Detection of Streptococcus Pneumoniae and elimination of carryover contamination by multiple cross displacement amplification coupled with antarctic thermal sensitive uracil-DNA-glycosylase

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Abstract

Background: *Streptococcus pneumoniae* is an important clinical pathogenic bacterium, which is the primary cause of meningitis, septicemia and community-acquired pneumonia. The mortality rate of pneumococcal disease was high, especially in children under 5 years of age. Rapid and accurate detection of *S. pneumoniae* is critical. **Methods:** A ply gene-based multiple cross displacement amplification (MCDA), which amplifies DNA under isothermal conditions (65 °C) for 40 min, was established for accurate and rapid detection of *S. pneumoniae*. Antarctic thermal sensitive uracil-DNA-glycosylase (AUDG) was applied for eliminating carryover contamination. Lateral flow biosensor (LFB) was used to indicate the amplification results. **Results:** The ply-MCDA assay can detect as little as 10 fg of *S. pneumoniae* DNA, as well as 447 CFU/mL of spiked sputum samples. The sensitivity of ply-MCDA assay in clinical samples was 100 times that of PCR. The specificity of MCDA primer targeting the ply gene was validated using 15 *S. pneumoniae* and 25 non-*S. pneumoniae*, suggesting that ply gene-based MCDA assay was highly selective for *S. pneumoniae*. Moreover, the ply-MCDA coupled with AUDG can effectively eliminate carryover contamination, and then prevent false-positive results. **Conclusion:** The ply-MCDA assay coupled with AUDG was a simple, rapid and accurate method in the diagnosis of *S. pneumoniae* infection.

Background

Streptococcus pneumoniae is a gram-positive bacterium and regularly colonizes in the upper respiratory tract, which is the most common pathogen of human meningitis, septicemia, community-acquired pneumonia, otitis media and sinusitis [1, 2]. According to the 2005 data of World Health Organization (WHO), *S. pneumoniae* infection causes 1.6 million deaths worldwide yearly, of which 700,000 to 1 million are children under 5 years of age, and mostly from developing countries[3]. Thus, rapid and accurate detection of *S. pneumoniae* is a primary task in preventing the spread of this pathogen and reducing the risk of pneumococcal disease.

The traditional detection technique for *S. pneumoniae* is bacterial culture-colonial morphology-biochemical examination, which is time-consuming, usually requiring three or more days. Rapid and accurate detection of *S. pneumoniae* is essential for clinical treatment. Polymerase chain reaction (PCR)-based molecular biology techniques have been used for *S. pneumoniae* detection through primers specific to *ply* gene [4-6]. Pneumolysin, which is encoded by the *ply* gene, is a virulence factor from *S. pneumoniae* and involved in the pathogenesis [7, 8], representing the potential diagnostic target. However, the PCR reaction system is complex, complicated to perform, and requiring expensive thermal cycle apparatus and professional technicians[9]. Thus, PCR-based assay is not available in some backward clinical laboratories and “on-site” detection.

In recent years, a novel isothermal amplification method of DNA, multiple cross displacement amplification (MCDA), was devised by Wang et al. [10]. Compared with PCR, MCDA has superior performance, which amplifies DNA under isothermal temperature (60°C-69°C) with higher efficiency,

specificity and only a simple thermostatic equipment is required. This method has been used for detecting *Klebsiella pneumoniae* [11], *Vibrio parahaemolyticus* [12], *Listeria monocytogenes* [13] and so on. The MCDA reaction system requires ten primers that specially recognize ten different regions of the target gene, and it can be completed within 40 min. Therefore, MCDA assay might be a valuable method for rapid and accurate detection of *S. pneumoniae*, especially when resource settings are limited. In addition, four detection methods of MCDA products, including colorimetric indicator, real-time turbidity, lateral flow biosensor (LFB) and gel electrophoresis have been adopted[14] .

In present study, MCDA assay was established for rapid and accurate detection of *S. pneumoniae*. *Ply* gene, *S. pneumoniae*-specific gene that encodes pneumolysin, was used as the target gene. We evaluated the detection performance of *ply*-MCDA assay for *S. pneumoniae*. Furthermore, antarctic thermal sensitive uracil-DNA glycosylase (AUDG) was applied for the *ply*-MCDA reaction system to eliminate carryover contamination.

Methods

Primer design

Pneumolysin, a virulence factor, encoded by the *ply* gene, is a specific target for *S. Pneumoniae* detection. Ten primers were designed targeting the *ply* gene of *S. pneumoniae* according to the primer design principle of MCDA [13]. Primer Primer 5.0 and PrimerExploer V4 were used. The pneumolysin (*ply*)-encoding primer sets are listed in **Table 3** and **Figure 5**. Blast analysis was performed to confirm the specificity of primer sets designed for *S. pneumoniae*. The 5'-ends of C1 was labeled with fluorescein isothiocyanate (FITC). The 5'-ends of D1 was labeled with biotin. Primers synthesis was done in Aoke Dingsheng Biotechnology Co., Ltd. (Beijing, China).

Bacteria strains

In this study, 15 *S. pneumoniae* and 25 non-*S. pneumoniae* were used (**Table 1**). The QIAamp DNA mini Kit (Hilden, Germany) was used for the extraction of genomic DNA from bacteria strains. Protocols were carried out according to the manufacturer's instructions. The *S. pneumoniae* ATCC49619 was used for performance confirmation, optimal temperature determination and sensitivity analysis. Serial dilutions of *S. pneumoniae* ATCC49619 DNA (10ng, 10pg, 1pg, 100fg, 10fg, 1fg and 0.1fg/μL) were used to analyze the sensitivity of *ply*-MCDA assay.

S. pneumoniae ply-MCDA assay

The *S. pneumoniae ply*-MCDA assay was conducted referring to previously published methods of Wang et al. [13]. Isothermal amplification kits (Haitaizhengyuan, Beijing, China) was used for MCDA assay. *S. pneumoniae ply*-MCDA assay was performed in a 25μl reaction system, including 12.5μl of 2×Reaction buffer, 0.4μM of F1 and F2, 1.6μM of CP1 and CP2, 0.8μM of C1 (C1*), R1, D1 (D1*), C2, R2 and D2, 1μl of *Bst* DNA polymerase (10U), and 1 μL of DNA template. Distilled water was blank control, and

Staphylococcus aureus DNA and *Salmonella typhi* DNA were negative controls. The reactions were isothermal amplified at 65°C 40 min, then 85 °C 5 min to stop the amplification.

In this study, four methods including colorimetric indicator (Malachite Green, MG), turbidimeter (Loopamp Realtime Turbidimeter LA-320C), lateral flow biosensor (LFB) and 2% agarose gel electrophoresis were used to detect the amplification results. The above four methods were performed as previously described [14].

Optimal temperature of *ply*-MCDA assay

The optimal temperature of *S. pneumoniae ply*-MCDA assay was measured at an isothermal temperature in the range of 62°C to 67°C at intervals of 1 °C. Distilled water was blank control. *Staphylococcus aureus* DNA and *Salmonella typhi* DNA were negative controls. The temperature that contributed to produce higher amplification products and occur turbidity earlier was considered for the optimal amplification temperature. This experiment repeated three times.

Sensitivity of *ply*-MCDA assay

Sensitivity of *ply*-MCDA assay was determined using serial dilutions of *S. pneumoniae* ATCC49619 DNA. A total of 7 different concentrations of DNA templates (10ng, 10pg, 1pg, 100fg, 10fg, 1fg, 0.1fg/μL) were acquired. 1 μL of dilution was adding to the mixture system and then amplified at optimal temperature for 40min. Colorimetric indicator, real-time turbidity, LFB detection and gel electrophoresis were carried out to analyze the *ply*-MCDA products and then determine the detection limit of *S. pneumoniae ply*-MCDA assay. Distilled water was blank control. The sensitivity experiment repeated in triplicate.

AUDG enzyme eliminates carryover contamination.

The *S. pneumoniae ply*-MCDA products without AUDG enzyme digestion were quantified and subsequently diluted in the range of 1×10⁻¹⁰ g/μL to 1×10⁻²⁰ g/μL. These dilutions were used as simulated carryover contamination and served as templates in the *S. pneumoniae ply*-MCDA assay. To demonstrate that the simulated carryover contamination (UTP-incorporated *ply*-MCDA products) can contaminate the *ply*-MCDA reaction and confirm that AUDG enzyme can eliminate the carryover contamination, the sensitivity of *ply*-MCDA-AUDG and *ply*-MCDA were compared by using the above serial diluted *S. pneumoniae ply*-MCDA products (1 μL). The ultraviolet spectrophotometer (NanoDrop ND-1000, Calibre, China) was used for quantification.

Specificity of *ply*-MCDA assay

To determine the specificity of *S. pneumoniae ply*-MCDA assay, genomic DNA from 40 bacteria strains including 15 *S. pneumoniae* and 25 non-*S. pneumoniae* were used for *ply*-MCDA assay (**Table 1**). The *ply*-MCDA products were analyzed using colorimetric indicator and LFB. The specificity experiment repeated three times.

Clinical sensitivity of *ply*-MCDA assay

The clinical sensitivity of *S. pneumoniae* *ply*-MCDA assay was evaluated by artificially adding different amounts of colony forming units (CFU) of *S. pneumoniae* ATCC49619 to the sputum samples. The single colony of ATCC49619 was enriched and cultured. Then, the number of ATCC49619 CFUs was counted and the culture suspension was added to *S. pneumoniae* negative sputum samples. The concentration of ATCC49619 in the sputum samples was adjusted to 4.47×10^0 , 4.47×10^1 , 4.47×10^2 , 4.47×10^3 , 4.47×10^4 , 4.47×10^5 , 4.47×10^6 CFU/mL. 100 μ L of each spiked sputum sample was taken for genomic DNA extraction and then eluted in 10 μ L of elution buffer (Qiagen, Germany). Next, 1 μ L of DNA template was used for *ply*-MCDA assay and PCR method was also adopted. The experiment was performed three times independently.

Results

Confirmation of *ply*-MCDA products.

To confirm the feasibility of the *ply* gene-targeted MCDA assay in detecting *S. pneumoniae*, *ply*-MCDA assay with and without target DNA were conducted at 65 °C for 40 min. The *ply*-MCDA products of *S. pneumoniae* ATCC49619 was visually detected as lake green color in the tube (**Fig.1A**), two red lines (control line and test line) on the LFB (**Fig.1B**) and ladder-like bands on the gel (**Fig.1C**), which indicated the amplification with target sequence. While the *ply*-MCDA products of negative controls and blank control were present as colorless (**Fig.1A**), one red line (control line) (**Fig.1B**) and non-ladder bands (**Fig.1C**). Our results suggested that *S. pneumoniae*-specific primers targeting the *ply* gene were suitable for MCDA assay.

Optimal temperature of *ply*-MCDA assay.

To measure the optimal temperature of the *ply*-MCDA assay, the DNA of *S. pneumoniae* ATCC49619 (1 pg/reaction) was amplified at intervals of 1 °C in the range of 62°C to 67°C, respectively. The real-time turbidity of different temperatures was monitored. From the Kinetic curves as shown in **Figure 2**, we found that the optimal amplification temperature was 65°C because the reaction turbidity occurred earlier and the amount of *ply*-MCDA products were higher than other temperatures (**Fig. 2D**). 65°C was used in subsequent studies.

Sensitivity of *ply*-MCDA assay.

To identify the detection limit, serial dilutions of *S. pneumoniae* ATCC49619 DNA (10ng, 10pg, 1pg, 100fg, 10fg, 1fg, 0.1fg/ μ L) were subjected to be amplified at 65°C. Our results indicated that the detection limit of *S. pneumoniae* *ply*-MCDA assay was 10fg (**Fig.3**). The positive amplicons were represented in the color indicator (**Fig.3A**), real-time turbidity (**Fig.3B**), LFB (**Fig.3C**) and gel electrophoresis (**Fig.3D**) as lake green color, turbidity curve, two red lines and ladder-like bands, respectively. Moreover, the results of the above four methods were completely consistent.

AUDG enzyme eliminates carryover contamination.

To determine whether the carryover contamination from the UTP-incorporated *ply*-MCDA products would contaminate the *ply*-MCDA reaction, the sensitivity of *ply*-MCDA-AUDG and *ply*-MCDA were evaluated by using serial dilutions of *ply*-MCDA products (between 1×10^{-10} g/ μ L and 1×10^{-20} g/ μ L). Our results showed that *ply*-MCDA-AUDG could detect simulated carryover contamination of 1×10^{-14} g/ μ L or more (Fig.4). However, *ply*-MCDA without AUDG enzyme could detect simulated carryover contamination of 1×10^{-19} g/ μ L (Fig.4). These results indicated that false positive results would occur when the concentration of carryover contamination reach 1×10^{-19} g/ μ L. In addition, the results demonstrated that the AUDG enzyme is able to eliminate the carryover contamination. Therefore, the possibility of false positive results can be significantly reduced by adding AUDG enzyme to the *ply*-MCDA system.

Specificity of *ply*-MCDA assay.

To identify the specificity of *ply*-MCDA assay with AUDG digestion in detecting *S. pneumoniae*, DNA from 15 *S. pneumoniae* and 25 non-*S. pneumoniae* were tested. The 15 strains of *S. pneumoniae* showed positive results, while other *Streptococcus* species and non-*Streptococcus* strains showed negative results (Table 1). The results suggested that *ply*-MCDA-AUDG assay was highly specific for *S. pneumoniae*.

Clinical sensitivity of *ply*-MCDA assay.

To determine the sensitivity of *ply*-MCDA assay with AUDG digestion in clinical samples, sputum samples artificially contaminated with serial dilutions of *S. pneumoniae* ATCC49619 were tested. Positive amplification occurred when the amount of *S. pneumoniae* ATCC49619 in the sputum samples reach 447 CFU/ml (equivalent to 4.47 CFU per reaction) (Table 2). Moreover, the sensitivity of *ply*-MCDA-AUDG assay was 100 times that of PCR, the detection limit of PCR was 44700 CFU/ml (equivalent to 447 CFU per reaction) (Table 2).

Discussion

S. pneumoniae, an important human pathogenic bacterium, causes high morbidity and mortality. Statistics show that in China, the cases of pneumococcal infection account for 12% of the global cases, which is also one of the countries with the highest deaths caused by pneumococcal infection in children under 5 years old [15]. Because of the severity of *S. pneumoniae* infection, simple and accurate methods are needed to detect *S. pneumoniae* and timely guide clinical treatment. Mitsuko et al. has reported a rapid method, loop-mediated isothermal amplification (LAMP), applying for detection of *S. pneumoniae* infection, which is highly selective due to its recognition of six different regions of the target gene by four primers [16, 17]. Studies have reported that increasing the primers of isothermal amplification could improve the sensitivity [18]. This study developed a novel method, MCDA in combination with AUDG, to specifically detect *S. pneumoniae* and prevent false positive results.

Within the present study, we designed five pairs of primers targeting ten distinct regions of the *ply* gene. *Ply* gene, which encodes pneumolysin, a well-characterized virulence factor of *S. pneumoniae* and

participating in the pathogenesis, has been used as the specific target gene of *S. pneumoniae* [7, 8]. Our results demonstrated that the primer sets are highly specific for *S. pneumoniae*. All of the strains of *S. pneumoniae* were positively amplified, while the other *Streptococcus* species and non-*Streptococcus* strains were not amplified (**Table 1**). Therefore, the specificity of *ply*-MCDA assay in the diagnosis of *S. pneumoniae* infection was extremely high, especially combining with clinical manifestations.

In addition, we determined the sensitivity of *ply*-MCDA assay by testing serial dilutions of *S. pneumoniae* ATCC49619 DNA. The detection limit of *ply*-MCDA assay was 10fg (**Fig.3**). It has been reported that LAMP can detect *S. pneumoniae* as few as 25 fg [19] or 20 fg [20]. We also assessed the clinical sensitivity of this assay by using spiked sputum samples. The *ply*-MCDA assay can detect *S. pneumoniae* as low as 447 CFU/ml (equivalent to 4.47 CFU per reaction), which is similar to that of LAMP [19]. Thus, *ply*-MCDA assay was a sensitive method for *S. pneumoniae* detection.

However, owing to the high sensitivity of *ply*-MCDA assay, false positive results caused by carryover contamination became a barrier to rapid and accurate detection of *S. pneumoniae*. Moreover, aerosols that containing high concentrations of products cannot be avoided because the opening indicating operations of *ply*-MCDA products (such as LFB). The results of this study suggested that trace amounts of carryover contamination (1×10^{-19} g/ μ L) can lead to false positive results. Thus, AUDG enzyme digestion was performed to prevent the false positive results caused by carryover contamination, which specifically cleave uracil bases and permit nature templates to be amplified normally [11, 19], and which can eliminate up to 1×10^{-15} g/ μ L of simulated contaminants. These results suggested that *ply*-MCDA-AUDG assay can effectively eliminate carryover contamination and then reduce false positive results.

About results analysis, colorimetric indicator, real-time turbidity, LFB and gel electrophoresis were used. Our results showed that these four methods were highly consistent. However, comparing with conventional analysis methods, LFB was probably the optimal monitoring method. In the MCDA system, one primer was labeled with FITC and one primer was labeled with biotin. Subsequently, positive amplicons are simultaneously labeled with FITC and biotin. In LFB, FITC and biotin labeled products combined with dye streptavidin coated on the control line and anti-FITC antibodies immobilized on the test line, respectively. The results are displayed as two red lines within 2 minutes. The advantages of LFB included simple, rapid, cost-effective, objective, and no equipment requirement. Thus, LFB is an effective method for analysis of MCDA products.

In summary, we established a simple, rapid and accurate *ply*-MCDA-LFB method in combination with AUDG for the diagnosis of *S. pneumoniae* and eliminating carryover contamination. LFB provided a potential tool for rapid and accurate detection of *S. pneumoniae*. The sensitivity, specificity and clinical sensitivity of *ply*-MCDA assay in the diagnosis of *S. pneumoniae* were successfully evaluated using cultured strains and sputum samples. Therefore, the *ply*-MCDA-LFB method coupled with AUDG is potentially a valuable tool for the detection of *S. pneumoniae*.

Abbreviations

AUDG: Antarctic thermal sensitive uracil-DNA glycosylase

CFU: colony forming unit

FITC: fluorescein isothiocyanate

LAMP: loop-mediated isothermal amplification

LFB: lateral flow biosensor

MCDA: multiple cross displacement amplification

MG: Malachite Green

PCR: polymerase chain reaction

Ply: pneumolysin

WHO: World Health Organization

Declarations

Acknowledgments

Not applicable

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Availability of data and materials

The datasets used during the current study are available from the corresponding author on reasonable request.

Authors' contributions

SH designed the study. LY, FZ and LN carried out the experiments and data analysis, and manuscript writing. YC, LW, XZ, YW and JN participated in the acquisition of materials. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

This study was carried out in accordance with the Declaration of Helsinki. This study was approved by the Ethics Committee of Peking University Shougang Hospital. All subjects gave written informed consent.

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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Tables

Table 1. Bacteria strains used in this study.

ID	Strains	Sources	MCDA result
1	<i>Streptococcus pneumoniae</i>	ATCC49619	Positive
2-15	<i>Streptococcus pneumoniae</i>	Isolated strains from sputum	Positive
16	<i>Shigatoxin-producing E. coli (STEC)</i>	<i>E. Coli</i> 933	Negative
17	<i>Enteraggregative E. coli (EAEC)</i>	<i>E. Coli</i> 042	Negative
18	<i>Enteroinvasive Escherichia coli (EIEC)</i>	<i>E. Coli</i> 44825	Negative
19	<i>Enteropathogen Escherichia coli (EPEC)</i>	<i>E. Coli</i> 2348/69	Negative
20	<i>Enterotoxigenic Escherichia coli (ETEC)</i>	<i>E. Coli</i> 10407	Negative
21	<i>Salmonella typhi</i>	Isolated strains from feces	Negative
22	<i>Citrobacter freundii</i>	Isolated strains from sputum	Negative
23	<i>Listeria ivanovii</i>	BAA678	Negative
24	<i>Listeria monocytogenes</i>	Isolated strains from blood	Negative
25	<i>Klebsiella pneumoniae</i>	ATCC2146	Negative
26	<i>Streptococcus suis</i>	Isolated strains from feces	Negative
27	<i>Proteus mirabilis</i>	Isolated strains from urine	Negative
28	<i>Streptococcus salivarius</i>	Isolated strains from dialysate	Negative
29	<i>Neisseria perflava</i>	Isolated strains (unknown)	Negative
30	<i>Staphylococcus cohnii</i>	Isolated strains from environment	Negative
31	<i>Pseudomonas aeruginosa</i>	Isolated strains from sputum	Negative
32	<i>Enterobacter cloacae</i>	ATCC700323	Negative
33	<i>Serratia marcescens</i>	Isolated strains from sputum	Negative
34	<i>Candida krusei</i>	Isolated strains from urine	Negative
35	<i>Bacillus cereus</i>	Isolated strains from blood	Negative
36	<i>Escherichia coli</i>	ATCC25922	Negative
37	<i>Acinetobacter baumannii</i>	Isolated strains from sputum	Negative
38	<i>Pseudomonas aeruginosa</i>	ATCC27853	Negative
39	<i>Escherichia coli</i>	ATCC35218	Negative
40	<i>Streptococcus agalactiae</i>	Isolated strains from sputum	Negative

Table 2. Clinical sensitivity of *ply*-MCDA assay.

Method	Detection limit	Sputum
<i>ply</i> -MCDA	4.47 CFU/reaction	447 CFU/ml
PCR	447 CFU/reaction	44700 CFU/ml

Table 3. Primers used in this study.

Primera	Sequence and modification (5'-3')	Length ^b
F1	AGGATTTAAAACAGAGAGGAATT	23nt
F2	CGCCCCCTAAAATAACCG	18nt
CP1	GAGATAGACTTGGCGCCCATTCTGCAGAGCGTCCTTTG	38mer
C1	GAGATAGACTTGGCGCCCAT	20nt
C1*	5'-FITC-GAGATAGACTTGGCGCCCAT-3'	20nt
CP2	AAGGAGTCAAGGTAGCTCCTCCCTTCACTTCTGTATTGTCCAA	43mer
C2	AAGGAGTCAAGGTAGCTCCTC	21nt
D1	GCAACACTCGAAATATAGAC	20nt
D1*	5'-Biotin-GCAACACTCGAAATATAGAC-3'	20nt
D2	AGACAGAGTGGAAGCAGA	18nt
R1	ATCACTCTTACTCGTGGT	18nt
R2	AGTAGAGGCTGCTTTTGAAG	20nt

a C1*, 5'-labeled with FITC; D1*, 5'-labeled with Biotin.

b nt, nucleotide; mer, monomeric.

Figures

Figure 1

Confirmation of ply-MCDA products. (A) Colorimetric indicator of *S. pneumoniae* ply-MCDA products (Malachite Green, lake green indicates positive and colorless indicates negative); (B) LFB for visually detection of *S. pneumoniae* ply-MCDA products (positive with two red lines, the control line and the test line); (C) Gel electrophoresis of *S. pneumoniae* ply-MCDA products (positive with ladder-like bands). Reaction 1, positive control, *S. pneumoniae* ATCC49619; Reaction 2, negative control (*Staphylococcus aureus*); Reaction 3, negative control (*Salmonella typhi*); Reaction 4, blank control.

Figure 2

Optimal temperature of ply-MCDA assay. The DNA templates of *S. pneumoniae* ATCC49619 (1pg/reaction) were amplified at 62°C to 67°C (1°C intervals), respectively. The real-time turbidities were monitored at 650 nm (A-F). *Staphylococcus aureus* DNA and *Salmonella typhi* DNA were negative controls, and distilled water were blank control. Turbidity>0.1 was defined as positive.

Figure 3

Sensitivity of ply-MCDA assay. Serial dilutions of *S. pneumoniae* ATCC49619 DNA (10ng, 10pg, 1pg, 100fg, 10fg, 1fg, 0.1fg/ μ L) were subjected to be amplified at 65°C for 40min. Distilled water was blank control (BC). (A) Colorimetric indicator (Malachite Green, lake green indicates positive and colorless indicates negative). (B) Real-time turbidity curves of eight reactions were recorded at 650 nm. Turbidity>0.1 was defined as positive. (C) LFB detection of ply-MCDA products (two red lines represents positive and one red line represents negative). (D) Gel electrophoresis of ply-MCDA products (ladder-like bands indicate positive). 1-8: 10ng, 10pg, 1pg, 100fg, 10fg, 1fg, 0.1fg and BC.

Figure 4

AUDG enzyme eliminates carryover contamination. The sensitivity of ply-MCDA assay with AUDG (AUDG+) and without AUDG (AUDG-) were evaluated using 10-fold serial diluted simulated carryover contamination (UTP-incorporated ply-MCDA products, concentrations between 1×10^{-10} and 1×10^{-20} g/ μ L). The products were analyzed by colorimetric indicator (A) and LFB (B).

Figure 5

Location of the primers on the ply gene used for MCDA assay. The right arrows showed the direction from 5' to 3' ends and the sense sequences, the left arrows indicated the complementary sequences.