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Note

Removal of contaminating DNA from commercial nucleic acid extraction kit reagents

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Abstract

Due to contamination of DNA extraction reagents, false-positive results can occur when applying broad-range real-time PCR based on bacterial 16*S* rDNA. Filtration of the nucleic acid extraction kit reagents with GenElute Maxiprep binding columns was effective in removing this reagent-derived contaminating DNA while the sensitivity of the assay was maintained.
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A broad-range PCR assay was recently developed, based on real-time PCR technology, to monitor bacterial contamination in platelet concentrates (PCs) (Mohammadi et al., 2003). The assay is rapid and enables the detection of 1 CFU equivalent/PCR. The assay combines the automated MagNA Pure DNA extraction and real-time amplification of a conserved sequence of the eubacterial 16*S* rRNA gene. The availability of fully automated extraction systems offers the benefit of standardization, high efficiency of purification and reduction of the potential risk of cross-contamination. However, the performance of the assay is compromised by a batch-dependent DNA contamination of the DNA isolation kit reagents (Peters et al., 2004). To achieve optimal sensitivity and reproducibility, it is important to remove this contaminating DNA. In the present study, a method to eliminate contaminating DNA in MagNA Pure Total Nucleic Acid kit reagents is described.

The real-time PCR assay was performed on 20 samples of PCs (derived from pools of 5 individual blood donors each). The presence of bacteria in these PCs was assessed by culture of a sample in the BacT/Alert (bioMérieux, The Netherlands). By this automated culture system, the PCs were deemed to be negative meaning that no bacteria were present.

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DNA was extracted from 200 μL of each sample with the commercially available MagNA Pure Total Nucleic Acid Extraction kit (Roche Diagnostics). Amplification and detection were performed simultaneously with the real-time PCR on the ABI Prism 7000 Sequence Detection System (Applied Biosystems). The PCR were performed in a final volume of 25 μL with the TaqMan Universal PCR Master Mix (1×TaqMan Universal PCR Master Mix, AmpliTaq Gold DNA Polymerase, AmpErase UNG, dTPs with dUTP, Passive Reference 1 and optimized buffer components; Applied Biosystems, Foster city, CA USA). The reaction mixtures included 900 nM of each of the universal primers (forward primer 5′-TCCTACGGGAGGCAGCAGT-3′, reverse primer 5′-GGACTACCAGGGTATCTAATCCTGTT-3′), 200 nM of the probe (6-FAM-5′-CGTATTACCGCGGCTGCTGGCAC-3′-TAMRA), and 5 μL template DNA. In each assay, isolation and PCR inhibition controls were incorporated. A negative control (no template control (NTC)) with water instead of template DNA was also included. Amplification conditions were 50 °C for 2 min, 95 °C for 10 min and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The results were evaluated based on the threshold cycle (C_T) values of each PCR reaction.

Unexpectedly, bacterial DNA was detected in all tested samples (including NTCs): C_T values ranging from 33 to 34 cycles were obtained.

Subsequently, the products of the PCR were purified and subjected to automated sequencing with the ABI Prism 310 Genetic Analyzer (Applied Biosystems). Sequencing was conducted with the same universal primers. Sequence analysis by using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/) revealed sequence similarities with bacteria inhabiting water and moist soil environments. Among these were *Burkholderia* spp., *Pseudomonas saccharophilia*, *Ralstonia* spp., *Alcaligenes* spp., and other related bacteria.

Since contaminating DNA in the PCR reagents was removed by digestion with *Sau*3AI (Mohammadi et al., 2003) and all measures to prevent cross-contamination were taken throughout the whole procedure from processing, DNA isolation to PCR amplification and detection, evidence was accumulating for a link between the MagNA Pure isolation reagents and the occurrence of false-positive results in the PCR assay.

Three approaches to remove contaminating DNA were evaluated and their effect on the sensitivity of the PCR was assessed.

First, the MagNA Pure Total Nucleic Acid kit reagents (Wash Buffer I, Wash Buffer II, Wash Buffer III, Lysis/Binding Buffer, and Elution Buffer of the extraction kit) were sonicated (Sanyo Soniprep 150). By sonication, DNA fragments were generated. Due to their minute size, these pieces of DNA could not be amplified by PCR. Only target DNA from genuine contamination of PCs would be then amplified and detected. The duration of sonication was varied between 2 and 30 min. This, however, did not sufficiently reduce the positive signals derived from the contaminating DNA (data not shown).

In a second attempt to eliminate contaminating DNA, a probe was designed which targeted a sequence of the 16*S* ribosomal RNA gene that is homologous in all *Burkholderia* spp. and related contaminants identified by sequencing. The sequence of the probe was (VIC)-5′-TAAGCACCGGCTAACTACGTGCCAGC-3′-(TAMRA). This probe was used in combination with the universal primers and probe set in a competitive real-time PCR. As the C_T values of the PCR reactions were almost equal when both probes were used, it was difficult to establish a clear difference between the targets. This strategy was therefore not pursued.

A third method consisted of filtration of the reagents of the Total Nucleic Acid kit with the aid of the GenElute Plasmid Maxiprep binding columns (Sigma-Aldrich, Germany). These silica-based membrane columns are constituents of a kit for the isolation of plasmid DNA from bacterial cultures. Except Magnetic Glass Particles and Proteinase K, all reagents of the MagNA Pure Total Nucleic Acid Extraction kit were transferred to the columns and centrifuged at 5000×*g* for 5 min. The flow-through was used in the MagNA Pure system to extract DNA from PCs. Compared to the C_T values obtained with the real-time PCR on DNA extracted with untreated reagents, the C_T values of DNA samples extracted with the treated reagents were higher (Table 1). This demonstrated the reduction of contaminating DNA. The background signals that were still obtained after filtration are attributable to the presence of contaminating bacterial DNA in the PCR reagents. This signal became completely negative (undetectable)

Table 1
Amplification of 16*S* rDNA performed with DNA isolated from five separate PCs using the indicated MagNA Pure extraction kits

Extraction kit	PCs samples[a]					
	1	2	3	4	5	NTC
Total Nucleic Acid	34.59±0.30	35.32±0.37	34.57±0.28	35.58±0.63	34.52±0.22	34.77±0.70
Total Nucleic Acid+filtration	37.30±0.5	37.3±0.21	37.58±0.60	37.73±0.77	37.07±1.61	37.13±0.11
M^{GRADE}	37.7±0.63	36.47±0.54	36.28±0.48	37.11±0.35	37.24±0.60	38.17±1.07

NTC: no template control.

[a] The results are expressed as C_T values. The mean±standard deviation of three independent runs is given.

after digestion of the PCR mixture with *Sau*3AI (Table 2). As described in a previous study (Mohammadi et al., 2003), this pretreatment was carried out routinely to remove contaminating DNA in the PCR reagents.

The MagNA Pure LC Microbiology kit (Roche Diagnostics) recently became commercially available, after which the manufacturer of the MagNA Pure reagents was notified of the problem of DNA contamination. All the reagents of this new kit are M^{GRADE} quality, ensuring that they are tested for and free of bacterial and fungal DNA contamination.

To confirm the purity of the reagents of this kit, DNA was isolated from the same samples as described above and the results were compared to the results obtained with the untreated- and the column-purified reagents. As shown in Table 1, the C_T values generated with DNA extracted with untreated reagents augmented substantially when the extraction from the same samples was conducted with the M^{GRADE} kit. Comparable results were also found when the reagents of the Total Nucleic Acid kit were first filtered with the columns. The included controls indicate the efficacy of DNA extraction and the absence of inhibitors of amplification. These results indicate that the use of commercial M^{GRADE} reagents or filtration of the reagents from the conventional MagNA Pure Total Nucleic Acid Extraction kit are equally effective methods to avoid contamination of PCR by DNA present in the extraction reagents.

To assess the effect of filtration on the sensitivity of the assay, PCs were spiked with bacterial suspensions to reach a final amount of 10^3, 10^1, and 10^0 CFU equivalent/PCR. The sensitivity of the assay performed with reagents that had been passed over the column was not altered: 1 CFU equivalent/PCR was still detectable (Table 2).

The use of GenElute columns was also effective for the elimination of contaminating DNA in QIAamp manual DNA extraction kits. Performance of PCR with DNA isolated from PCs without any pretreatment of the extraction reagents showed a mean C_T value of 36.84±0.9 (three independent experiments) although contaminating DNA in the PCR reagents was removed. When reagents of QIAmp DNA Blood Mini kit reagents (Qiagen) were filtered with the columns

Table 2
C_T values determined by real-time PCR for PCs samples extracted with Total Nucleic Acid kit, Total Nucleic Acid kit+filtration, and M^{GRADE} kit

Extraction kit	NTC	PCs samples[a]	PCs spiked with *E. coli* (No. CFU equivalent/PCR)[b]		
			1×10^3	1×10^1	1×10^0
Total Nucleic Acid	33.84 (38.93)	34.91 (37.71)	23.74 (23.16)	30.24 (30.01)	32.41 (32.25)
Total Nucleic Acid+filtration	39.28 (und)[c]	37.39 (und)	23.50 (21.41)	31.70 (28.72)	34.96 (34.34)
M^{GRADE}	37.48 (und)	36.96 (und)	24.80 (22.59)	32.22 (31.31)	36.77 (36.32)

[a] Culture-negative specimen was used as a negative control.

[b] Real-time PCR results are expressed as C_T values. Template DNA was isolated from PCs using Total Nucleic Acid kit, Total Nucleic Acid kit+filtration, and M^{GRADE} kit. The effect of filtration on the sensitivity of the assay was assessed by amplifying DNA extracted from PCs spiked with different amounts of *E. coli* suspensions. Numbers in parentheses are the C_T values obtained after digestion of the PCR mixture with *Sau*3AI. This pretreatment was carried out to remove contaminating DNA in the PCR reagents.

[c] No amplification signal was detectable.

prior to their use for the isolation of DNA from PCs (negative by culture), no amplification products were observed (mean C_T 39.48±0.4) indicating that they were free from contaminating DNA. A complete absence of amplification signals was observed when both filtration of the extraction reagents and digestion of the PCR reagents with *Sau*3AI were carried out. This method may therefore also be employed to eliminate DNA from reagents in other diagnostic PCR-based assays with manual DNA extraction methods.

Newly developed, high-throughput standardized automated extraction methods that substantially improve conventional manual methods are becoming increasingly popular in clinical laboratories. However, it is of utmost importance to consider potential pitfalls of these methods especially those concerning of false-positive results due to reagent contamination. Van der Zee and Crielaard (2002) and Evans et al. (2003) have previously demonstrated the contamination of Qiagen DNA extraction kits with *Legionella* DNA. Hence, DNA contamination should be taken into account when applying both automated and manual extraction methods in routine clinical microbiology.

Contamination derived from extraction reagents can result from a number of sources. In this study, the identified bacteria are inhabitants of a variety of aquatic environments. Other authors have reported before that these bacteria are indigenous to ultrapure water in industrial systems (Kulakov et al., 2002; Taghavi et al., 1996). Therefore, it is likely that this problem originates from the distilling systems of the manufacturer.

The method introduced here to overcome the problem of contaminating DNA in the MagNA Pure Total Nucleic Acid kit reagents is easy-to-perform and rapid. There is no significant loss of volume after filtration of the reagents. The use of Genelute columns may also prove useful for the filtration of other MagNA Pure kits reagents for example the Large Volume Total Nucleic Acid kit. In addition it is less expensive to use the columns compared to the M^{GRADE} kit at present. Moreover, the M^{GRADE} kit is only suited for the processing of 100 μL sample volumes, whereas the Total Nucleic Acid kit and the Large Volume Total Nucleic Acid kit are designed for DNA extraction from samples of 200 and 1000 μL, respectively. Finally, the results indicate that purification of the reagents with the columns did not hamper the sensitivity of the PCR assay.

In conclusion, purification of reagents to be used in DNA-based assays by centrifugation over GenElute columns is a generally applicable, easy, and fast procedure that can be incorporated in large number of protocols, especially in case of possible false-positive results. It can be then decided to use the GenElute columns to purify the reagents from contaminating DNA. For this pretreatment, modification of optimized procedures and protocols are not necessary.

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