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Protocol and Matters for Consideration for the Treatment of Polymerase Chain Reaction Contamination in Next-Generation Sequencing Laboratories

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Abstract

***Objective**: The contamination of polymerase chain reaction (PCR) samples in molecular diagnostic laboratories can cause serious consequences. Internal quality control efforts are often inadequate, especially in clinical next-generation sequencing (NGS) laboratories. **Methods**: In this study, we retrospectively investigated an incidence of PCR contamination and its decontamination process in a clinical laboratory. We performed a series of measures for decontamination. Taqman fluorescence quantification was carried out to determine the presence of contaminating DNA. SYBR-Green PCR was conducted to evaluate the effect of chlorine disinfectant on NGS library preparation. **Results**: Through a series of elimination measures undertaken over 8 weeks, the decontamination process was verified as reliable. Almost no contamination was detected. Chlorine disinfectant should be forbidden in Illumina NGS laboratories because it may cause the failure of library preparation.**Conclusion**: Our prevention and decontamination strategies could effectively eliminate PCR amplicons. Chlorine disinfectants should not be used in Illumina NGS laboratories.*

***Keywords**: laboratory contamination; chlorine disinfectants; high-throughput library*

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Introduction

The polymerase chain reaction (PCR) technique is one of the most frequently applied methods in diagnostic and molecular biology laboratories. Molecular detection via PCR could be used for the qualitative or quantitative detection of bacteria, viruses, tumour cells, gene mutations associated with diseases and therapeutic response genes. However, false-positive results caused by DNA contamination is a fatal drawback in nucleic acid testing (1; 2). Amplicon contamination remains a major problem in laboratories despite the wide acceptance of PCR for the detection of target DNA.

PCR has the characteristics of high sensitivity and specificity for the *in vitro* amplification of target nucleotide sequences. PCR contamination could originate from several different sources, particularly positive plasmids and reverse tran-

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scription operations; the source of error is user-dependent(3). High copy numbers of DNA that exist in air or on the surfaces of objects could cause serious consequences(4). Nucleic acid aerosol pollution (airborne solid or liquid particles) when gas and liquid surfaces interact is the most common way of contamination. For example, aerosols can form and pollute the environment when reaction tubes are violently shaken during operation or centrifugation, PCR products are opened, and PCR samples are repeatedly aspirated. Aerosol easily diffuses and can cause the contamination of the whole PCR laboratory. This situation is very difficult to address and may even result in the closure of the PCR laboratory in serious cases. Hypochlorite reagents could be highly efficient in the removal of nucleic acid contamination and as a cleaning agent for work areas and equipment(5); 0.08% hypochlorite (*w*/*v*, 5 min) is recommended to eliminate fragments as small as 76 base pairs (bp). Our laboratory has been performing next-generation sequencing (NGS) as part of its clinical molecular diagnostic services for approximately 6 years. NGS laboratory disinfection is different from microbiology and cell culture laboratory disinfection because chlorine disinfectant could cause the failure of high-throughput library preparation.

Our laboratory recently suffered from the contamination of nucleic acid amplification products. After 2 months of treatment, the contamination was almost eliminated. In today's molecular diagnostic laboratories, many nucleic acid experiments are performed at the same time and in the same space, and many precautions should be taken in routine work. Our experience and lessons are summarised below.

Materials and Methods

Laboratory profile

We describe an episode of PCR contamination in the genetic diagnostic laboratory of the Northwest and Women's Hospital in Shaanxi Province, China, from June 20, 2021 to August 19, 2021. Our laboratory is mainly divided on the basis of functions into the reagent preparation, specimen handling, gene amplification and product analysis rooms. The laboratory also includes dressing, sample receiving, information analysis, NGS and chip analysis rooms. In accordance with the regulations of the conventional PCR laboratory, staff and materials flow in a one-way direction. Each area is independent, and facilities are used separately. Reagents and reaction mixes are transferred via transfer windows.

Methods of assessment

The primers and probes for the detection of the phenylketonuria-related variant in exon 12 of the *PAH* gene were synthesised by Sangon Biotech (Shanghai, China). All PCR reagents were obtained from TaKaRa (Dalian, China). The PCR program was 95 °C for 2 min, followed by three cycles of 95 °C for 15 s and 64 °C for 15 s; three cycles of 95 °C for 15 s and 62 °C for 15 s; three cycles of 95 °C for 15 s and 60 °C for 15 s; three cycles of 95 °C for 15 s and 59 °C for 15 and 45 cycles of 95 °C for 15 s and 60.5 °C for 15 s (fluorescence collection). PCR was conducted by using an ABI StepOne Plus Instrument (Applied Biosystems, Life Technology, USA) in a biological safety cabinet (model no: HFsafe-900, Shanghai Lishen, China)

Discovery of contamination

The internal control (IC) gene was amplified in all negative controls with Ct value < 26 cycles. The primers and reagents were replaced with new ones, and we found that all negative controls (DNA samples in pure water) also had the IC gene, indicating that the laboratory suffered from amplified fragment contamination. However, the source of contamination was unknown.

Tracing of the contamination source

The dressing room (sampling site 1), reagent preparation room (sampling site 2), specimen

preparation room 1 (sampling site 3), specimen preparation room 2 (sampling site 4), operated biosafety cabinet (sampling site 5), and NGS room (sampling site 6) were sampled to measure the degree of contamination. A total of 1 ml of pure water in an opened 1.5 ml tube was used as the collector. Each area was sampled for 1 h with three duplications. We discarded all reagents that had been opened, all supplies (e.g., tips, tubes, and boxes) were replaced and pipettors were completely disassembled and disinfected with ethyl alcohol and pure water. The following steps were performed: first, we washed every component with pure water, fully saturated them with 75% ethyl alcohol and allowed them to dry naturally.

Measures of decontamination

PCR amplicons in the laboratory can cause widespread aerosol contamination and can contaminate working surfaces, equipment, and personnel. The following measures for DNA decontamination were performed: (1) the surfaces of objects and equipment were wiped with 75% ethyl alcohol by using disposable rags to remove settled particles; (2) centrifuge rotors were disassembled, and PCR equipment was wiped with absolute ethyl alcohol; (3) air particles in the whole laboratory air were precipitated by using watering cans with pure water from the ceiling; (4) rooms and equipment surfaces were irradiated with ultraviolet (UV) light for 1 h; (5) disinfected work suits were worn before starting a new experiment; (6) floors were wiped with water and (7) fixed cleaning tools were used for each room and were not mixed. The above procedures were followed thrice each day for approximately 2 months, and the level of contamination at the six sampling sites was measured.

Assessment of the effectiveness of the DNA decontamination procedure

When the decontamination procedure was completed, comparison with a reference laboratory was performed. The comparison was based on the gradient dilutions of the PCR product of exon 12 of the *PAH* gene. We prepared 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}, and 10^{-10} dilutions of 1 µl of PCR products as the template. The procedure was the same as that described above. We used the 10 diluted samples and three pure water samples (negative control) as the templates to assess the similarity of the two laboratories. The collected results were statistically compared.

Chlorine disinfectant caused the failure of high-throughput library preparation

When we used chlorine disinfectant (500 mg/l) to wipe the surfaces of facilities, high-throughput library construction did not reach the required concentration. This situation happened twice in our laboratory, and 80 library samples were tested each time. However, when we used 75% ethyl alcohol solution and pure water to wipe the facilities, the libraries were unaffected. We applied a SYBR green kit (KAPA Library Quant Kit, Roche) to measure library quality. We collected data on tests that failed twice (160 samples) and tests that succeeded twice (160 samples). We performed the *t* test to evaluate the effect on the Ct value when chlorine disinfectant was used.

Statistical analysis

Statistical analysis was performed by using SPSS13.0. Data were analysed through the *t* test, and similarity analysis was conducted with the R^2 value and Pearson correction coefficients. *P* value < 0.05 was considered statistically significant.

Results

Determination of contamination sources

When we found DNA contamination in our experiments, we analysed the reason for this inci-

dent. Amplified PCR tubes were opened for gradient dilution (1 µl of PCR products was extracted for gradient dilution) in a biosafety cabinet. This situation could be the reason for contamination. All sites showed DNA amplification. The Ct value was approximately at 23 cycles. Sampling sites 1, 2, and 3 showed high amplification efficiency. However, PCR was not performed at these sites, nor were these sites in contact with related objects. The contamination could have occurred through aerosol (Figure 1).

Implementation of the decontamination procedure

By following the decontamination protocol, we focused on eliminating aerosol in the air and cleaning the surface contamination of the PCR-contacting rooms 4, 5 and 6. The most seriously contaminated site was site 5, followed by site 4, indicating that the source of DNA contamination were sites 4 and 5. We exactly diluted PCR products in the biosafety cabinet (site 5). Sites 4 and 5 are located in one room, and the biosafety cabinet is an A2 type, which recirculates 70% of the airflow within the cabinet while exhausting 30% of the airflow into the room(6). We used 75% ethyl alcohol and pure water to clean the inside and outside of the cabinet and replaced all supplies.

We used 75% ethyl alcohol and pure water to disinfect disassembled equipment and watering cans to precipitate air particles. We performed UV irradiation and air exchange. The Ct values gradually decreased each week over 4 weeks and presented the opposite trend as the PCR amplicon concentrations (Figure 1). Sites 1, 2, and 6 were further away from the source of contamination and demonstrated lower contamination loads than the other sites. The contamination at these sites was first eliminated in the first 2 weeks and was consistently maintained at negative levels. Sampling site 3 was adjacent to sam-

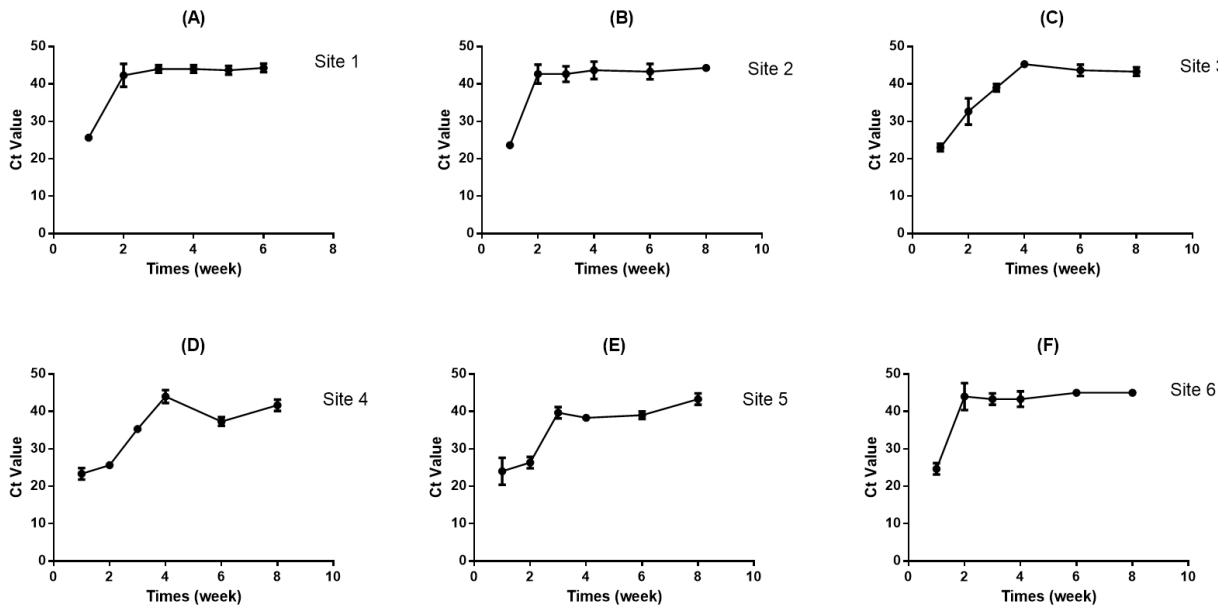

Fig. 1. Efficiency analysis of the surface decontamination in each site at six sampling times. Ct values were used as an index to evaluate the level of contamination.

pling point 4. The contamination at this site was slowly eliminated over 4 weeks. The PCR DNA levels at sites 4 and 5 decreased more slowly than those at other sites, and low levels of amplicons could be continuously detected at 6 weeks. Therefore, trace contamination remained. Contamination will not be completely removed within a short period (Table 1).

Assessment of the reliability of the decontamination method

We detected 10 gradient dilutions of PCR products and three pure water samples and compared the results of our laboratory to those of a reference laboratory (Figure 2). The statistical comparison of amplification results by similarity analysis produced the R^2 value of 0.9, which

Table 1. Assessment of contamination in different sites

Weeks	Dressing room (site 1)	Reagent preparation room (site 2)	Specimen preparation room 1 (site 3)	Specimen preparation room 2 (site 4)	Operated biosafety cabinet (site 5)	NGS room (site 6)
1	***	***	***	***	***	***
2	/	/	**	***	***	/
3	/	/	*	**	*	/
4	/	/	/	/	*	/
6	/	/	/	*	*	/
8	/	/	/	/	/	/

*** represent: Ct value≦30， ** represent: 30<Ct value≦35, * represent: 35<Ct value≦40.
/ represent: non-amplified result.

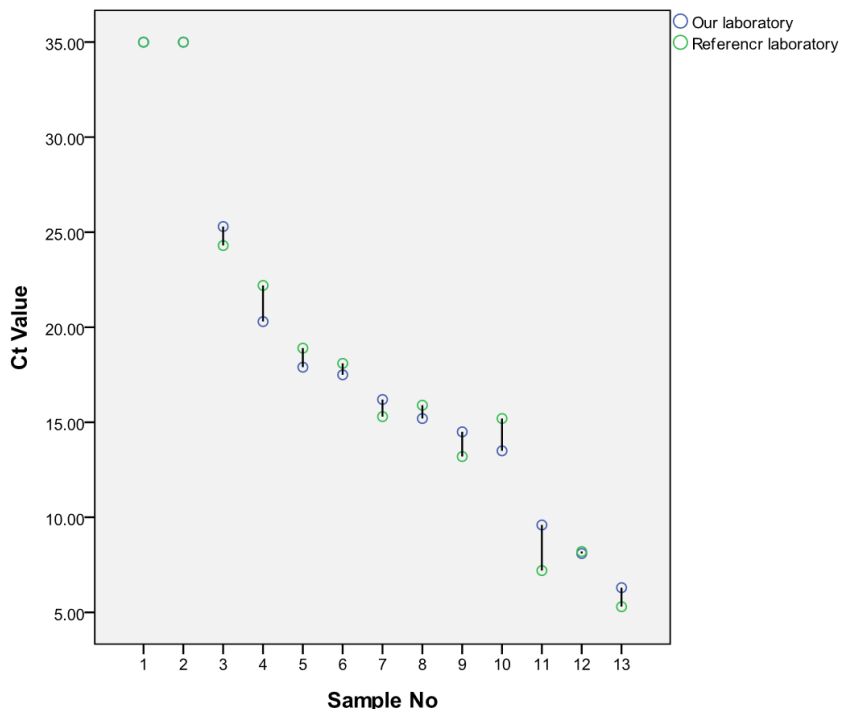

Fig. 2. Comparison of gradient dilution samples between two laboratories. Similarity analysis results produce an R^2 value of 0.9. Pearson correlation analysis showed a correlation coefficient of 0.991 (P<0.01)

implied high similarity. Pearson correlation analysis also showed a high similarity with the correlation coefficient of 0.991 ($P < 0.01$). The results demonstrated that our decontamination methods were effective.

Hypochlorite disinfectant

Our laboratory performs many medical tests, including NGS, which is applied to detect peripheral blood free DNA in pregnant women. We backtracked our experimental records to January 13-16, 2016. The incident first occurred when the cleaner used chlorine disinfectant to clean the environment. We also consulted other Illumina laboratories. These other laboratories also found that chlorine disinfectant could cause the failure of library preparation. The pooling concentration in library preparation was close to zero. When we used 75% ethyl alcohol solution and pure water to wipe the facilities on the second day, the library was unaffected. As a result of the careless use of chlorine disinfectant to clean floors and tables again in the NGS room, library preparation failed again (this PCR contamination accident). Hence, we validated that chlorine disinfectant could cause library failure. To the best of our knowledge, this study is the first to report that chlorine disinfectant could lead to the failure of library preparation in NGS. Statistical comparison was conducted on the use of chlorine and nonchlorine disinfectants in the environment. The obtained P value of 0.00 (significance threshold of $P > 0.05$) was considered statistically significant. The results demonstrated that chlorine disinfectant should be forbidden in library preparation using the Illumina NGS system (Figure 3).

Discussion

In the present study, we retrospectively studied a PCR contamination incident and its decontamination process in our PCR laboratory. Air samples were collected and analysed over 8 weeks. By using our cleaning and disinfection strategy, we effectively eliminated DNA contamination. In the disinfection procedures, we found that chlorine disinfectant could cause the failure of library preparation in NGS. Overall, we established an effective disinfection protocol that can be adapted by NGS laboratories.

The pathogen surveillance of the environment was considered to evaluate the efficacy of daily cleaning and disinfection in the hospital. Disinfection procedures may not be efficiently performed and may fail to sterilise pathogenic bacteria. In most clinical PCR laboratories, the diagnostic detection of pathogens is common. False-positive results or contamination is frequently reported in microbiology laboratories, and this phenomenon has far-reaching consequence for patients.

Our laboratory is mainly engaged in genetic diagnosis. PCR contamination had never occurred prior to the incident reported in this paper. In this incident, PCR was contamination attributed to the *PAH* gene mutation in phenylketonuria. During the period of the contamination incident, all negative controls started to yield false-positive results, and this problem could not be resolved by the extensive cleaning of the working areas and the use of new reagents.

Although the prevention of DNA contamination is a priority, contamination should be eliminated when it occurs. Many suggestions have already been published. Procedures that eliminate contaminating DNA in general include irradiation, enzymatic treatment, sodium hypochlorite disinfection, the HCl uracil–DNA–glycosylase/dUTP approach, and irradiation with the addition of psoralen(6). Nevertheless, the application of one of these classical decontamination approaches alone does not achieve complete or efficient results. For example, the uracil–*N*-glycosylase–dUTP approach is used to combat the substitution of dUTP for dTTP in the carry-over contamination of amplification products. UV irradiation is a common approach for surface

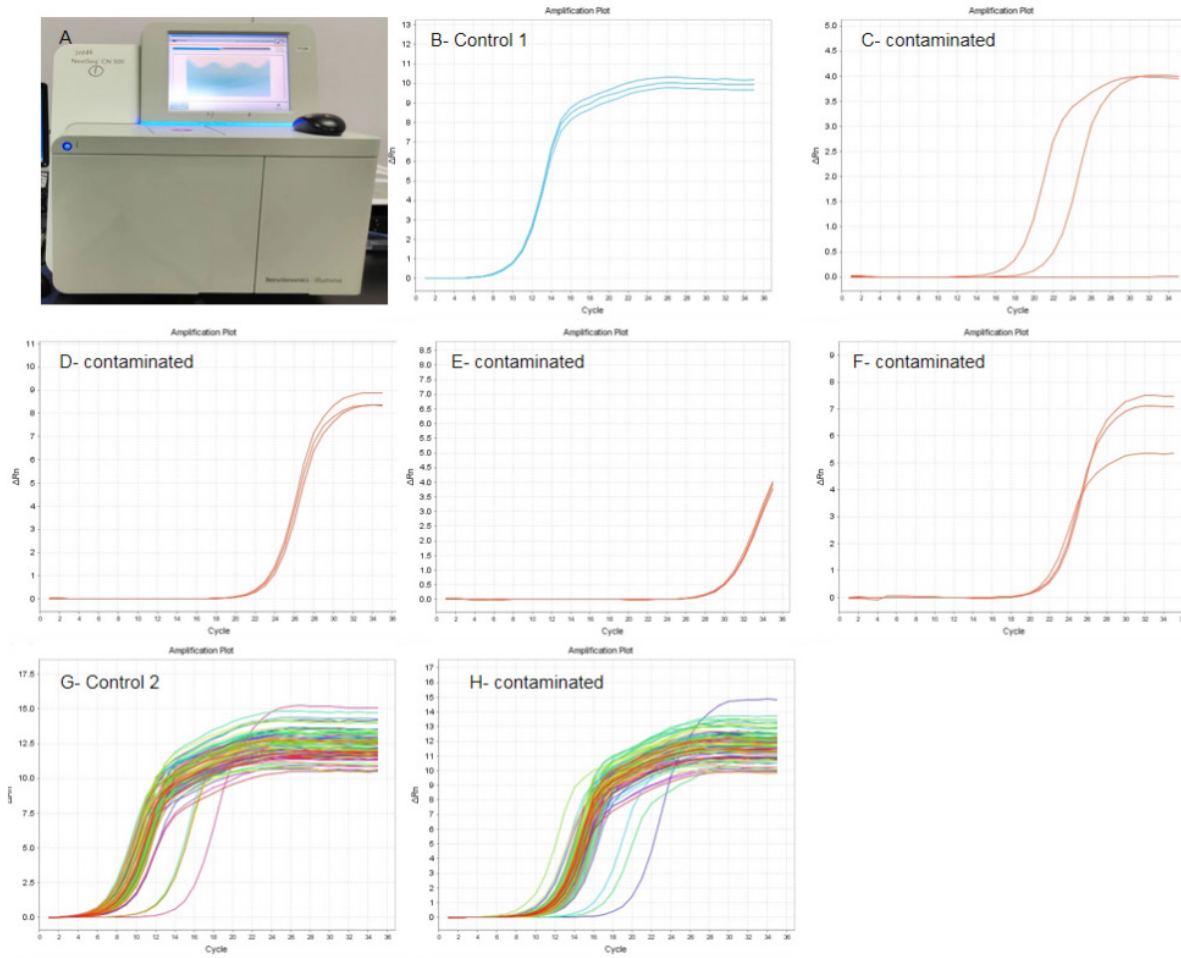

Fig. 3. Chlorine affects NGS lab library preparation in Illumina sustem. (A) Illumina system CN500 are our NGS equipment. (B-F) indicated that we used SYRB-Green to detect our high-throughput library concentration. B was a control the Ct Value <8 cycles. C-F were contaminated experiments, the Ct value: >16 cycles. The concentration of pooling in library preparation is closed to zero. This contamination was happened about in January 13-16, 2016. G an H was recently happened accident. When the PCR contamination, careless use of chlorine lead to failure of library preparation.

disinfection. However, the appropriate wavelength, intensity, duration of exposure time, and effects on the sensitivity of PCR of UV have to be determined empirically. UV irradiation does not completely eliminate PCR products > 200 bp in length(8). Although UV is recommended for eliminating contaminating DNA from surfaces, such as benches, floors and instruments, it is ineffective for decontaminating internal equipment components and dried DNA. Hence, we used watering cans to spray water from high to low areas, in which aerosol particles were intercepted, captured and finally fell onto the floor. This approach also increased the humidity in the rooms and maximised DNA degradation caused by subsequent UV irradiation.

Hypochlorite disinfectant is a cleaning agent for work areas and equipment. However, this

chemical solution can cause metal corrosion. In the general laboratory, the use of 0.05% sodium hypochlorite is recommended for sterilisation and incubation for 5 min to obtain good results. Hypochlorite solution would reduce DNA/RNA stability. NGS has rapidly evolved as part of routine clinical laboratory activities, and the use of hypochlorite solution in wet bench processes affects DNA library preparation, thus causing the failure of library preparation. Hence, hypochlorite should be forbidden in association with NGS. Through our laboratory decontamination process, the following measures could be employed to eliminate amplicons, especially those present as aerosol pollution:

1. Contaminated tests should be suspended, and whether other tests are affected should be confirmed.
2. All associated reagents and supplies (e.g., tips, tubes, and boxes) should be discarded.
3. The surfaces of some equipment, such as centrifuges and PCR instruments, should be wiped with 75% ethyl alcohol, and absolute ethyl alcohol could be used to treat internal or metal components. Disposable rags are recommended for application.
4. The use of disposables for cleaning tables, stools and floors is recommended.
5. By using watering cans to spray water from the ceiling, aerosol particles can be intercepted, captured and finally fall onto the floor. After precipitation, the floor should be cleaned.
6. The room should be irradiated with UV light for 1 h after watering can spraying.
7. Windows should be opened or ventilation fans should be used for air exchange.

Conclusions

PCR contamination can have far-reaching consequences, and the sources of contaminants are diverse and sometimes highly unexpected. Internal quality control is a perennial topic in the management of molecular laboratories, especially clinical NGS laboratories. Our strategies for the prevention and removal of nucleic acid contamination may serve as references to help improve laboratory practices and reduce the number of false-positive amplification results.

Authors' contribution

LW and HZ conceived and designed the study.
BH contributed to patient recruitment and phenotypic information collection.
CM performed the experiments.
RQ performed data analysis and interpretation.
LW drafted the manuscript.
All authors read and approved the final manuscript.

Competing Interests

The authors report no conflicts of interest.

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