated strain) and *S. pneumonia* (Isolated strain) were used as negative controls, and reaction mixtures with 1 μL of double distilled water (DW) were used as a blank control. A total of four monitoring techniques, including colorimetric indicator (HNB), real time turbidity (turbidimeters, LA-320C), gel electrophoresis and lateral flow biosensor detection, are selected for confirming the amplification of LAMP-based assays.

2.7 Optimization of the temperature for SAMRS-LAMP assay

We examine the optimal amplification temperature of SAMRS-LAMP, which are conducted at a constant temperature ranging from 58°C to 65°C for 1.5 h, and then heated at 85°C for 5 minutes to stop the SAMRS-LAMP reaction.

2.8 Sensitivity of SAMRS-LAMP assay

In order to evaluate the sensitivity of SAMRS-LAMP assay, DNA templates extracted from *M. tuberculosis* H37Rv are used to make serial dilutions (100 ng μL^{-1}~2×10^{7} copies μL^{-1}, 100 pg μL^{-1}~2×10^{4} copies μL^{-1}, 10 pg μL^{-1}~2×10^{3} copies μL^{-1}, 1 pg μL^{-1}~2×10^{2} copies μL^{-1}, 100 fg μL^{-1}~2×10^{1} copies μL^{-1}, 10 fg μL^{-1}~2×10^{0} copies μL^{-1} and 1 fg μL^{-1}~2×10^{-1} copies μL^{-1}). Using the molecular weight per genome as determined from the whole genome sequence of the H37Rv strain (a molecular size of 4.4 Mbp for H37Rv), we calculate that 5 fg of H37Rv DNA correlates to an estimated genome equivalent of one cell [8, 9]. A volume of 1μL of each dilution is used as the template for SAMRS-LAMP amplification, and three tests were performed at each concentration of genomic templates.

2.9 Simulating carryover contamination

In the current study, SAMRS-LAMP products generated form 2×10^{6} copies μL^{-1} in the absence of AUDG is quantitated using ultraviolet spectrophotometer (NanoDrop

ND-1000, Calibre, Beijing, China) to make a 10-fold serial dilution (1×10^{-13} μL^{-1}, 1×10^{-14} μL^{-1}, 1×10^{-15} μL^{-1}, 1×10^{-16} μL^{-1}, 1×10^{-17} μL^{-1}, 1×10^{-18} μL^{-1} and 1×10^{-19} g μL^{-1}), which are used as the source of simulating carryover contaminants. A volume of 1 μL of diluted SAMRS-LAMP products served as the template DNA.

2.10 Elimination of carryover contamination by AUDG-SAMRS-LAMP

In order to assess the ability of the established AUDG-SAMRS-LAMP assay to eliminate false-positive results due to carryover contaminants in detecting MTC, we carried out AUDG-SAMRS-LAMP reactions by adding a volume of 1 μL of diluted DNA templates (100 ng μL^{-1}~2×10^{7} copies μL^{-1}, 100 pg μL^{-1}~2×10^{4} copies μL^{-1}, 10 pg μL^{-1}~2×10^{3} copies μL^{-1}, 1 pg μL^{-1}~2×10^{2} copies μL^{-1}, 100 fg μL^{-1}~2×10^{1} copies μL^{-1}, 10 fg μL^{-1}~2×10^{0} copies μL^{-1} and 1 fg μL^{-1}~2×10^{-1} copies μL^{-1}) and 1 μL of simulated carryover contamination of 1×10^{-18} g μL^{-1} (approximately equivalent to a 0.2 μm-diameter aerosol droplet) in the same reaction tube. Total mass (1×10^{-18} g) of the simulated carryover contamination for each reaction was not efficiently prevented by either fibrous pipette tip filters or high efficiency particulate air filters in the biosafety cabinets [10, 11]. Limit of detection (LoD) of SAMRS-LAMP with and without AUDG treatment before LAMP reaction is compared to confirm whether the established AUDG-SAMRS-LAMP technique can efficiently remove false-positive results.

2.11 Specificity of AUDG-SAMRS-LAMP assay

In order to evaluate the specificity of AUDG-SAMRS-LAMP assay, samples of genomic DNA (at least 10 ng of genomic templates) from a panel of 71 mycobacterial strains and 32 non-mycobacterial species were tested (**Table 1**).

2.12 AUDG-SAMRS-LAMP assay detects MTC in clinical samples

An evaluation of AUDG-SAMRS-LAMP assay was conducted using 47 sputum specimens derived from suspected cases of pulmonary TB. The sputum specimens were detected by smear microscopy for the presence or absence of acid-fast bacilli and by conventional culture for growth of MTC. Genomic DNAs were extracted from clinical samples using boiling method according to the description from Afghani and coworkers, and a 5 μL aliquots of templates was used in the AUDG-SAMRS-LAMP assay [12]. Moreover, one negative control is included for each two clinical sample reactions for controlling the possible cross-contamination, and the results from AUDG-SAMRS-LAMP assay were compared with smear microscopy test and conventional culture method.

3. Results and Discussion

3.1 Establishment of LAMP-LFB assay

Schematic outline of the principle of LAMP-LFB assay is displayed in **Figure 1A**. In the LAMP-LFB system, only a single primer (F3, B3, FIP, BIP, LF and LB) involved in LAMP reaction is modified at the 5’ end with hapten, and biotin-14-dCTP is added into

the amplification mixture. In this report, the loop forward primer (LF) is used as the model primer to demonstrate the availability of LAMP-LFB strategy, and a digoxigenin (Dig) tag is modified at the 5' end of the LF primer, termed as LF*. During the isothermal reaction stage, forward inner primer (FIP) anneals to the target templates and is extended by *Bst* 2.0 polymerase (**Figure 1A**, step 1). The newly synthesized strand derived from FIP is displaced by the upstream synthesis from the forward primer F3, then, three primers (LF*, backward inner primer BIP, backward primer B3) anneal to the strand generated from FIP (**Figure 1A**, step 2). *Bst* 2.0 polymerase extends in tandem producing two different strands (**Figure 1A**, step 3). LF* product serves as the templates for subsequent extension by FIP primer (**Figure 1A**, step 4), and biotin-14-dCTP is successfully incorporated into product. As a result, a double-labeled detectable product, which is biotin and Dig labeled, is successfully formed by using biotin-14-dCTP and Dig labeled primer (LF*). Details of the amplification process for BIP product have been descripted in previous study [4].

The structure of lateral flow biosensor (LFB) and the principle of LFB visualization of LAMP amplicons are exhibited in **Figure 1B**. LAMP reaction mixture (0.5 μL) is loaded on the sample pad of the biosensor, and a 80 μL aliquots of running buffer is also loaded on the sample pad. Running buffer rehydrates and releases the visual reagents (dye streptavidin coated polymer nanoparticles, SA-DNPs) from the conjugate pad to reaction area by capillary migration. At the detection zone (NC membrane), the double-labeled detectable amplicons (biotin and Dig labeled products) are specially captured by anti-Dig

antibody, which is immobilized at test line (TL) and recognizes Dig tag at the end of LAMP amplicons yielding from Dig labeled primer (LF* primer). Biotins, which can bind SA-DNPs (crimson, 129 nm) through biotin/streptavidin interaction for visualization, are successfully incorporated into LAMP amplicons by using biotin-14-dCTP. Thus, the detection reagents (SA-DNPs) accumulate in the test line zone of biosensor, resulting a visual crimson red line at the TL, which provide a response to the presence/absence of the target amplicons. The excess detection reagents (SA-DNPs) can bind biotin-BSA conjugates, which are immobilized at the control line (CL), generating another crimson red line. In all examination results, the CL must appear on in the reaction area zone (NC member), which demonstrates that the biosensor is working well. The appearance of two crimson red bands at both control and test lines is considered as a positive result, and the appearance of only one band at the control line is regarded as a negative result (**Figure 1C**).

Lateral flow biosensor is characterized by its simple use, low cost, rapid result and long shelf life, and has been widely used to detect LAMP amplicons [13, 14]. In these detection systems, two primers involved in LAMP reaction were labeled, one with hapten and one with biotin. LAMP amplicons were labeled simultaneously with hapten and biotin during the amplification stage [5, 6, 15-18]. However, a double-labeled detectable product can be form by hybridization from two labeled primers without amplification, which can generate a false-positive result. In the current study, we established a strategy to form a detectable LAMP amplicon, and only one primer involved in LAMP

amplification was modified with hapten at the 5’ end. During the amplification stage, the biotin-14-dCTP is incorporated into the target amplicons when the labeled primer (LF* primer) extends, thus the LAMP amplicons were labeled simultaneously with hapten and biotin (**Figure 1A**). Due to use only a labeled primer in LAMP system, the false positive result yielding from the hybridization of two labeled primers was efficiently prevented. Moreover, the lateral flow-based nucleic acid biosensor presented in this study can directly analyze of labeled amplicons without any post-hybridization procedure. Comparing with these monitoring techniques (gel electrophoresis, colorimetric indicator reagents, real time turbidity and fluorescence) developed for detection of LAMP product, LFB is sometimes easy to perform, disposable and provide a quick (test done within minutes) and visual result. Therefore, LFB is more suitable than other detection techniques for application in field, point-of-care and poor-resource settings.

3.2 Confirmation of LAMP and SAMRS-LAMP products

By colorimetric indicator reagent (HNB), the color shift of positive results in LAMP and SAMRS-LAMP tubes from violet to blue sky can be directly observed with naked eyes within 60-minute incubation period (**Figure 2A**, **top row**; and **2B**, **top row**). The LAMP and SAMRS-LAMP products were analyzed by agarose gel electrophoresis, and the typical ladder-like pattern could be seen in positive amplifications, but not in the negative and blank controls (**Figure 2A**, **middle row**; and **2B**, **middle row**). The LAMP and SAMRS-LAMP products also were detected by LFB, and two crimson red lines (CL and

TL) were visible in positive results, but only the CL was seen in negative and blank controls (**Figure 2A**, **bottom row**; and **2B**, **bottom row**). These results indicated that the primer sets targeting the IS6110 sequence for MTC detection is good candidate for development of LAMP and SAMRS-LAMP methods.

3.3 Performance of LAMP and SAMRS-LAMP using different strains and double distilled water

Sixty-four LAMP reactions, including one positive control (PC), thirty-one blank controls (BC) and thirty-two negative controls (NC), were performed at a constant temperature (63°C) for 1h. A total of five false positive results were obtained from LAMP reactions (**Figure 3**, Signals 14, 21, 33, 44 and 56). Two false positive results (Signals 14 and 21), termed self-amplification, were produced from bland controls, in which no template was added. Another three undesired amplifications (Signals 33, 44 and 56), named non-specific amplification, were yielded from negative controls, in which non-MTC templates were added. These results indicated that LAMP method had the shortcomings (self-amplification and non-specific amplification), which have been reported in previous study [19]. Under normal conditions, LAMP reaction did not generated the non-specific amplification within 30 minutes. However, the LAMP amplification of low-abundance nucleic acid (present at less than 10 copies per reaction) requires more than 30 minutes for producing positive signals that were extremely similar with those obtained in the non-specific reactions. Hence, there is the possibility that a

sample may be somewhat ambiguous to the researcher when the minute amounts of genomic template are added into LAMP reaction mixtures. In order to overcome these barriers, the detection methods, including melting curves of products and agarose gel electrophoresis, were applied to differentiate specific amplification from undesired amplification, while these strategies did not eliminate the use of complicated equipment and additional post-procedure [19].

3.4 Performance of SAMRS-LAMP using different strains and double distilled water

In the SAMRS-LAMP system, natural T, C, A and G nucleobases of LAMP primers are replaced at some sites with T* (2-thiotymine), C* (N^4-ethylcytosine), A* (2-aminopurine) and G* (hypoxanthine), respectively (**Table 2**) [20]. Natural A pairs with T*, T pairs with A*, C pairs with G* and G pair with C*, but A*:T* and C*:G* base pairs do not form the stability of the helix due to they either contribute one or 1.5 bonds [21-23]. The pairing principles mean that the SAMRS molecules (nucleotide analogues T*, C*, A*, and G*) do not interact with other SAMRS molecules (even when they exhibit 100% complementarity), and SAMRS oligonucleotides bind solely to their intended targets (natural complements). LAMP primers containing SAMRS components can effectively prevent the false positive results, which are obtained from self-amplification (amplification yielded from interactions between or within LAMP primers) and non-specific amplification (amplification yielded from interactions between LAMP primers and non-target sequences). As shown in **Figure 4**, with the LAMP primers

containing SAMRS components, only a positive result was seen in positive control. No false positive results were generated form negative controls and blank controls even when the SAMRS-LAMP reaction lasted for 90 minutes. These results indicated that the SAMRS-LAMP assay can successfully removed the false positive results. Therefore, SAMRS-mediated strategy can completely eliminate the undesired LAMP amplification (**Figure 4**), while others strategies (melting curves of products and agarose gel electrophoresis) are employed for only distinguishing specific LAMP reactions from non-specific LAMP reactions [19].

3.5 Optimal temperature of the SAMRS-LAMP assay

During SAMRS-LAMP optimization, 10 pg of DNA extracted from *M. tuberculosis* H37Rv was used as a template to optimize assay temperature. SAMRS-LAMP reactions were performed at eight different temperatures (58°C-65°C, with 1°C interval) for 90 minutes. Although eight reaction temperatures yielded the robust signals, the faster amplification were obtained when the temperature varied between 59°C-62°C, and 60°C was optimal (**Figure 5**). Thus, the optimal temperature (60°C) was used for the rest of SAMRS-LAMP reactions conducted in this study.

3.6 Sensitivity of SAMRS-LAMP in pure culture

The analytical sensitivity of SAMRS-LAMP assay was evaluated using serially diluted templates extracted from pure culture of *M. tuberculosis* H37Rv. After 90-minute

reaction, SAMRS-LAMP assay revealed a detection limit of 100 fg of template DNA, and the results obtained using real time turbidity (**Figure 6A**, **top row**) and were in conformity with LFB analysis (**Figure 6A**, **bottom row**).

In this report, the analytical sensitivity of the SAMRS-LAMP assay was compared with these of LAMP detected products by real time turbidity (**Figure 6B**, **top row**) and LFB (**Figure 6B**, **bottom row**). After 60-minute amplification, LAMP assay revealed a detection limit of 100 fg of template DNA, which was in complete accordance with that seen in SAMRS-LAMP assay. Comparing with traditional LAMP technique, additional 30 minutes was required by SAMRS-LAMP technique for efficiently amplifying the same levels of genomic templates (**Figure 6A**, **top row**; 6B, **top row**). Thus, a reaction time of 90 minutes was recommended as the optimal time for the SAMRS-LAMP assay during the reaction stage, because a reaction time of 60 minutes was use as the optimal time for the traditional LAMP. The whole procedure (sample processing, 25 minutes; isothermal amplification, 90; result indicating, 2 minutes) could be completed within 2 h. In particular, SAMRS-LAMP can effectively prevent the false-positive results yielding from negative controls and blank controls, thus provides the reliable results for detecting target nucleic acids (**Figure 3**, **4**).

3.7 AUDG-SAMRS-LAMP assay

In this study, we also devised the integration of SAMRS-LAMP amplification with AUDG digestion in a one-pot, closed-tube reaction to remove false-positive results

yielding from carryover contaminants. In order to effectively prevent the carryover contamination, the antarctic thermal sensitive uracil-DNA-glycosylase-supplemented SAMRS-LAMP (AUDG-SAMRS-LAMP) method requires two additional components (dUTP and AUDG) relative to standard SAMRS-LAMP assay. In the first stage, dUTP is added into all SAMRS-LAMP amplification mixtures, thus uracil is successfully incorporated into all SAMRS-LAMP amplicons by *Bst* 2.0 DNA polymerase (**Figure 7**, stage 1). In the second stage, AUDG digestion of carryover contaminants and SAMRS-LAMP amplification are carried out in a one-pot process (**Figure 7**, stage 2). Prior to SAMRS-LAMP amplification, the reaction mixture is treated using a heat-labile enzyme (AUDG) at room temperature for only 5 minutes. AUDG enzyme specifically cleaves uracil based from any uracil-containing double-stranded or single-stranded SAMRS-LAMP amplicons, leaving behind abasic sites. At elevated temperature, the resulting abasic sites are susceptible to hydrolytic cleavage. However, the natural DNA templates, which are uracil-free target sequences, are not digested by AUDG enzyme and remain completely unaffected. During the SAMRS-LAMP amplification stage (60°C), these abasic sites cause rapid degradation of the contaminating products via hydrolysis at the phosphate backbone, thus block replication by *Bst* 2.0 DNA polymerase. As a result, the carryover contaminants generating from previous SAMRS-LAMP reactions are effectively eliminated, thus they cannot be used as the templates for re-amplification. Furthermore, to form the hapten-biotin doubled-labeled SAMRS-LAMP products and enable them analysis on the lateral flow biosensors, two components, including

biotin-14-dCTP and hapten-labeled primer, also are added into SAMRS-LAMP amplification mixtures (**Figure 7**).

In the AUDG-SAMRS-LAMP system, AUDG has the ability to catalyze the release of free uracil from uracil-containing DNA at relatively low temperature (i.e., room temperature), thus is selected for development of our AUDG-SAMRS-LAMP assay [24]. Importantly, the heat-labile AUDG enzyme is automatically, rapidly and completely deactivated at an elevated temperature (i.e., above 50°C). The use of AUDG enzyme enables the AUDG-SAMRS-LAMP assay to be conducted in a single closed tube, because AUDG enzyme is deactivated when the AUDG-SAMRS-LAMP reaction is carried out at assay temperature (i.e., 60°C). As a result, genuine products subsequently yielded from the target sequences during AUDG-SAMRS-LAMP reaction do not be cleaved, permitting amplification to proceed normally (**Figure 7**). The AUDG-SAMRS-LAMP amplicons can be detected using various monitoring methods (such as colorimetric indicator, gel electrophoresis, real time turbidity and LFB) when the AUDG-SAMRS-LAMP reaction is finished. Hence, the use of dUTP and AUDG enable carryover contamination from previous reactions to be effectively removed, and the use of hapten-labeled and biotin-14-dCTP enables the AUDG-SAMRS-LAMP assay to couple with lateral flow biosensor.

In order to remove the false-positive results arising from carryover contamination, some groups have tried to use special enzymatic cleavage of contaminant products from previous reaction [25-27]. However, these techniques devised for prevention of carryover

contamination require enzymatic digestion and amplification reaction to be carried out in separate steps, which necessitates opening the reaction tubes and exposing the reaction tubes to carryover contaminants. The amplification methodologies (such PCR- and LAMP-based assays) has been revealed to be highly specific and sensitive techniques in the various fields, while they were also found to be highly susceptible to contaminants due to their outstanding sensitivity. Opening of the reaction tubes can easily produce aerosol droplets of distinct sizes that contain high concentrations of amplification products, thus cause the false-positive results due to reintroduction of the contaminants from previous reactions and environment [28]. Therefore, the strategies presented by previous reports may not be effective because they are not the one-pot reactions (enzymatic digestion and amplification reaction are not conducted in a closed vessel). In our study, AUDG-SAMRS-LAMP is a one-pot and closed-tube reaction for specific identification of target nucleic acid, which can effectively prevent undesired results generating from carryover contaminants.

3.8 AUDG-SAMRS-LAMP and SAMRS-LAMP detect simulated carryover contamination

To verify that carryover contamination is sufficient to contaminate SAMRS-LAMP reactions, the dUTP-incorporated amplicons generating from an AUDG-free SAMRS-LAMP reaction were used as templates to perform AUDG-SAMRS-LAMP and SAMRS-LAMP reactions. The analytical sensitivity of SAMRS-LAMP and

AUDG-SAMRS-LAMP assay are compared using serial diluted dUTP-incorporated amplicons with concentrations ranging from 1×10^{-13}, 1×10^{-14}, 1×10^{-15}, 1×10^{-16}, 1×10^{-17}, 1×10^{-18} and 1×10^{-19} g μL^{-1}. In samples without AUDG (SAMRS-LAMP assay), the method amplifies as little as 1×10^{-18} g (equivalent to a 0.2 μm-diameter aerosol droplet) of simulated carryover contaminant per vessel (**Figure 8B**). The result indicates that a source contaminant (a 0.2 μm-diameter aerosol droplet ~1×10^{-18} g of contaminants), which is not be efficiently blocked by fibrous pipette tip filters, is sufficient to cause false-positive results in SAMRS-LAMP assay [29, 30]. These results clearly verify the danger of marginal quantities of carryover contamination in SAMRS-LAMP reactions, where even extremely low concentrations of contaminants can contaminate new SARMS-LAMP reactions. In contrast, AUDG-SAMRS-LAMP assay (in samples with AUDG enzyme) only amplifies 1×10^{-14} g of simulated carryover contaminant per vessel. Results confirm that the AUDG enzyme is capable of cleaving carryover contaminants with extremely high efficiency. Hence, AUDG is able to prevent amplification of up to 1000-fold higher concentrations of carryover contaminant templates, which significantly decrease the likelihood of false-positive results of SAMRS-LAMP analysis.

3.9 AUDG-SAMRS-LAMP prevents false-positive results

To further verify that AUDG-SAMRS-LAMP has the ability to efficiently reduce the likelihood of false-positive amplifications yielding from contaminants, we examine the sensitivity of AUDG-SAMRS-LAMP and SAMRS-LAMP with serial dilution of the *M.*

tuberculosis H37Rv DNA, and the aliquots of 1×10^{-18} g of contaminants was added into each reaction tube. In the presence of AUDG treatment of AUDG-SAMRS-LAMP assay, the sensitivity was consistent with the aforementioned sensitivity evaluation (**Figure 6** and **Figure 9A**). As shown in **Figure 9B**, all tested samples produce positive results in the absence of AUDG treatment of SAMRS-LAMP assay. In particular, the positive amplifications, which were yielded from these samples with undetectable levels of DNA (less than 10 fg μL^{-1}~2×10^{0} copies μL^{-1}), were regarded as false-positive results. Herein, we could not obtain the genuine sensitivity of the SAMRS-LAMP assay without AUDG treatment. Herein, the AUDG-SAMRS-LAMP approach established here could effectively eliminate false-positive amplifications yielding from carryover contamination.

Similar to conventional LAMP assay, the SAMRS-LAMP results can be confirmed by gel electrophoresis, LFB or other monitoring techniques. In these processes, the opening of amplification tube is needed, thus the high concentrations of products easily enter into environment in form of aerosol droplets [27]. Consequently, false-positive results are obtained when amplified DNA products from previous SAMRS-LAMP reactions occur in new SAMRS-LAMP reaction mixtures and become templates for re-amplification. Our report confirmed that marginal amounts of amplicons (1×10^{-18} g of carryover contaminants) could serve as templates to produce false-positive results (**Figure 8**). Usually, eliminating carryover contamination only depends on careful operation, because there is currently no effective strategy for preventing LAMP carryover

contamination. Previous publications have developed some technologies for removing carryover contamination in nucleic acid amplification assays, especially for PCR-based methods, and they majorly included pre- and post-amplification strategies (primer hydrolysis, UV light irradiation and chemical/mechanical barrier) [25, 27, 31-33]. However, these techniques may not have the ability to completely eliminate carryover contamination, and are costly, time-consuming and extremely complicated [34, 35]. In our study, we devised the AUDG-SAMRS-LAMP assay, which is able to digest dUTP-incorporated contaminants and is extremely simple and effective, alleviating the use of substantial modification of existing protocols.

3.10 Specificity of the AUDG-SAMRS-LAMP assay

Among the mycobacteria tested by the AUDG-SAMRS-LAMP assay, forty-seven species were unrelated to MTC and four species were members of the MTC (**Table 1**). After a 90-minute reaction at 60°C, all positive results were yielded only with the chromosomal DNA templates extracted from mycobacteria, which were the members of MTC. Thus, the specificity of AUDG-SAMRS-LAMP assay was of 100% (**Table 1**). Moreover, no cross-reactions to non-MTC and non-mycobacteria were found according to the specificity analysis. These results indicated that the AUDG-SAMRS-LAMP assay displayed high selectivity for detection of MTC.

3.11 Applicability of AUDG-SAMRS-LAMP assay to clinical samples

A total of 47 clinical sputum double-blind samples using liquid culture and AFB smear test results were detected by National Tuberculosis Reference Laboratory, Chinese Center for Disease Control and Prevention. After AUDG-SAMRS-LAMP reaction, we confirmed the results by LFB, and these results were compared with those obtained from conventional culture and AFB smear test. AUDG-SAMRS-LAMP assay was able to correctly diagnose MTC in all 31 sputum specimens of the TB patients. Comparing with traditional culture technique, the AUDG-SAMRS-LAMP detection accuracy results obtained is 100%. However, 3 sputum samples that were regarded as negative results using the AFB smear test had a low sputum score. Therefore, MTC in sputum samples with a low bacillary load was more difficult to detect by AFB smear test, while it could be detected by traditional culture assay and AUDG-SAMRS-LAMP technique. These preliminary results indicated that AUDG-SAMRS-LAMP assay developed here used for detection of MTC in clinical sputum samples. In order to further verify the feasibility of the AUDG-SAMRS-LFB method, a large number of sputum specimens and different kinds of clinical samples should be evaluated.

4. Conclusion

In sum, we successfully devised a new LAMP assay that is SAMRS components and AUDG enzyme-assisted, named AUDG-SAMRS-LAMP. LAMP primers containing SAMRS components can completely eliminate the false positive amplifications arising from off-target hybrids and undesired interactions between (hetero-dimer) or within

(self-dimerization) primers. Then, we integrated SAMRS-LAMP with AUDG cleavage in a one-pot, closed-tube reaction to great avoid false-positives from carryover contaminants. Furthermore, a new analysis strategy was developed and intended for the analysis of amplification products using LFB. Due to only require a single labeled primer, the analysis system removed the false-positive results arising from hybridization (the labeled primer and probe, or between two labeled primers). Herein, the SAMRS components, AUDG and LFB convert traditional LAMP from a technique suited for the research laboratory into one that has practical value in the field of diagnosis. As a demonstration, we used AUDG-SAMRS-LAMP to detect genomic DNA from these pathogens (MTC), which are the causes of human TB. The performance of AUDG-SAMRS-LAMP method in detecting MTC from pure culture and clinical samples is successfully demonstrated. The proof-of-concept approaches can be reconfigured to detect various nucleic acids by redesigning the specific primers.

Acknowledgements

We acknowledge the financial supports of the grants (Mega Project of Research on the Prevention and Control of HIV/AIDS, Viral Hepatitis Infectious Diseases 2013ZX10004-101 to Changyun Ye) from the Ministry of Science and Technology, People's Republic of China, and grant (2015SKLID507 to Changyun Ye) from State Key Laboratory of Infectious Disease Prevention and Control, China CDC.

References

[1] Y. Wang, Y. Wang, A.-J. Ma, D.-X. Li, L.-J. Luo, D.-X. Liu, D. Jin, K. Liu, C.-Y. Ye, Rapid and sensitive isothermal detection of nucleic-acid sequence by multiple cross displacement amplification, Scientific reports, 5 (2015).

[2] Y. Zhao, F. Chen, Q. Li, L. Wang, C. Fan, Isothermal amplification of nucleic acids, Chem. Rev, 115 (2015) 12491-12545.

[3] H. Deng, Z. Gao, Bioanalytical applications of isothermal nucleic acid amplification techniques, Analytica chimica acta, 853 (2015) 30-45.

[4] Y. Wang, Y. Wang, R. Lan, H. Xu, A. Ma, D. Li, H. Dai, X. Yuan, J. Xu, C. Ye, Multiple endonuclease restriction real-time loop-mediated isothermal amplification: a novel analytically rapid, sensitive, multiplex loop-mediated isothermal amplification detection technique, The Journal of Molecular Diagnostics, 17 (2015) 392-401.

[5] Y. Wang, H. Li, Y. Wang, L. Zhang, J. Xu, C. Ye, Loop-Mediated Isothermal Amplification Label-Based Gold Nanoparticles Lateral Flow Biosensor for Detection of Enterococcus faecalis and Staphylococcus aureus, Frontiers in microbiology, 8 (2017).

[6] A.N. Najian, E.E.N. Syafirah, N. Ismail, M. Mohamed, C.Y. Yean, Development of multiplex loop mediated isothermal amplification (m-LAMP) label-based gold nanoparticles lateral flow dipstick biosensor for detection of pathogenic Leptospira, Analytica chimica acta, 903 (2016) 142-148.

[7] J.H. Jung, S.J. Oh, Y.T. Kim, S.Y. Kim, W.-J. Kim, J. Jung, T.S. Seo, Combination of multiplex reverse-transcription loop-mediated isothermal amplification with an immunochromatographic strip for subtyping influenza A virus, Analytica chimica acta, 853 (2015) 541-547.

[8] T. Iwamoto, T. Sonobe, K. Hayashi, Loop-mediated isothermal amplification for direct detection of

Mycobacterium tuberculosis complex, M. avium, and M. intracellulare in sputum samples, Journal of clinical microbiology, 41 (2003) 2616-2622.

[9] E. Aryan, M. Makvandi, A. Farajzadeh, K. Huygen, P. Bifani, S.-L. Mousavi, A. Fateh, A. Jelodar, M.-M. Gouya, M. Romano, A novel and more sensitive loop-mediated isothermal amplification assay targeting IS6110 for detection of Mycobacterium tuberculosis complex, Microbiological research, 165 (2010) 211-220.

[10] E. Le Rouzic, Contamination-pipetting: relative efficiency of filter tips compared to Microman® positive displacement pipette, Nature Methods| Application Notes, (2006).

[11] R.S. Barhate, S. Ramakrishna, Nanofibrous filtering media: filtration problems and solutions from tiny materials, Journal of membrane science, 296 (2007) 1-8.

[12] B. Afghani, H.R. Stutman, Polymerase Chain Reaction for Diagnosis ofM. tuberculosis: Comparison of Simple Boiling and a Conventional Method for DNA Extraction, Biochemical and molecular medicine, 57 (1996) 14-18.

[13] D. Quesada-González, A. Merkoçi, Nanoparticle-based lateral flow biosensors, Biosensors and Bioelectronics, 73 (2015) 47-63.

[14] P.C. Foo, Y.Y. Chan, M. Mohamed, W.K. Wong, A.B. Nurul Najian, B.H. Lim, 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, Analytica Chimica Acta, 966 (2017) 71-80.

[15] A. Chua, C.Y. Yean, M. Ravichandran, B. Lim, P. Lalitha, A rapid DNA biosensor for the molecular diagnosis of infectious disease, Biosensors and Bioelectronics, 26 (2011) 3825-3831.

[16] L. Cui, Y. Ge, X. Qi, G. Xu, H. Li, K. Zhao, B. Wu, Z. Shi, X. Guo, L. Hu, Q. You, L.H. Zhang, A.N. Freiberg, X. Yu, H. Wang, M. Zhou, Y.W. Tang, Detection of severe fever with thrombocytopenia

syndrome virus by reverse transcription-cross-priming amplification coupled with vertical flow visualization, J Clin Microbiol, 50 (2012) 3881-3885.

[17] Y. Wang, H. Li, Y. Wang, H. Li, L. Luo, J. Xu, C. Ye, Development of multiple cross displacement amplification label-based gold nanoparticles lateral flow biosensor for detection of Listeria monocytogenes, Int J Nanomedicine, 12 (2017) 473-486.

[18] Y. Wang, Y. Wang, L. Zhang, J. Xu, C. Ye, Visual and multiplex detection of nucleic acid sequence by multiple cross displacement amplification coupled with gold nanoparticle-based lateral flow biosensor, Sensors and Actuators B: Chemical, 241 (2017) 1283-1293.

[19] N. Uemura, K. Makimura, M. Onozaki, Y. Otsuka, Y. Shibuya, H. Yazaki, Y. Kikuchi, S. Abe, S. Kudoh, Development of a loop-mediated isothermal amplification method for diagnosing Pneumocystis pneumonia, J Med Microbiol, 57 (2008) 50-57.

[20] N. Sharma, S. Hoshika, D. Hutter, K.M. Bradley, S.A. Benner, Recombinase-based isothermal amplification of nucleic acids with self-avoiding molecular recognition systems (SAMRS), Chembiochem, 15 (2014) 2268-2274.

[21] V.B. Pinheiro, D. Loakes, P. Holliger, Synthetic polymers and their potential as genetic materials, Bioessays, 35 (2013) 113-122.

[22] S.A. Benner, N.B. Karalkar, S. Hoshika, R. Laos, R.W. Shaw, M. Matsuura, D. Fajardo, P. Moussatche, Alternative Watson-Crick Synthetic Genetic Systems, Cold Spring Harb Perspect Biol, 8 (2016).

[23] S. Hoshika, F. Chen, N.A. Leal, S.A. Benner, Self-Avoiding Molecular Recognition Systems (SAMRS), Nucleic Acids Symp Ser (Oxf), (2008) 129-130.

[24] M.C. Longo, M.S. Berninger, J.L. Hartley, Use of uracil DNA glycosylase to control carry-over

contamination in polymerase chain reactions, Gene, 93 (1990) 125-128.

[25] A. Borst, A.T. Box, A.C. Fluit, False-positive results and contamination in nucleic acid amplification assays: suggestions for a prevent and destroy strategy, Eur J Clin Microbiol Infect Dis, 23 (2004) 289-299.

[26] E.J. Kil, S. Kim, Y.J. Lee, E.H. Kang, M. Lee, S.H. Cho, M.K. Kim, K.Y. Lee, N.Y. Heo, H.S. Choi, S.T. Kwon, S. Lee, Advanced loop-mediated isothermal amplification method for sensitive and specific detection of Tomato chlorosis virus using a uracil DNA glycosylase to control carry-over contamination, J Virol Methods, 213 (2015) 68-74.

[27] J. Aslanzadeh, Preventing PCR amplification carryover contamination in a clinical laboratory, Ann Clin Lab Sci, 34 (2004) 389-396.

[28] N. Tomita, Y. Mori, H. Kanda, T. Notomi, Loop-mediated isothermal amplification (LAMP) of gene sequences and simple visual detection of products, Nat Protoc, 3 (2008) 877-882.

[29] H.-C. Wang, G. Kasper, Filtration efficiency of nanometer-size aerosol particles, Journal of Aerosol Science, 22 (1991) 31-41.

[30] C.-s. Wang, Y. Otani, Removal of Nanoparticles from Gas Streams by Fibrous Filters: A Review, Industrial & Engineering Chemistry Research, 52 (2013) 5-17.

[31] P.N. Rys, D.H. Persing, Preventing false positives: quantitative evaluation of three protocols for inactivation of polymerase chain reaction amplification products, J Clin Microbiol, 31 (1993) 2356-2360.

[32] G. Sarkar, S.S. Sommer, Parameters affecting susceptibility of PCR contamination to UV inactivation, Biotechniques, 10 (1991) 590-594.

[33] M. Nimesh, D. Joon, M. Varma-Basil, D. Saluja, Development and clinical evaluation of sdaA

loop-mediated isothermal amplification assay for detection of Mycobacterium tuberculosis with an approach to prevent carryover contamination, J Clin Microbiol, 52 (2014) 2662-2664.

[34] J. Pang, J. Modlin, R. Yolken, Use of modified nucleotides and uracil-DNA glycosylase (UNG) for the control of contamination in the PCR-based amplification of RNA, Mol Cell Probes, 6 (1992) 251-256.

[35] Y. Hu, Regulatory Concern of Polymerase Chain Reaction (PCR) Carryover Contamination, in: A. Samadikuchaksaraei (Ed.) Polymerase Chain Reaction for Biomedical Applications, InTech, Rijeka, 2016, pp. Ch. 04.

Tables

Table 1. Bacterial strains used in this study

Bacteria	Strain no. (source of strains) [b]	No. of strains	MCDA Plus result [c]
Mycobacterial species			
M. tuberculosis [a]	H37Rv	1	P
M. tuberculosis	Isolated strains (ZG-CDC)	50	P
M. bovis [a]	Isolated strains	1	P
M. africanum [a]	Isolated strains	1	P
M. microti [a]	Isolated strains	1	P
M. abscessus [a]	ATCC 19977	1	N
M. aurum [a]	ATCC 23366	1	N
M. avium [a]	ATCC 25291	1	N
M. chelonae [a]	ATCC 14472	1	N
M. fortuitum [a]	ATCC 6481	1	N
M. gastri [a]	ATCC 15754	1	N
M. gordonae [a]	ATCC 14470	1	N
M. gilyum [a]	ATCC 43909	1	N
M. intracellulare [a]	ATCC 13950	1	N
M. kansasii [a]	ATCC 12478	1	N
M. marinum [a]	ATCC 927	1	N
M. malmoense [a]	ATCC 29571	1	N

M. nonchromogenicum [a]	ATCC 19530	1	N
M. phlei [a]	ATCC 11758	1	N
M. smegmatis [a]	ATCC 19420	1	N
M. terrae [a]	ATCC 19619	1	N
M. xenopi [a]	ATCC 19250	1	N
Non- Mycobacterial species			
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
Vibrio cholerae	ATCC14035	1	N
Streptococcus pneumonia	Isolated strains (ICDC)	1	N
Plesiomonas shigelloides	Isolated strains (ICDC)	1	N
Aeromonas hydrophila	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
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
Streptococcus suis	Isolated strains (ICDC)	1	N
Campylobacter jejuni	ATCC33291	1	N
Pseudomonas aeruginosa	Isolated strains (ICDC)	1	N
Staphylococcus aureus	Isolated strains (ICDC)	1	N
Staphylococcus epidemidis	Isolated strains (ICDC)	1	N
Klebsiella pneumoniae	Isolated strains (ICDC)	1	N

[a] Bacterial strains were kindly provided by Prof. Yanlin Zhao, National Tuberculosis Reference Laboratory, Chinese Center for Disease Control and Prevention, Changping, Beijing 102206, PR, China.

[b] ZG-CDC, Zigong Center for Disease Control and Prevention; ICDC, National Institute for Communicable

Disease Control Disease Control and Prevention, Chinese Center for Disease Control and Prevention;

ATCC, American Type Culture Collection;.

[c] P, positive; N, negative. Only mycobacteria belonging to the MTC could be detected by the

AUDG-SAMRS-LAMP assay, indicating the extremely high selectivity of the method.

Table 2. The primers used in this study

Primers name [a]	Sequences and modifications [b]	Length [c]
F3	ATCGAGCAAGCCATCTGG	18 nt
B3	TCGACATCCTCGATGGAC	18 nt
FIP	GGATCGATGTGTACTGAGATCCCGCGTACTCGACCTGAAAG	41 mer
BIP	AGCGGTCGGAAGCTCCTATGTTGATCAGCTCGGTCTTG	38 mer
LF	Dig-CTATCCGTATGGTGGATAAC	20 nt
LB	AATGCACTAGCCGAGACG	18 nt
SAMRS-F3	ATCGAGCAAGCCAT*C*T*G*G	18 nt
SAMRS-B3	TCGACATCCTCGAT*G*G*A*C	18 nt
SAMRS-FIP	GGATCGATGTGTACTGAGATCCCGCGTACTCGACCTG*A*A*A*G	41 nt
SAMRS-BIP	AGCGGTCGGAAGCTCCTATGTTGATCAGCTCGGT*C*T*T*G	38 nt
SAMRS-LF	Dig-CTATCCGTATGGTGGA*T*A*A*C	20 nt
SAMRS-LB	AATGCACTAGCCGA*G*A*C*G	18 nt

[a] SAMRS, self-avoiding molecular recognition system.

[b] Dig, digoxigenin. LF*, 5'-labeled with Dig; SAMRS-LF*, 5'-labeled with Dig. A*, 2-aminopurine; T*, 2-thiothymine; C*, N^4-ethylcytosine; G*, hypoxanthine.

[c] nt, nucleitid; mer, monomeric.

Figure legends

Figure 1. The outline of loop-mediated isothermal amplification (LAMP) combined with lateral flow biosensor (LFB) assay (LAMP-LFB)

A, schematic depiction of LAMP with LF* (5'-labeled with Dig when used in LAMP-LFB assay) and biotin-14-dCTP. B, outline of the principle of LFB for visualization of LAMP products. C, interpretation of the results: I, positive (two crimson lines at the readout region) and II, negative (only the control line region showed a crimson red bands).

Figure 2. Confirmation and detection of LAMP and SAMRS-LAMP products

A, Confirmation of LAMP products (top row: color change of LAMP reaction tubes; middle row: 2%agarose gel electrophoresis applied to LAMP products; bottom row: LFB applied for visual detection of LAMP products); **B**, Confirmation of SAMRS-LAMP products (top row: color change of LAMP reaction tubes; middle row: 2%agarose gel electrophoresis applied to LAMP products; bottom row: LFB applied for visual detection of LAMP products). Tube 1(biosensor 1, lane 1), positive amplification; Tube 2 (biosensor 2, lane 2), negative amplification (*M. kansasii*); Tube 3 (biosensor 2, lane 2), negative amplification (*S. aureus*); Tube 4 (biosensor 3, lane 3), negative amplification (S. *pneumoniae*); Tube 5 (biosensor 4, lane 4), blank control (DW). Lane M, DL 100-bp DNA marker.

Figure 3. Performance of traditional LAMP using double distilled water (DW) and different strains

Signal 1, positive control (H37Rv); Signals 2-32, DW; Signals 33-64, *Enterohemorrhagic E.*

coli, *Enteropathogenic E. coli*, *Enterotoxigenic E. coli*, *Enteroaggregative E. coli*, *Enteroinvasive E. coli*, *Vibrio cholera*, *Streptococcus pneumonia*, *Plesiomonas shigelloides*, *Aeromonas hydrophila*, *Shigella dysenteriae*, *Shigella boydii*, *Shigella flexneria*, *Shigella sonneri*, *Salmonella*, *Enterococcus faecalis*, *Enterococcus faecium*, *Yersinia enterocolitica*, *Enterobacter cloacae*, *Bntorobater sakazakii*, *Bacillus cereus*, *Listeria monocytogenes*, *Listeria ivanovii*, *Listeria grayi*, *Listeria innocua*, *Listeria welshimeri*, *Listeria seeligeri*, *Streptococcus suis*, *Campylobacter jejuni*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Staphylococcus epidemidis*, *Klebsiella pneumonia*.

Figure 4. Performance of SAMRS-LAMP using double distilled water (DW) and different strains

Signal 1, positive control (H37Rv); Signals 2-32, DW; Signals 33-64, *Enterohemorrhagic E. coli*, *Enteropathogenic E. coli*, *Enterotoxigenic E. coli*, *Enteroaggregative E. coli*, *Enteroinvasive E. coli*, *Vibrio cholera*, *Streptococcus pneumonia*, *Plesiomonas shigelloides*, *Aeromonas hydrophila*, *Shigella dysenteriae*, *Shigella boydii*, *Shigella flexneria*, *Shigella sonneri*, *Salmonella*, *Enterococcus faecalis*, *Enterococcus faecium*, *Yersinia enterocolitica*, *Enterobacter cloacae*, *Bntorobater sakazakii*, *Bacillus cereus*, *Listeria monocytogenes*, *Listeria ivanovii*, *Listeria grayi*, *Listeria innocua*, *Listeria welshimeri*, *Listeria seeligeri*, *Streptococcus suis*, *Campylobacter jejuni*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Staphylococcus epidemidis*, *Klebsiella pneumonia*.

Figure 5. Optimal reaction temperature for SAMRS-LAMP

The SAMRS-LAMP amplifications for detection of MTC were monitored by real-time measurement of turbidity and the corresponding curves of concentrations of DNA were marked in the figures. The threshold value was 0.1 and the turbidity of >0.1 was considered to be positive. Eight kinetic graphs (A-H) were obtained at various temperatures (58-65℃, 1^{o}C intervals) with target pathogens DNA (H37Rv) at the level of 10 pg per reaction and the graphs from B to E showed robust amplification.

Figure 6. Sensitivity of LAMP and SAMRS-LAMP methods using serially diluted genomic DNA with H37Rv

A, sensitivity of SAMRS-LAMP; **B**, sensitivity of LAMP. Two monitoring methods, including real time turbidity (**top row**) and lateral flow biosensor (**bottom row**), were applied for analyzing the reaction products. Genomic DNA levels of 100 ng, 100 pg, 10 pg, 1 pg and 100 fg per reaction produced the positive reactions.

Figure 7. Schematic illustration of the principle of AUDG-SAMRS-LAMP technique for eliminating carryover contamination

AUDG-SAMRS-LAMP removes carryover contamination in two stages. During the first stage, all SAMRS-LAMP amplifications are carried out in the presence of *Bst* 2.0 DNA polymerase and dUTP, such that all SAMRS-LAMP products contain uracil bases. Then, all subsequence SAMRS-LAMP amplifications are treated with AUDG to prevent carryover contaminants by removing uracil from amplicons from previous SAMRS-LAMP amplifications, but having no effect on target templates (natural DNA). During the SAMRS-LAMP amplification (60°C), the

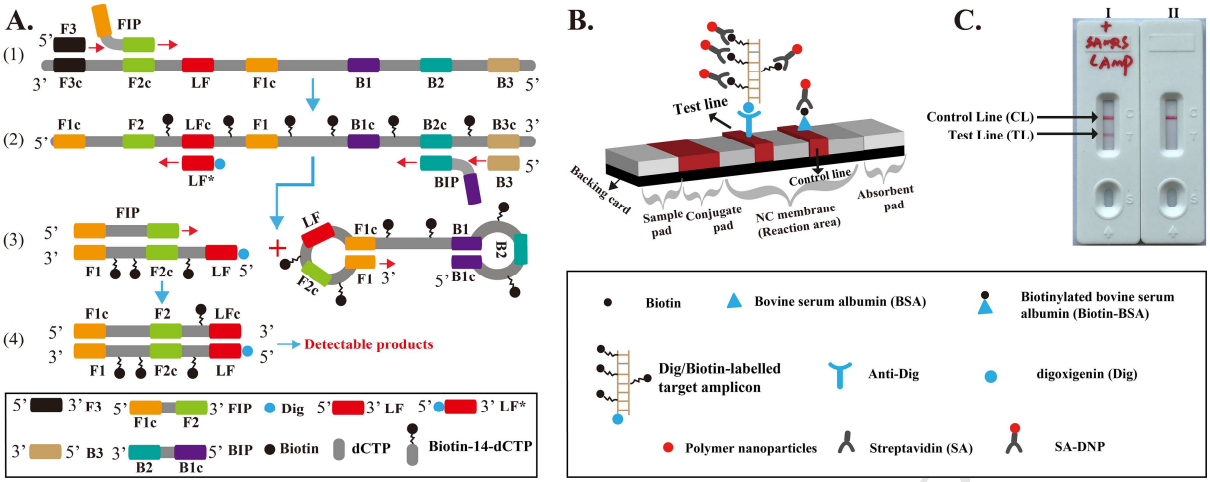
cleaved contaminants are degraded and the AUDG enzyme is heat-inactivated, guaranteeing that only the target nucleic acids are amplified. Moreover, to construct the hapten-biotin doubled-labeled SAMRS-LAMP products and enable them analysis on the lateral flow biosensors, two components, including biotin-14-dCTP and hapten-labeled primer, are added into SAMRS-LAMP amplification mixtures

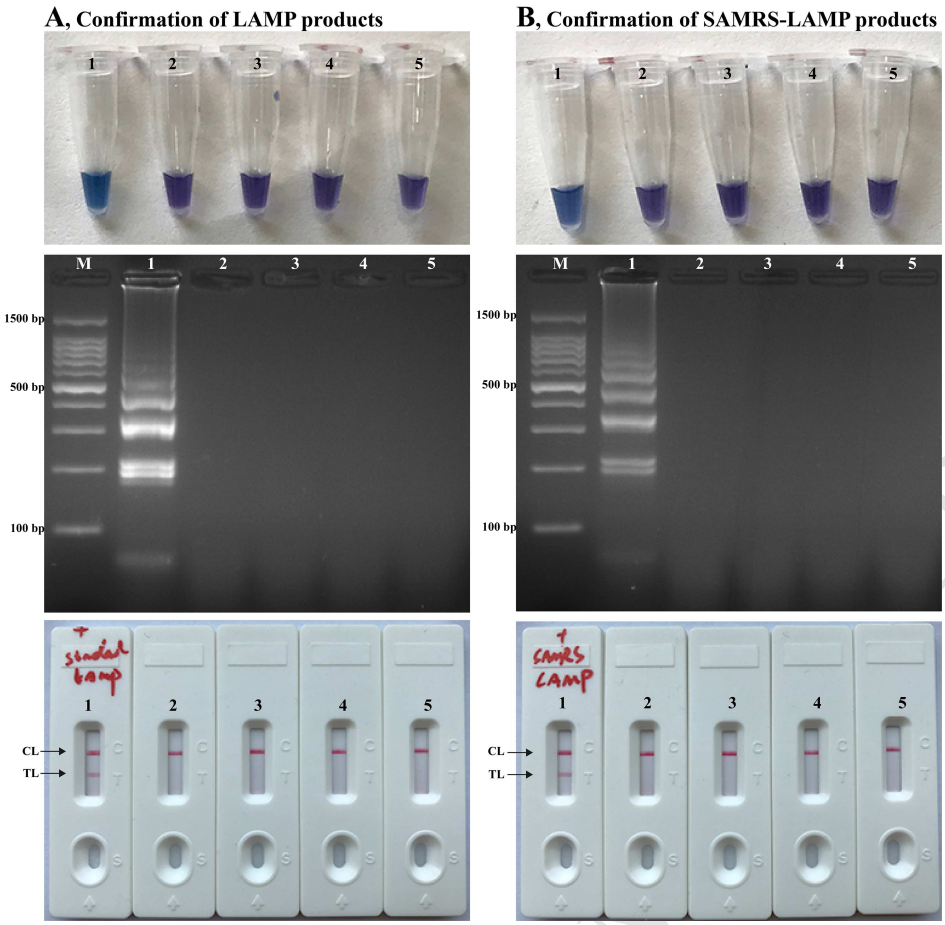
Figure 8. Control of carryover contamination in AUDG-SAMRS-LAMP assay.

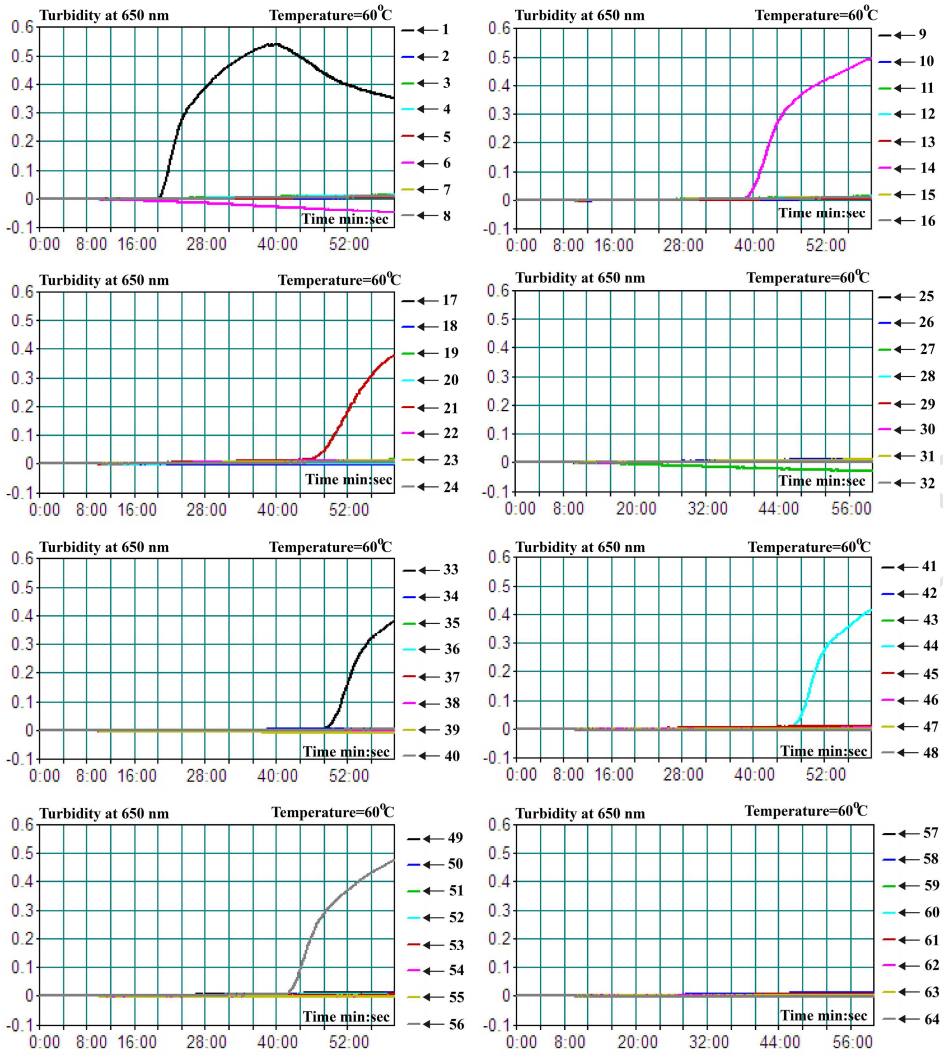
Sensitivity examination of AUDG-SAMRS-LAMP (**A**) and SAMRS-LAMP (**B**) using 10-fold serial dilutions of simulated carryover contamination (dUTP-incorporated amplicons DNA, concentration diluted from 1×10^{-13}, 1×10^{-14}, 1×10^{-15}, 1×10^{-16}, 1×10^{-17}, 1×10^{-18} and 1×10^{-19} g μL^{-1}) as determined using real time turbidity (**top row**) and lateral flow biosensor (**bottom row**).

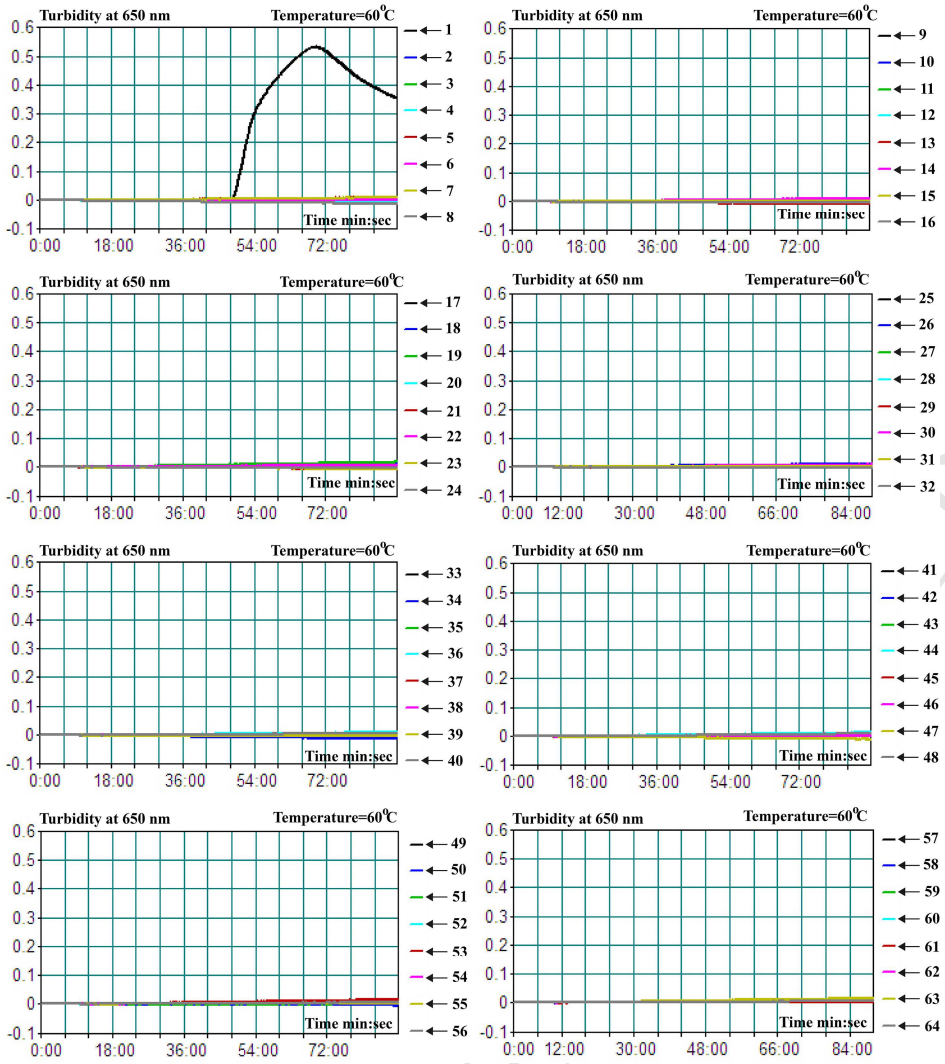
Figure 9. AUDG-SAMRS-LAMP technique eliminates false-positive detection due to carryover contamination

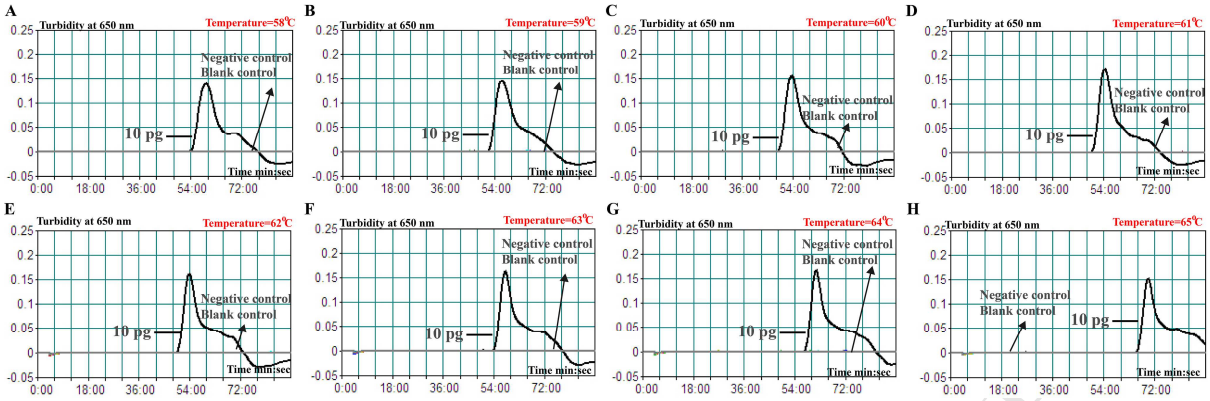
Sensitivity examination of AUDG-SAMRS-LAMP (**A**) and SAMRS-LAMP (**B**) assay using serial dilutions (100 ng μL^{-1}, 100 pg μL^{-1}, 10 pg μL^{-1}, 1 pg μL^{-1}, 100 fg μL^{-1}, 10 fg μL^{-1} and 1 fg μL^{-1}) of H37Rv and 1×10^{-18} g μL^{-1} of simulated carryover contamination (dUTP-incorporated amplicons DNA) as determined using real time turbidity (**top row**) and lateral flow biosensor (**bottom row**).



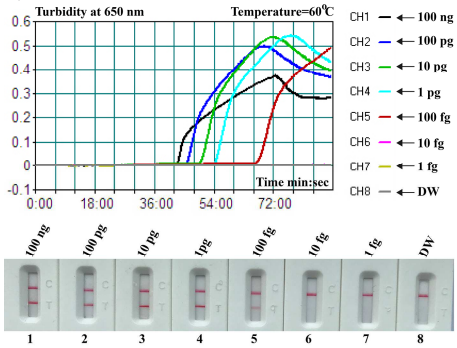




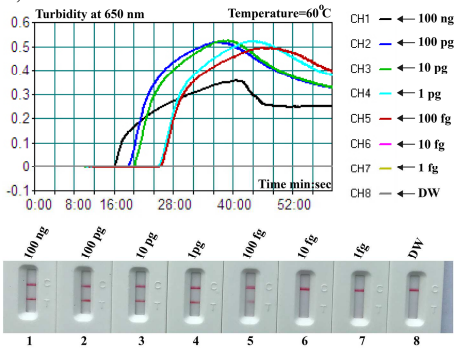




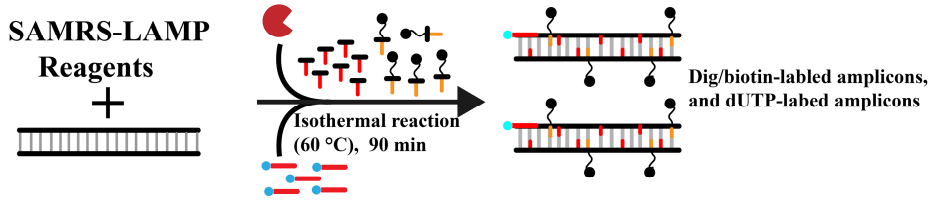
A, SAMRS-LAMP



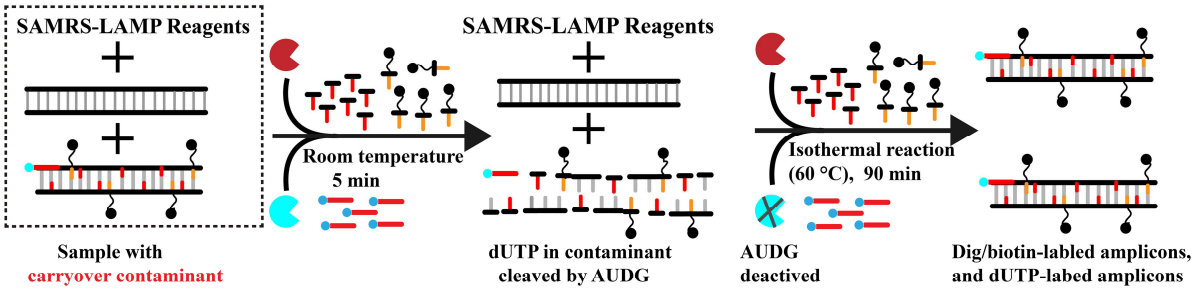
B, LAMP

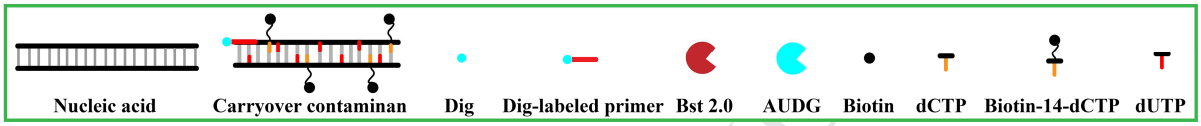


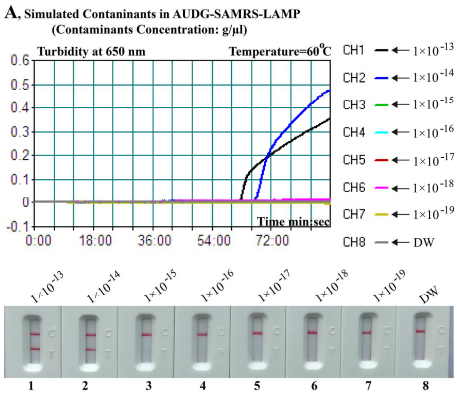
Stage 1: Incorporation of dUTP into SAMRS-LAMP amplicons

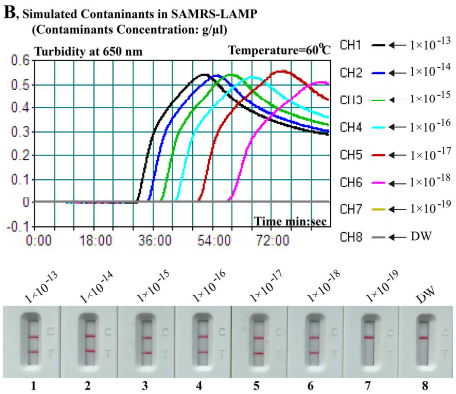


Stage 2: AUDG treatment and AUDG-SAMRS-LAMP reaction

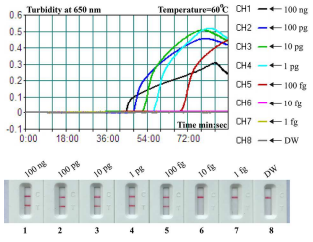




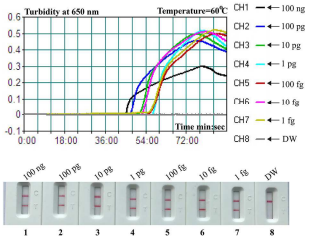




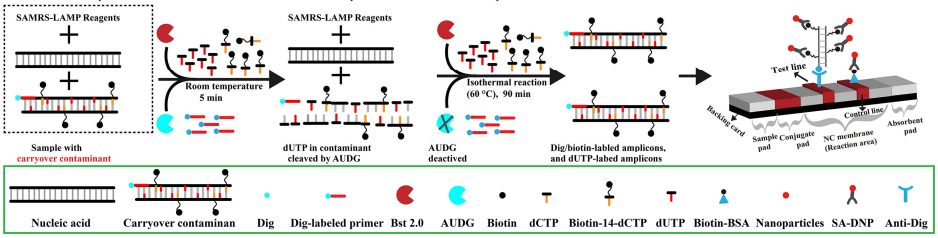
A, Sensitivity examination of the AUDG-SAMRS-LAMP by adding the Simulated Contaminants (Concentration of H37Rv: 100 ng/μl ~ 1 fg/μl)



B, Sensitivity examination of SAMRS-LAMP by adding the Simulated Contaminants (Concentration of H37Rv: 100 ng/μl ~ 1 fg/μl)



Elimination of carryover contamination and detection of nucleic acid by AUDG-SAMRS-LAMP assay



Highlights

A new LAMP was devised for detection of nucleic acid and prevention of carryover contamination

SARMS were used for improving the specificity of LAMP, and AUDG enzyme for carryover contamination

The assay was successfully used for detecting *Mycobacterium tuberculosis* complex (MTC)

The proof-of-concept assay can be reconfigured to detect various sequences by redesigning primers