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Rapid, sensitive and reliable detection of *Klebsiella pneumoniae* by label-free multiple cross displacement amplification coupled with nanoparticles-based biosensor

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ABSTRACT

Klebsiella pneumoniae (*K. pneumoniae*), as an important hospital-acquired bacterium, is responsible for severe morbidity and mortality among the elderly, newborn and immune-compromised people. We established a *rcsA* gene-based label-free multiple cross displacement amplification (MCDA) assay for rapid, simple and sensitive detection of *K. pneumoniae* by using lateral flow biosensor (LFB). MCDA reaction was conducted at a fixed temperature (65 °C) for only 30 min, and amplification results were directly indicated using LFB. The results showed that reaction products were detectable from as little as 100 fg and 4.8 CFU of pure *K. pneumoniae* templates, and from approximately 480 CFU in 1 mL of spiked clinical samples. All *K. pneumoniae* strains examined were positive for label-free MCDA-LFB analysis, and all non-*K. pneumoniae* strains used in the report were negative for label-free MCDA-LFB assay, indicating the high selectivity of the label free MCDA-LFB assay. Furthermore, to remove false-positive results, the label-free MCDA-LFB assay was supplemented with antarctic thermal sensitive uracil-DNA-glycosylase (AUDG) to eliminate the carryover contamination. Thus, label-free MCDA-LFB assay complemented with AUDG enzyme was a rapid, simple, sensitive and reliable technique for detection of target pathogen, which has the ability to effectively avoid carryover contamination, and can be a valuable tool for "on-site" detection, clinical diagnosis, and primary quarantine purposes.

1. Introduction

Klebsiella pneumoniae (*K. pneumoniae*), as a Gram-negative bacterium, can asymptomatically colonize in the skin, nose, throat and intestinal tract of healthy individuals (Holt et al., 2015). Widely considered as an opportunistic organism, *K. pneumoniae* causes pneumonia, bronchitis, bacteremias, wound, soft tissue and urinary tract infections. Recently, *K. pneumoniae* has gained notoriety as an etiological agent due to a growth in the number of severe infections, especially in older adults, newborn infants and immuno-compromised patients (Paczosa and Mecsas, 2016). Moreover, the *K. pneumonae* infections acquired in hospitals are difficult to treat, as many *K. pneumoniae* strains have acquired additional genetic traits and become highly resistant to treatment with broad-spectrum aminoglycosides and cephalosporins (Wang et al., 2015a,b). Hence, the rapid, accurate and sensitive identification of this bacterium is required for guiding the appropriate therapy and controlling the spread of the pathogen.

Traditional laboratory techniques for *K. pneumonae* diagnosis, including microscopic determination, biochemical examination and the use of automatic bacterial detection apparatus, such as VITEK® 2 system, are labor-intensive and time-consuming, typically requiring one day to three days of incubation (Ko et al., 2017). Recently, a wide variety of molecular biological methodologies have been devised and used to identify *K. pneumoniae*. Particularly, polymerase chain reaction (PCR)-based assays (such as conventional PCR, multiplex PCR and real-time PCR methods) have gained a wide popularity as the diagnostic tools for detection of *K. pneumoniae* (Sun et al., 2010). However, these techniques require expensive equipment, trained personnel, and a stable power supply, thus these technologies are best suited to diagnostic reference laboratories and are not suitable for "on-site" detection and low resource-setting areas. In addition, the application of PCR-based methods for the direct detection of *K. pneumoniae* in biological samples is limited, as *Taq* polymerase (the enzyme for amplification of nucleic acid in PCR-based techniques) is usually inhibited by various

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compounds, such as cellular debris, hemoglobin, and other ingredients (Kumar et al., n.d.).

Several isothermal amplification methodologies have been widely reported for amplification of nucleic acids in a simple heating block or water bath. Particularly, multiple cross displacement amplification (MCDA) assay, originally devised by *Wang* et al., is a rapid and simple technique that allows nucleic acid amplification at a fixed temperature (58 °C–69 °C) within 40-min (Wang et al., 2015a,b). In the amplification system, MCDA utilizes ten specific primers that are designed by ten distinct regions of the target sequence. The MCDA assay eliminates the use of much technique skill, special laboratory facilities and skilled personnel, but only for a simple isothermal instrument. Thus, these benefits make MCDA-based methods suitable for diagnostic application in "on-site" testing, filed detection and poor-resource settings. A wide variety of monitoring methods, including colorimetric indicators, gel electrophoresis, real time fluorescence and turbidity techniques, have been employed to analyze MCDA products (Wang et al., 2017a,b). However, these monitoring techniques are not specific to target sequences, thus they cannot differentiate specific amplification and non-specific amplification (Wang et al., 2017a,b). To avoid these shortcomings posed by traditional monitoring methods and speed up the total time for MCDA amplicons analysis, lateral flow biosensor (LFB) has been devised and applied to MCDA-amplified products detection, which makes the amplification technique more suitable for molecular diagnosis, especially for point-of-care testing, "on-site" detection and in resource-limited areas (Wang et al., 2017a,b,c,d, Wang et al., 2018b).

In this report, label-free MCDA technique coupled with LFB (label-free MCDA-LFB) was developed for rapid, sensitive and specific diagnosis of all *K. pneumoniae* strains. *RcsA* gene, a specific sequence to all *K. pneumoniae* strains, regulates the synthesis CPS (capsular polysaccharide), thus is used as the target gene in our study (Goncalves Mdos et al., 2014). Moreover, the opening of reaction vessels is an essential step for indicating label-free MCDA results by LFB, which carries high risk of carryover contamination (because the MCDA-based methods are highly sensitive to reaction products). Hence, the label-free MCDA-LFB assay is integrated with antarctic thermal sensitive uracil-DNA-glycosylase (AUDG) cleavage to eliminate the contaminants, and the false-positive results arising from carryover contamination are prevented. Analytical sensitivity, specificity and feasibility of the label-free MCDA-LFB assay for detection *K. pneumoniae* were successfully evaluated using pure cultures, spiked sputum samples and clinical samples.

2. Materials and methods

2.1. Primer design

MCDA primers used for *K. pneumoniae* detection were designed targeting *rcsA* gene (https://www.ncbi.nlm.nih.gov/nuccore/NC_016845.1?report=fasta&from=3416457&to=3417080), a well-conserved sequence of *K. pneumoniae*, which make it an ideal target gene for MCDA primer design (Goncalves Mdos et al., 2014). A total of ten primers, including two displacement primers (F1 and F2), two cross primers (CP1 and CP2), six amplification primers (C1, C2, D1, D2, R1 and R2), were generated using primer software PRIMER PREMIER 5.0, which recognizing ten different regions of on the target sequence. The details of MCDA primers used were given in Fig. 1 and Table 1. All MCDA primers were commercially synthesized by Ruibo-Xingke biotech Co., Ltd. (Beijing, China).

2.2. Bacterial strains and genomic DNA preparation

A total of 73 bacterial strains, including 41 strains of *K. pneumoniae* and 32 strains of non-*K. pneumoniae* bacteria, were used in this report (Table 2). Genomic DNA was extracted using DNA extraction kits (QIAamp DNA minikits, Hilden, Germany) in accordance with the manufacture's instructions, and extracted DNA was quantified with ultraviolet spectrophotometer (Nano drop ND-1000, Calibre, Beijing, China) at A260/280. The strain of *K. pneumoniae* ATCC BAA-2164 was used for confirmation performance, optimal temperature and sensitivity study conducted in the current study. Gnomic templates of *K. penumoniae* ATCC BAA-2164 was serially diluted (10 ng, 10 pg, 1 pg, 100 fg, 10 fg, 1 fg, and 100 ag/mL) and a volume of 1 μl of each dilution was added into the label-free MCDA reactions.

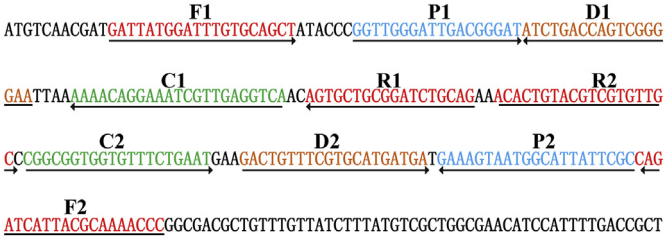

Fig. 1. The scheme shows the sequences and design of MCDA primers. The nucleotide sequences of the sense strand of *rcsA* gene were listed. The locations of the primer sites are marked with different colors. Right and left arrows indicate the sense and complementary sequences that are used.

2.3. Label-free MCDA reaction

Label-free MCDA reactions is performed in a one-step reaction in a 25-μl mixture containing 2.5 μl 10× of the supplied buffer (New England Biolabs), 0.4 μM each of displacement primers F1 and F2, 0.8 μM each of amplification primers C1, C2, R1, R2, D1 and D2, 1.6 μM each of cross primers CP1 and CP2, 0.8 M betaine (Sigma-Aldrich), 1.4 mM dATP, 1.0 mM dCTP, 1.4 mM dGTP, 0.7 mM dUTP, 0.5 μl of 0.1 mM FITC-aha-dUTP, 0.5 μl of 0.4 mM biotin-14-dCTP, 1 μl (8 U) of *Bst* 2.0 DNA polymerase (New England Biolabs), 0.3 μl (0.3 U) of AUDG and 1 μl DNA template.

Monitoring techniques, including colorimetric indicator (Malachite Green, MG) and lateral flow biosensor detection, are employed for confirming and verifying the amplification of label-free MCDA reaction. The method of visualizing label-free product on LFB was adapted from previous studies (Wang et al., 2017c).

Then, amplification temperatures ranging from 60 °C to 68 °C at 1 °C intervals were compared for optimal reaction amplification. Amplification mixtures with 1 μl of DNA template of *S. aureus* (ICDC-NPSau001) and *S. pneumonia* (ATCC700674) were used as negative controls (NC), and 1 μl of double distilled water (DW) were used as a blank control (BC).

2.4. Sensitivity of label-free MCDA assays

Limit of detection (LoD) of label-free MCDA technique was determined using serial dilutions of genomic DNA of *K. penumoniae* ATCC BAA-2164. The initial concentration of genomic templates was 100 ng/mL, which was used to make serial dilutions (10 ng, 10 pg, 1 pg, 100 fg, 10 fg, 1 fg, and 100 ag/mL). Aliquots (1 μl) of each dilution were examined by label-free MCDA assay. Testing was repeated three times.

In addition, the sensitivity of label-free MCDA-LFB assay also was evaluated using serial dilutions of colony forming units (CFUs). The number of CFUs (*K. pneumoniae* ATCC BAA-2146) was tested by using plate count, and overnight cultures were serially diluted from starting inocula of 4.8×10^5 CFU/mL to 4.8×10^0 CFU/mL. Then, aliquots (100 μl) of each dilution were subjected to extract the templates, which were eluted in 20 μl of elution buffer. Aliquots (2 μl) of templates were used for label-free MCDA reactions. Testing was repeated three times.

Then, we examined the optimal duration of time required for the label-free MCDA assay during the amplification stage. A total of 4 reaction times, including 10 min, 20 min, 30 min and 40 min, were compared at the optimal temperature, and the label-free MCDA

Table 1
The primers used in this study.

Primers name	Sequences and modifications	Length[a]
F1	5′-GATTATGGATTTGTGCAGCT-3′	20 nt
F2	5′-GGGTTTTGCGTAATGATCTG-3′	20 nt
CP1	5′-TGACCTCAACGATTTCCTGTTTTGGTTGGGATTGACGGGAT-3′	41 mer
CP2	5′-CGGCGGTGGTGTTTCTGAATGCGAATAATGCCATTACTTTC-3′	41 mer
C1	5′-TGACCTCAACGATTTCCTGTTTT-3′	23 nt
C2	5′-CGGCGGTGGTGTTTCTGAAT-3′	20 nt
D1	5′-TTCCCCGACTGGTCAGAT-3′	18 nt
D2	5′-GACTGTTTCGTGCATGATGA-3′	20 nt
R1	5′-CTGCAGATCCGCAGCACT-3′	18 nt
R2	5′-ACACTGTACGTCGTGTTGC-3′	19 nt

[a] nt, nucleitid; mer, monomeric.

Table 2
Bacterial strains used in this study.

Bacteria	Strain no. (source of strains)[a]	No. of strains	MCDA result [b]
K. pneumoniae	ATCC BAA-2146	1	P
	ATCC BAA-1705	1	P
	Isolated strains (ICDC)	39	P
Non-K. pneumoniae			
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
Vibrio alginolyticus	Isolated strains (ICDC)	1	N
Vibrio parahaemolyticus	Isolated strains (ICDC)	1	N
Listeria monocytogenes	ATCC EGD-e	1	N
Listeria ivanovii	ATCC BAA-678	1	N
Listeria grayi	ATCC 25402	1	N
Listeria innocua	Isolated strains (ICDC)	1	N
Listeria welshimeri	Isolated strains (ICDC)	1	N
Listeria seeligeri	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
Plesiomonas shigelloides	Isolated strains (ICDC)	1	N
Aeromonas hydrophila	Isolated strains (ICDC)	1	N
Streptococcus pneumoniae	ATCC 700674	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	ATCC 33291	1	N
Pseudomonas aeruginosa	Isolated strains (ICDC)	1	N
Staphylococcus aureus	Isolated strains (ICDC-NPSau001)	1	N
Staphylococcus epidemidis	Isolated strains (ICDC)	1	N
Yersinia enterocolitica	ATCC 23715	1	N

[a] ATCC, American Type Culture Collection; ICDC, National Institute for Communicable Disease Control Disease Control and Prevention, Chinese Center for Disease Control and Prevention.

[b] P, positive; N, negative. Only genomic templates extracted from *K. pneumoniae* strains could be detected by the label-free MCDA assay, indicating the extremely high specificity of the approach.

products were analyzed using lateral flow biosensor (FB).

2.5. Simulating carryover contamination

In the absence of AUDG enzyme, the label-free MCDA amplicons generated from 10 pg μl^{-1} are quantitated using ultraviolet spectrophotometer (NanoDrop ND-1000, Calibre, Beijing, China), which is used for making serial dilution from 1×10^{-13} to 1×10^{-20} g μL^{-1}. Aliquots (1 μl) of each dilution were used as templates in label-free MCDA reactions, which severed as the source of simulating carryover contaminants.

2.6. Prevention of carryover contamination by AUDG enzyme

To demonstrated the ability of AUDG to eliminate the false-positive results due to carryover contaminants in diagnosing target sequences, label-free MCDA reactions with AUDG and without AUDG were conducted by adding 1 μl of diluted DNA templates (10 ng, 10 pg, 1 pg, 100 fg, 10 fg, 1 fg, and 100 ag/mL/mL) and 1 μl of simulated carryover contamination of 1×10^{-18} g/μl in the same reaction tube. The total mass (1×10^{-18} g) of simulated carryover contamination for each reaction is approximately equivalent to a 0.2 μm-diameter aerosol droplet, which cannot be efficiently hampered by either high efficiency particulate air filters or fibrous pipette tip filters in the biosafety cabinets (Barhate and Ramakrishna, 2007; Le, 2006; Wang et al., 2017b). Analytical sensitivity of label-free MCDA with AUDG and without AUDG treatment before amplification reaction was compared to confirm whether the AUDG enzyme is ability to efficiently prevent carryover contamination in the label-free MCDA assays.

2.7. Specificity of label-free MCDA assay

To examine the assay specificity, DNA templates (at least 10 ng/mL) from 41 *K. pneumoniae* strains and 32 non-*K. pneumoniae* strains are used for performing the label-free MCDA reactions (Table 2). All experimental results were obtained from LFB detection and direct observations of color change. All tests were repeated three times.

2.8. Evaluation of the feasibility of label-free MCDA assay

To test the lowest number of *K. pneumoniae* cells detectable by the label-free MCDA assay, serial dilutions of *K. pnumoniae* cells were applied for spiking sputum samples. To prepare the artificially seeded sputum samples, five *K. pneumoniae*-negative sputa (from five different patients) were collected from Clinical laboratory of Peking University Shougang Hospital, with patients' informed consent. An equal volume of 0.1% dithiothreitol (Sigma) was added into five sputum samples, vortexing for 10 min, and incubating for 10 min at room temperature. Then, the homogenate (spiked sputum samples) was divided into 1-ml, which were adapted to 0.48 to 480,000 *K. pnumoniae* cells per milliliter. Aliquots (100 μl) of the artificially contaminated sputa were applied to extract the genomic templates, and the DNA templates were eluted in 20 μl of elution buffer. A volume of 2 μl of extracted templates was used for label-free MCDA reactions.

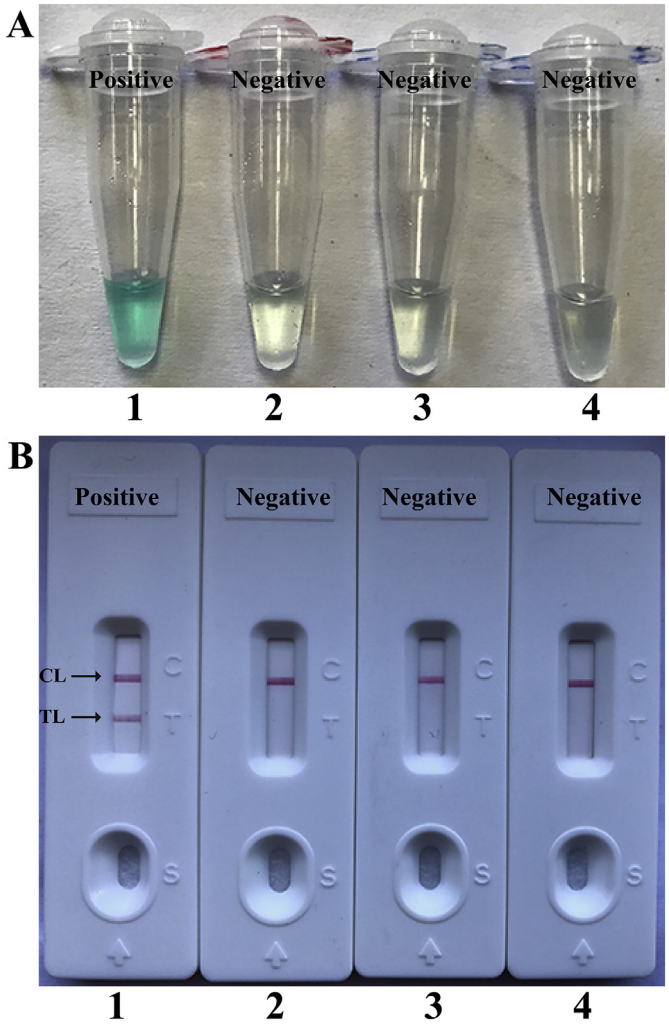

Fig. 2. Confirmation and detection of *K. pneumoniae*-label free multiple cross displacement amplification (MCDA) products.
A, Amplification products of *K. pneumoniae*-label free MCDA assay were visually detected by observation of the color change. B, lateral flow biosensor (LFB) applied for visual observation of *K. pneumoniae*-label free MCDA products. Tube 1/biosensor 1, positive amplification of *K. pneumoniae* strain (ATCC BAA-2164); Tube 2/biosensor 2, negative control of *S. aureus* strain (ICDC-NPSau001); Tube 3/biosensor 3, negative control *S. pneumoniae* (ATCC 700674); Tube 4/biosensor 4, blank control (double distilled water, DW). TL, test line; CL, control line.

3. Results

3.1. Detection and confirmation of K. pneumoniae label-free MCDA products

To confirm the availability of MCDA primers designed in this report, *K. pneumoniae* label-free MCDA assays with genomic templates from pure cultures were performed at 65 °C for 60-min. Positive amplification occurred with genomic DNA from *K. pneumoniae* (ATCC BAA-2164), but not with *S. aureus* (ICDC-NPSau001), *S. pneumoniae* (ATCC 700674) and DW control (Fig. 2). Hence, the MCDA primer set is a good candidate for establishment of label-free MCDA assay for *K. pneumoniae*.

3.2. Optimization of the reaction temperature for K. pneumoniae label-free MCDA assay

To obtain the optimal temperature, the label-free MCDA reactions were conducted from 60 °C to 68 °C (with 1 °C interval) for 60-min in a real-time turbidity machine (LA-320C). As shown in Fig. 3, the label-free MCDA for *K. pneumoniae* could be carried out at any reactions temperatures from 60 °C to 68 °C, and the most suitable reaction temperature range was 63–67 °C. Among the most suitable temperatures, the faster amplification was obtained from assay temperature 65 °C, thus we selected 65 °C as the optimal amplification temperature (Fig. 3).

3.3. Sensitivity for K. pneumoniae detection by label-free MCDA assay

Two serial dilutions of (10 ng to 100 fg/mL, and 4.8×10^5 CFU/mL to 4.8×10^0 CFU/mL) genomic DNA from *K. pneumoniae* ATCC BAA-2146 were used as templates for label-free MCDA assay to determine its sensitivity. In the label-free MCDA assay, *K. pneumoniae* were detected after the genomic templates were diluted up to 100 fg/mL (Fig. 4A and C). Moreover, label-free MCDA assay was able to detect down to 4.8×10^2 CFU/mL of target pathogen (equivalent to 4.8 CFU per reaction) (Fig. 4B and D). The results obtained using colorimetric indicator (Fig. 4C and D) were consistent with the LFB analysis (Fig. 4A and B).

Then, we determined the optimum time for label-free MCDA assay, and the reaction time ranging from 10 min to 40 min (at 10 min interval) were compared at optimal amplification temperature (65 °C). As shown in Fig. 5, the most suitable amplification time was found when the label-free MCDA lasted for 30 min (The lowest template level could be detected when the label-free MCDA reactions only lasted 30 min at 65 °C). Herein, a reaction time of 30 min was used as the optimal time for the rest label-free MCDA assays conducted in this study.

3.4. Label-free MCDA detects simulated carryover contamination

In order to demonstrate that simulated carryover contamination from dUTP-incorporated label-free MCDA products has the ability to contaminate subsequent reaction, we compared the analytical sensitivity of label-free MCDA reactions with AUDG enzyme and without AUDG enzyme using serial diluted amplification products with concentrations ranging from 1×10^{-13} to 1×10^{-20} g μL^{-1}. As shown in Fig. 6, the label-free MCDA without AUDG treatment is capable of detecting as little as 1×10^{-19} g μL^{-1} of simulated carryover contamination per vessel (Fig. 6B), and the label-free MCDA with AUDG digestion can only detect 1×10^{-13} g μL^{-1} or more of simulated carryover contamination per tube (Fig. 6A). These results demonstrated that the carryover contamination at extremely low concentrations (1×10^{-18} g μL^{-1}∼ a 0.2 μm-diameter aerosol droplet) or less (1×10^{-19} g μL^{-1}) is able to cause false positive results. Moreover, our results also verified that AUDG enzyme has the ability to prevent the amplification of up to 100,000-fold higher concentrations of carryover contamination template (1×10^{-19} g), thus the label-free MCDA assay with AUDG treatment can significantly reduce the likelihood of false-positive amplifications of *K. pneumoniae*.

3.5. Label-free MCDA assay with AUDG treatment can remove false-positive results

In this report, we then confirmed that label-free MCDA assay with AUDG cleavage is able to efficiently remove undesired results due to carryover contaminants. Sensitivity examination of label-free MCDA with AUDG and without AUDG treatment is performed using serial dilutions (10 ng, 10 pg, 1 pg, 100 fg, 10 fg, 1 fg and 100 ag/mL) of *K. pneumoniae* (ATCC BAA-2164) templates, and aliquots (1×10^{-18} g) of the simulated carryover contamination also were added into the same reaction tube. As shown in Fig. 7, we did not obtain the LoD of the label-free MCDA assay without AUDG treatment, and all examination samples shown positive results, even including these samples with undetectable levels of target DNA (10 fg, 1 fg and 100 ag/mL) (Fig. 7B).

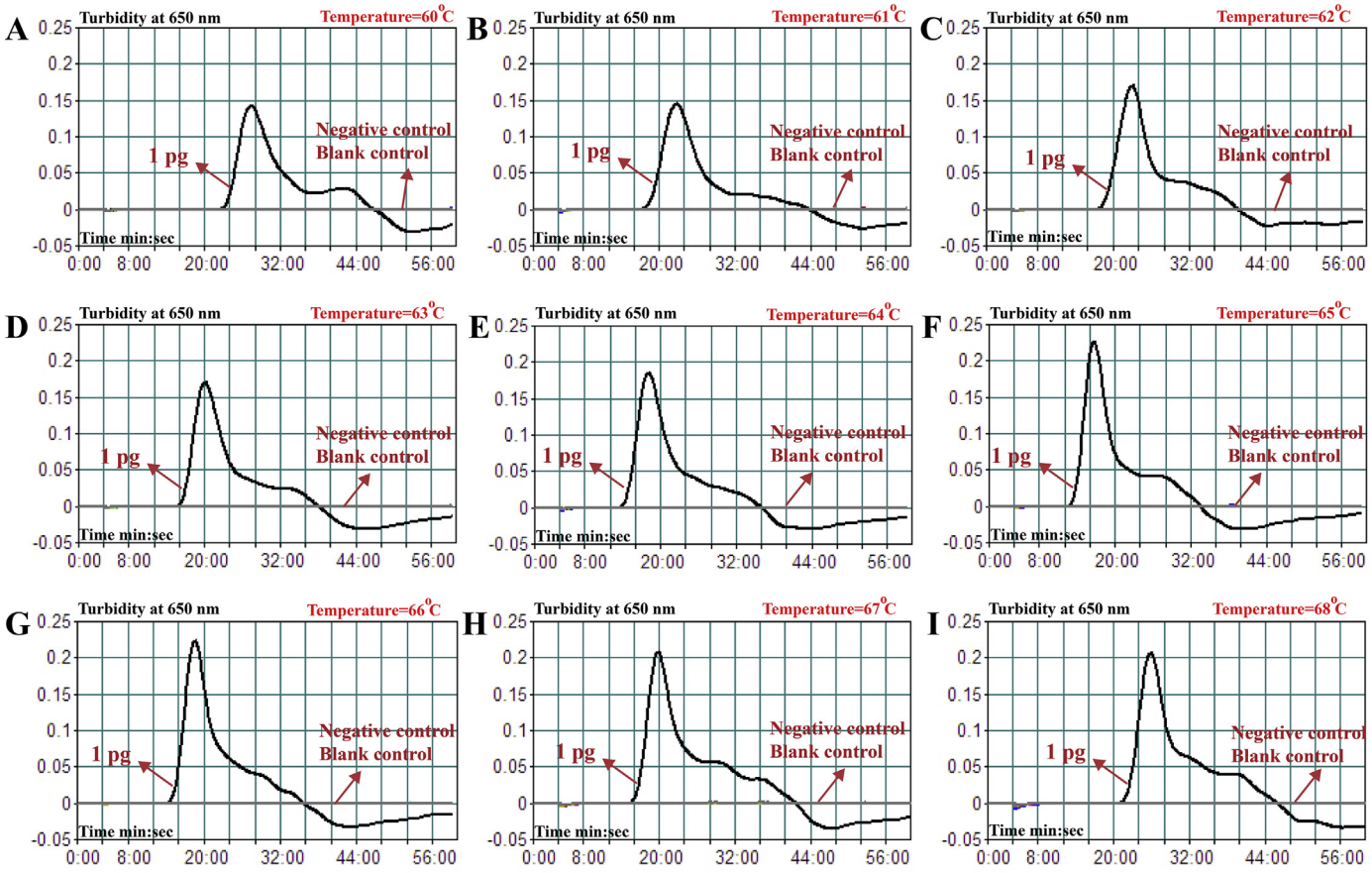

Fig. 3. Optimal amplification temperature for *K. pneumoniae*-label free MCDA primer set.
The label free MCDA reactions for detections of *K. pneumoniae* were monitored by real-time measurement of turbidity and the corresponding curves of concentration of templates were marked in the figure. The threshold value was 0.1, thus the turbidity of > 0.1 was regarded to be positive. Nine kinetic graphs (A–I) were yielded at various temperatures (60–68 °C, 1 °C intervals) with *K. pneumoniae* template at the level of 10 pg per reaction. The graphs from (D–H) showed robust amplification.

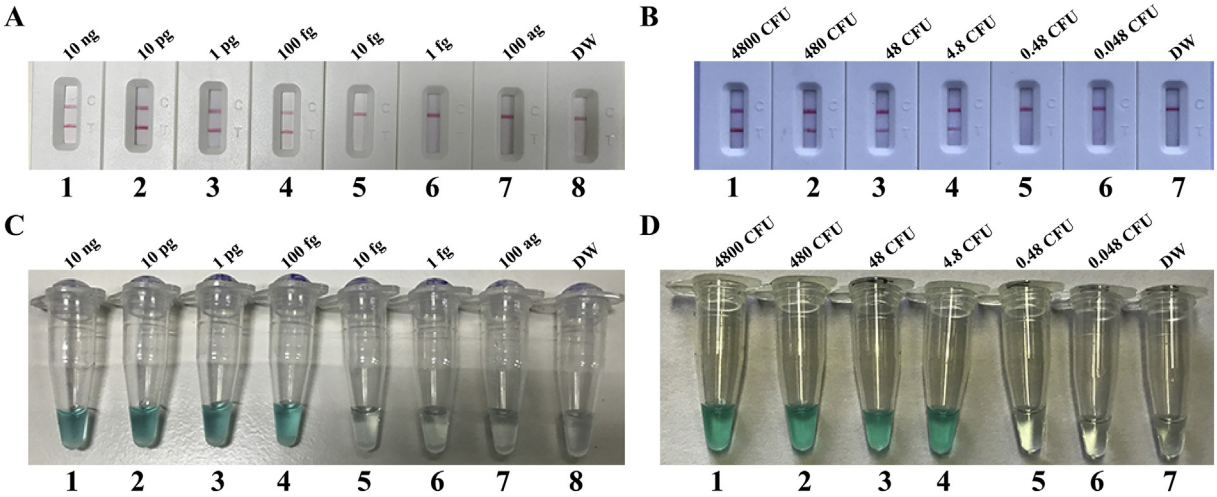

Fig. 4. Sensitivity of label-free MCDA-LFB assay using serially diluted genomic templates with *K. pneumoniae* strain ATCC BAA-2164.
Biosensors (A)/Tubes (C) 1–8 represented the DNA levels of 10 ng, 10 pg, 1 gp, 100 fg, 10 fg, 1 fg, 100 ag per reaction and blank control (DW). The genomic DNA levels of 10 ng to 100 fg per reaction produced the positive reactions. Biosensors (B)/Tubes (D) 1–7 represented the DNA levels of 4800 CFU, 480 CFU, 48 CFU, 4.8 CFU, 0.48 CFU and 0.048 CFU and blank control (DW). The genomic DNA levels of 4800 CFU (equivalent to 4.8×10^5 CFU/mL) to 4.8 CFU (equivalent to 4.8×10^2 CFU/mL) produced the positive reactions.

Thus, these results were considered as false-positive amplifications. In contrast, we could determine the LoD of label-free MCDA assay with AUDG treatment, which was in complete accordance with the aforementioned sensitivity study (Figs. 4 and 7A). Such amplifications were considered as the genuine positive results. These results indicated that the label-free MCDA assay with AUDG treatment could successfully avoid carryover contamination.

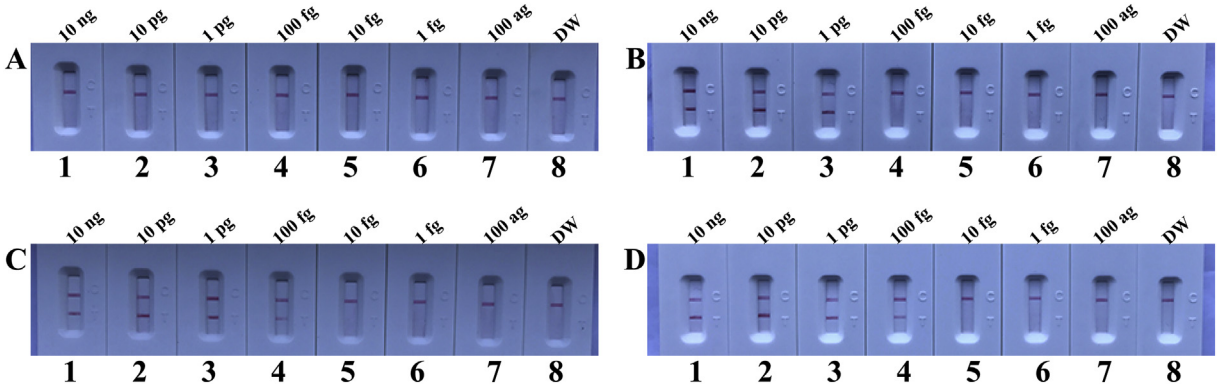

Fig. 5. Optimal duration of time required for *K. pneumoniae*-label-free MCDA assay.
Four different reaction times (A, 10 min; B, 20 min; C, 30 min; D, 40 min) were evaluated and compared at 65 °C. Biosensors 1, 2, 3, 4, 5, 6, 7 and 8 represent DNA levels of 10 ng of *K. pneumoniae* templates, 10 pg of *K. pneumoniae* templates, 1 pg of *K. pneumoniae* templates, 100 fg of *K. pneumoniae* templates, 10 fg of *K. pneumoniae* templates, 1 fg of *K. pneumoniae* templates, 100 a gram of *K. pneumoniae* templates and blank control (DW). The best sensitivity was observed when the amplification lasted for 30 min (C).

3.6. Analytical specificity for K. pneumoniae detection by label-free MCDA assay

The analytical specificity of label-free MCDA with AUDG digestion was determined using the genomic templates from 41 *K. pneumoniae* strains and 32 non-*K. pneumoniae* strains. The test was positive only for the 41 *K. pneumoniae* strains, and was negative for 32 non-*K. pneumoniae* strains (Table 2). These results indicated that the label-free MCDA assay with AUDG treatment has a 100% analytical specificity for *K. pneumoniae* detection.

3.7. Practical application of label-free MCDA assay to K. pneumoniae in spiked sputum samples

To demonstrate the feasibility of label-free MCDA assay with AUDG treatment as a nucleic detection tool, we analyzed the artificially contaminated sputum samples with *K. pneumoniae* by using label-free MCDA assay. The label-free MCDA method yielded the positive results when the spiked numbers of *K. pneumoniae* were > 480 CFU/mL (equivalent to 9.6 CFU per reaction) (Fig. 8). The negative results were obtained from negative control and when the spiked numbers of *K. penumoniae* were < 48 CFU/mL (equivalent to 0.96 CFU per reaction).

3.8. Feasibility of label-free MCDA-LFB assay to K. pneumoniae in clinical samples

Of total of thirty-two clinical sputum samples were obtained for label-free MCDA-LFB surveillance of target pathogen from clinical patients with suspected infection in Peking University Shougang Hospital, with patients' informed consent. Two sputum samples were obtained from healthy people as controls. Both culture-based assay and label-free MCDA-LFB were involved to examine these sputum samples, and the culture-based test was performed in the Clinical Laboratory of Peking University Shougang Hospital. As shown in Table 3, of the thirty-four sputum samples, label-free MCDA method detected seven positive samples. In particular, seven *K. pneumoniae* strains were successfully cultured from positive sputum samples. These results demonstrated that detection of the clinical sputum samples using label-free MCDA-LFB assay was as sensitive with culture-based assay. Moreover, the control samples all tested negative in each of the methods.

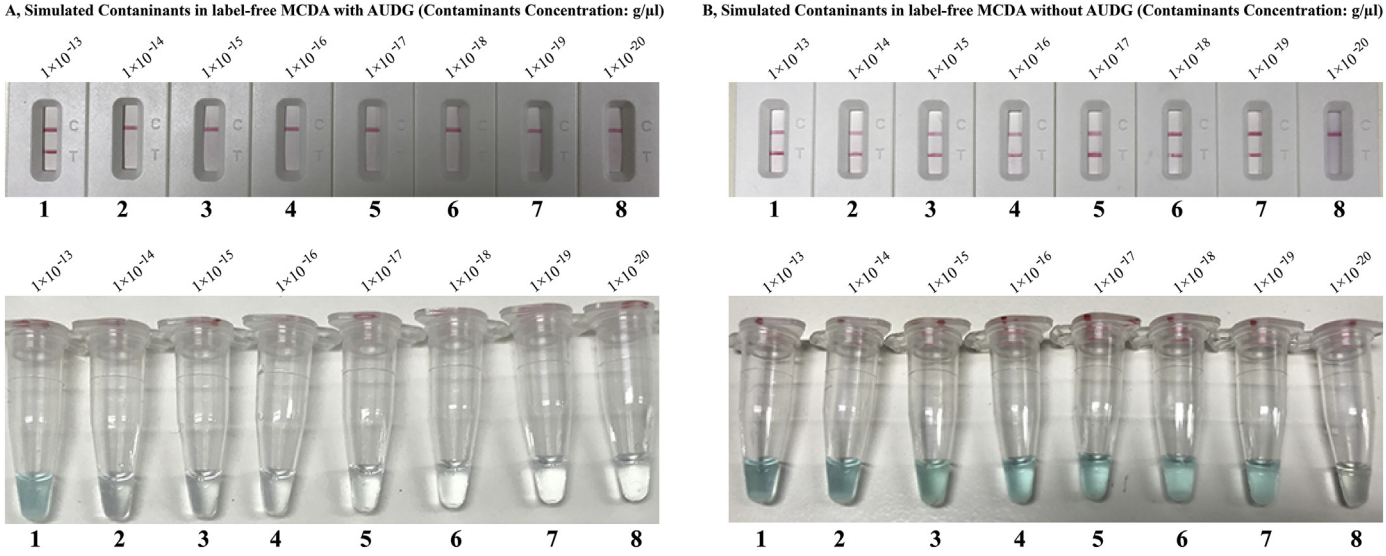

Fig. 6. Control of carryover contamination in label-free MCDA assay with AUDG enzyme.
Sensitivity evaluation of label-free MCDA with AUDG (A) and without AUDG (B) using 10-fold serial dilutions of simulated carryover contamination (dUTP-incorporated amplicons DNA, concentration diluted from 1×10^{-13}, 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 MG (bottom row).

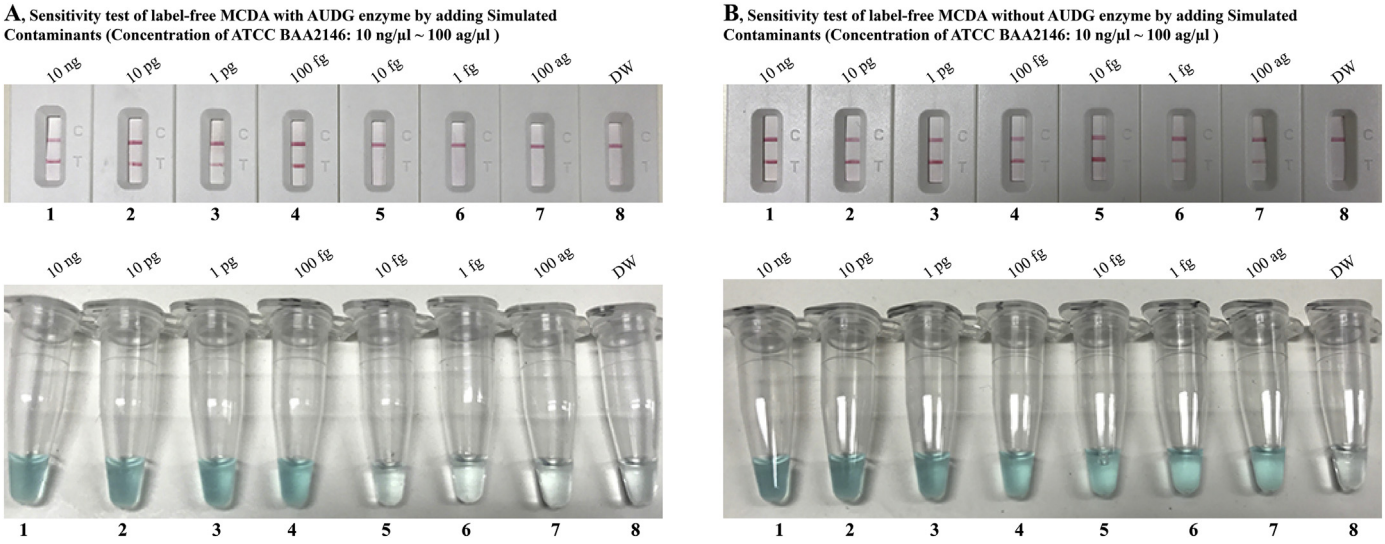

Fig. 7. Label-free MCDA assay with AUDG treatment eliminates false-positive results due to carryover contamination.
Sensitivity evaluation of label-free MCDA with AUDG (A) and without AUDG (B) using serial dilutions (10 ng/μl, 10 pg μl^{-1}, 1 pg μl^{-1}, 100 fg μl^{-1}, 10 fg μl^{-1}, 1 fg μl^{-1} and 100 ag μl^{-1}) of ATCC BAA-2146 and 1×10^{-18} g μL^{-1} of simulated carryover contamination (dUTP-incorporated amplicons DNA) as determined using LFB (top row) and MG (bottom row).

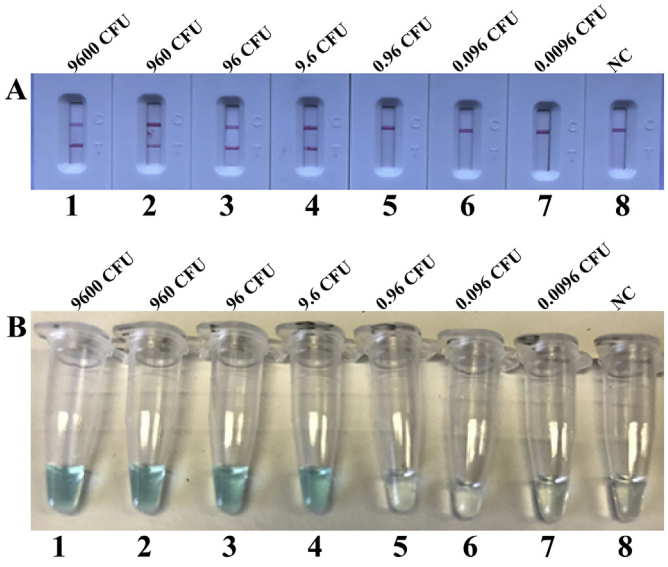

Fig. 8. Sensitivity of label-free MCDA with AUDG for detecting target pathogen in spiked sputum samples.
A total of two monitoring method, including later flow biosensor (A), and colorimetric indicator (MG, B), were applied for analyzing the amplification products. The serial dilutions of target templates were subjected to label-free MCDA reactions. Strips (A)/Tubes (B) 1–8 represented the DNA levels of 9600 CFU, 960 CFU, 96 CFU, 9.6 CFU, 0.96 CFU, 0.096, 0.006 CFU per reaction, negative control (non-contaminated sputum samples). The genomic DNA levels of 9600 CFU, 960 CFU, 96 CFU and 9.6 CFU per reaction produced the positive reactions.

Table 3
Comparison of culture-based assay and label-free MCDA-LFB method for the detection of *K. penumoniae* in sputum samples.

Detection methods	Sputum samples (n = 34)	
	Positive	Negative
Culture-based assay	7	27
Label-free MCDA-LFB assay	7	27

4. Discussion

K. penumoniae, as a widespread nosocomial bacterium, causes severe mortality and morbidity, especially in certain well-defined groups (such as neonates, persons of advanced age and immune-compromised populations) (Paczosa and Mecsas, 2016). Recent years, the bacteremia caused by *K. penumoniae* has significantly increase impatient mortality, and the organism has been known as the predominant species isolated from these persons with pyogenic liver abscess globally, surpassing *Escherichia coli* (Chetcuti Zammit et al., 2014; de et al., 2014). In particular, it has been increasingly difficult to treat carbapenem-resistant *K. pneumoniae*, because the pathogen acquired the antibiotic-resistant genes, including those encoding bla_{TEM}, bla_{NDM-1}, and bla_{KPC-2}. Hence, the early detection of *K. pneumoniae* has become extremely important. To accomplish the above, the label-free MCDA-LFB with AUDG treatment were applied to diagnose all *K. pneumoniae* strains and prevent the false-positive results arising from carryover contamination.

The label-free MCDA assay comprised ten primers (F1, F2, CP1, CP2, C1, D1, R1, C2, D2 and R2) targeting ten regions located in the conserved region of the *rscA* gene to ensure high specificity for *K. pneumoniae* detection. Our data demonstrated that the primers in label-free MCDA assay only amplified the genomic templates from *K. pneumoniae* strains and did not amplify the DNA from non-*K. pneumoniae* strains and blank control (Double-distilled water was used for replacing the DNA templates) (Table 2). Hence, the label-free MCDA method provided high selectivity for detecting the target sequence. In addition to its sufficient specificity, the label-free MCDA assay is capable of detecting as little as 100 fg and 4.8×10^2 CFU/mL (equivalent to 4.8 CFU per reaction) of *K. pneumoniae* template extracted from a pure culture (Figs. 4 and 7). The *K. pneumoniae* label-free MCDA was as sensitive as *K. pneumoniae*-LAMP approach, which detected 0.115 pg per vessel (Figs. 4 and 7) (Dong et al., 2015). Moreover, the label-free MCDA assay was successfully evaluated by spiked sputum samples, and the LoD of label-free MCDA for target pathogen detection was approximately 480 CFU/mL (equivalent to 9.6 CFU per reaction) (Fig. 8).

To conduct the MCDA amplification, only a simple instrument (such as water bath or heater) that maintains a fixed temperature is sufficient. Hence, this permits reduction of costs and simplification of instrument (Wang et al., 2015b). Lateral flow biosensor (LFB), as a product analysis tool, is employed to perform rapid, user-friendliness and cost-effective detection of *K. pneumoniae* DNA by merely seeing a colorimetric signal

with the unaided eye. In particular, the cost of LFB used in this report is estimated to be approximately $2 USD per examination (Wang et al., 2017a,b). Combined with the alleviation of labor costs due to the requirements for complex apparatus, trained personnel and expensive detection tools in a certified laboratory, label-free MCDA-LFB technique becomes more cost-effectives (Wang et al., 2017a). After 30 min of label-free MCDA reaction, the resultant products were rapidly analyzed by the LFB detection within 2 min.

Usually, MCDA-based amplicons were analyzed by gel electrophoresis, real-time turbidity and colorimetric indicator (Wang et al., 2015b). The use of gel electrophoresis to analyze amplicons is a complex procedure, and increases the risk of carryover contamination and degradation. The real-time turbidity analysis of MCDA amplicons requires an additional instrument, and suffers from background interference. The assessment of MCDA reactions by color with the unaided eye is potentially subjective, and it is possibility that a biological sample may be somewhat ambiguous to the naked eye when the concentration of target sequence is low. Here, we used a simple LFB utilizing a lateral flow dipstick housed in a plastic device to objectively analyze label-free MCDA amplicons, eliminating the use of an electrophoresis apparatus, a tubidimeter, and colorimetric indicator. Importantly, the label-free MCDA assay did not require labeled primers or probes, and only two additional components, including FITC-aha-dUTP and biotin-14-dCTP, were added into the amplification mixture to label the amplicons (Wang et al., 2017c). Within the biosensor, the FITC-labeled products were captured by anti-FITC antibody located on the test line, and the biotin-labeled amplicons could bind to dye streptavidin-coated polymer nanoparticles to visualize the results (Wang et al., 2017c, 2018a). Usually, to incorporate MCDA products and LFB analysis, two primers involved in MCDA reaction were labeled, one with biotin and one with FITC. Thus, a detectable product, which was simultaneously modified with FITC and biotin, was constructed using the labeled primers during the amplification stage. However, an undesired hybridization between two labeled primers also could form a detectable product simultaneously labeling with FITC and biotin, which yielded a false-positive signal without MCDA amplification (Wang et al., 2017d). Using the label-free strategy, the false-positive results arising from hybridization between two labeled primers, can were efficiently avoided (Wang et al., 2017c, 2018b).

Because of the extremely high sensitivity of the MCDA assay, a very small quantity of contaminant can cause false-positive signals, thus removing carryover contamination is an extremely important factor for reliable and accurate identification of target sequence. The further step, indicating label-free MCDA results by LFB, required opening of the reaction tube, thus the aerosol droplets containing high concentration of products were generated. In this report, our data suggested that a minute amount of contaminants (1×10^{-19} g per reaction) is capable of causing undesired amplification due to its high sensitivity (Fig. 7). Hence, we employed the AUDG-mediated digestion of dUTP-incorporated contaminant amplicons, which was simple and inexpensive strategy, and eliminated the use of substantial medication of existing protocols (Borst et al., 2004; Wang et al., 2017c). The label-free MCDA with AUDG treatment can specially degrade as much as 1×10^{-13} g/reaction of simulated contaminants (dUTP incorporated label-free amplicons), but has no effect on nature template. As a result, elimination of contaminants and performance of label-free MCDA are conducted in a single closed vessel, which efficiently prevent the reintroduction of contaminants from previous reaction and environment.

In summary, based on the conserved *rcsA* gene of *K. pneumoniae* strains, we employed label-free MCDA assay coupled with lateral flow biosensor (LFB) detection and antarctic thermal sensitive uracil-DNA-glycosylase (AUDG) to simultaneous diagnosis of *K. pneumoniae* and prevention of carryover contamination. Using the LFB detection, the label-free MCDA assay provided a simple, objective and convenient way for rapid detection of target pathogen. Analytical sensitivity, specificity, and feasibility of label-free MCDA assay were successfully evaluated using pure cultures, spiked sputum samples and clinical samples. Hence, the label-free MCDA assay developed here could be used as a valuable tool for clinical screening, especially for point-of-care detection, or in case of poor resources.

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