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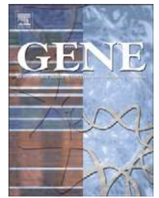

Short Communication

Improved method for the isolation of RNA from bacteria refractory to disruption, including *S. aureus* producing biofilm

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ABSTRACT

The development of fast, reliable and inexpensive phenol protocol is described for the isolation of RNA from bacterial biofilm producers. The method was tested on *Staphylococcus aureus* (*S. aureus*) and other biofilm-producing gram-negative microorganisms and provided the highest integrity of RNA recovery in comparison to other methods reported here. In parallel experiments, bacterial lysis with Qiagen, NucleoSpin RNAII, InnuREP RNA Mini, Trizol and MasterPure RNA extraction Kits using standard protocols consistently gave low RNA yields with an absence of integrity. The boiling method presented here yielded high concentration of RNA that was free from 16S and 23S rRNA, contained 5S RNA. Higher yields due to improved biofilm bacterial cell lysis were achieved with an added hot phenol incubation step without the need for a bead mill or the enzyme. This method when used in conjunction with the Qiagen RNeasy Mini kit, RNA isolation was a success with greater integrity and contained undegraded 16S and 23S rRNA and did not require further purification. Contaminating DNA was a problem with the RNA processing samples; we used quantitative real-time PCR (RT-qPCR) to measure the recovery of RNA from bacterial biofilm cells using the method described here.
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1. Introduction

Existing methods for the isolation of bacterial intact RNA are problematic especially when the important human pathogens *S. aureus* exuding extracellular glycocalyx that buries the organism in dense and coccoid-studded biofilms, became progressively more dense over time in vitro (Mangan et al., 1997). In addition, other compositions such as lipoteichoic acid and peptidoglycan increase the rigidity of the cell wall causing difficulty disrupted by standard method or detergent solutions (Buxton et al., 1987). Rapidity of lysis is the most important parameter in RNA extraction as the half life of bacterial mRNA is low short (Lewin, 1990). Current methods of RNA isolation from Gram-positive and Gram-negative bacteria are based on enzymatic lysis, or required cell wall disruption procedures (Lydia et al., 2010; Chomczynski and Sacchi, 2006). These methods have been effected and containing the model does not work well with bacterial producing biofilm. They are labor-intensive due to difficult handling and the need to perform extra precipitation steps which affect the total RNA isolation and result in low-integrity RNA. These effects profoundly alter the outcome of gene expression results (Kidon et al., 2003; Imbeaud et al., 2005). Meanwhile, the observed difference in gene expression data between intact and degraded RNA samples has led many authors to propose performing RNA quality control in order to obtain more accurate and reliable results (Courtney et al., 2008). This motivated us to find a simple protocol to isolate an intact RNA (including 5S RNA), which would work for the bacterial biofilm production with a high intercellular polysaccharide content. Therefore, the aim of this work was to describe a fast and easy-to-use phenol protocol that is time-saving for the recovery of RNA from the potential biofilm producing pathogen *S. aureus*.

Abbreviations: ATCC, American Type Culture Collection; BHI, brain heart infusion broth; cDNA, complementary DNA; DNase, deoxyribonuclease; *E. coli*, *Escherichia coli*; EDTA, Ethylenediaminetetraacetic acid; gDNA, wipeout buffer; MRSA, Methicillin-resistant *Staphylococcus aureus*; MSSA, Methicillin-sensitive *Staphylococcus aureus*; RNA, Ribonucleic acid; RT-qPCR, Quantitative Real-Time Polymerase Chain Reaction; *S. aureus*, *Staphylococcus aureus*; *S. epidermidis*, *Staphylococcus epidermidis*; SEM, scanning electron microscopy; TBE, Tris/Borate/EDTA; *P. aeruginosa*, *Pseudomonas aeruginosa*; RNase, Ribonuclease; ND-1000, NanoDrop; *16SrRNA*, 16S ribosomal RNA.

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2. Materials and methods

2.1. Bacterial strains and growth conditions

Four gram-positive (positive biofilm producer ATCC 35556, ATCC 29278 for methicillin-sensitive *S. aureus* (MSSA), ATCC 700698 for

methicillin-resistant *S. aureus* (MRSA) and *Staphylococcus epidermidis* (*S. epidermidis*) and two gram-negative bacteria (*Escherichia coli* (*E. coli*), *Pseudomonas aeruginosa* (*P. aeruginosa*) were subjected to RNA isolation procedure. The bacterial strains tested were obtained from the clinical microbiology laboratory at the University Putra Malaysia. The cells were each grown for 24 h in brain heart infusion broth (BHI, Difco Labs) at 37 °C, before they were diluted to 1:100 in fresh BHI medium containing 1% glucose. They were grown statically in 6-well polystyrene tissue culture plates for additional standard biofilm times of 48 h. The medium was poured and washed three times with distilled water to remove non-adherence free cells and replaced by fresh preheated medium every day. After the incubation time, the plates were washed ten times by submerging them in deionized water. The remaining attached bacteria were confirmed for their ability to produce biofilm using a scanning electron microscopy. The attached bacteria were suspended with 1 ml of RNase-free distilled water (Qiagen), while the cell densities were adjusted to 0.1 or 0.5 (600 nm optical) that corresponded to 1×10^9 cells/ml. They were pelleted by centrifugation for 3 min at 8000×*g*. RNA isolation were carried out by subjecting the pellets to five commercial RNA extraction kits together with the boiling and alternative simple phenol method described in this study.

2.2. RNA isolation by the commercial extraction method

The performance of five commercial RNA extraction kits was evaluated: RNeasy Mini Kit (Qiagen, Germany), NucleoSpin RNAII (Macherey-Nagel, Germany), InnuREP RNA Mini (Jena, Germany), Trizol (Invitrogen) and MasterPure RNA Purification Kits (EPICENTRE Biotechnologies). Three of the kits, except for Trizol and MasterPure kits, use spin-column based on silica membranes technology and the principles for RNA purification of these kits are very similar. The Trizol and Master Pure kits use a protocol, instead of a column, to purify RNA and capture the small RNA molecules that tend to be washed out in column based methods (Lydia et al., 2010). All RNA extractions were done in triplicate.

2.3. RNA extraction by the boiling method

The pallet biofilm cells were suspended with 1 ml of RNase-free distilled water and pelleted by centrifugation at 8000×*g* for 3 min in a 4 °C refrigerated centrifuge. The supernatant was discarded. The pallet was re-suspended with 100 μl of RNase-free H_2O and added with 5 μl of lysostaphin (10 mg/ml in sterile sodium acetate, Sigma, St. Louis, MO). After incubation at 37 °C for 10 min, 2.5 μl proteinase K (20 mg/ml, Qiagen) was added. The suspension was incubated at 37 °C for a further 10 min, boiled for 10 min and transferred to an ice bath for 1 min and centrifuged at 12,000×g for 10 min. The supernatant was transferred to an equal volume of chloroform. The tubes were inverted 1 min and centrifuged at 12,000×*g* for 10 min. The aqueous phase was collected and an equal volume of isopropanol was added to precipitate RNA. The mixture was inverted and vigorously vortexed for 3 min and centrifuged at 12,000×*g* for 10 min. The pellet was washed twice with 100 μl of 70% ethanol. After centrifugation, the pellet was air-dried for 3 min and dissolved in 25 μl of RNase-free distilled water. Total RNA was quantified using a NanoDrop® ND-1000 Spectrophotometer (NanoDrop, USA). The RNA integrity was checked by loading 5 μl of the total RNA into 1.4% agarose gel (Vivantis) containing 0.5× TBE buffer and electrophoresed at 120 V for 35 min.

2.4. Simple phenol method

Three steps were involved in the simple phenol method after carefully removing non-adherent cells. The remaining biofilm attached cells were suspended well with 1 ml of RNase-free distilled water and pelleted by centrifugation at 8000×*g* for 3 min in a 4 °C refrigerated centrifuge. The pellet was re-suspended in 100 μl of RNase free water. The tube was vigorously vortexed for 3 min and 100 μl of acid phenol was added with chloroform (1:1). It was vortexed for 1 min and incubated at 70 °C for 30 min. The vortex process was repeated periodically every 5 min. Subsequently, the tube was centrifuged at 12,000×*g* for 10 min and 100 μl from the aqueous (top only) phase was transferred into a new tube. This step is optional, i.e. it is to be included if commercial kits with column-based silica are used for RNA extraction. Seven hundred microliters of lysis buffer was added into the aqueous phase (700 μl was the best for use, i.e. optimized amount) and the subsequent steps were done according to the manufacturer's protocol of RNA kit, or following the method described here. Two hundred microliters of isopropanol was added into the aqueous phase to precipitate RNA. The tube was vigorously vortexed for 3 min and centrifuged at 12,000×*g* for 10 min. Two hundred microliters of 70% ethanol was added to clean the precipitated pellet from phenol and isopropanol and centrifuged at 8000×*g* for 5 min, and this step was repeated twice. The ethanol was removed and the tube was left upside down to dry for 3 min before 25 μl of RNase free water was added. The summary of this method is illustrated in Fig. 1. The RNA integrity was checked by loading 5 μl of the total RNA into 1.4% agarose gel containing 0.5× TBE buffer and electrophoresed at 120 V for 35 min. In addition, RNA concentration and purity were measured using the ND-1000 Spectrophotometer.

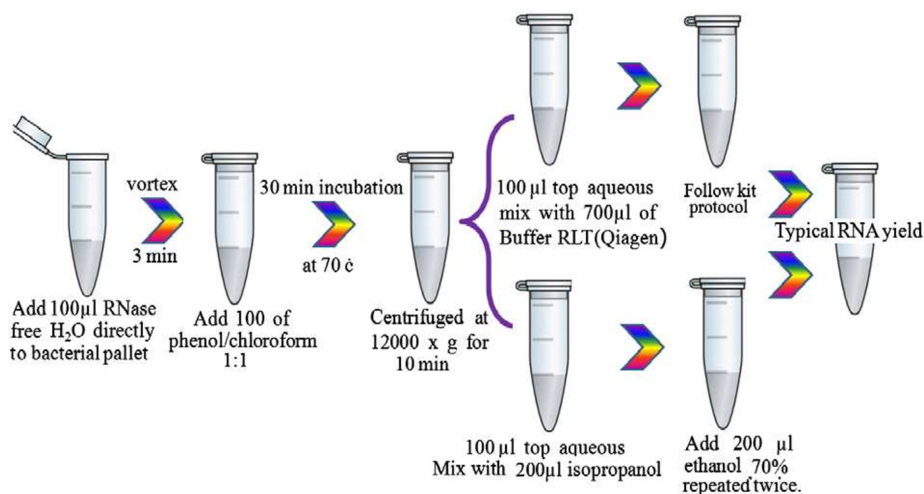

Fig. 1. The method of phenol for the RNA isolation from biofilm *S. aureus* producing.

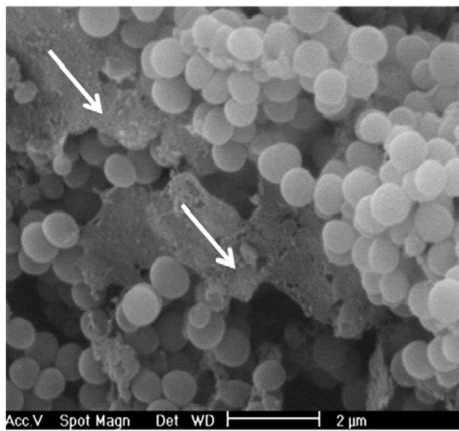

Fig. 2. A scanning electron micrograph revealing a heavy thick biofilm layer production of *S. aureus* ATCC 35556 reference strain.

2.5. Quantitative real-time PCR methods (RT-qPCR)

The equal amount of RNA concentration extracted by different methods was checked for the presence of DNA contaminating before and after treatment with 5 µl of DNase 1 and 5 µl of gDNA wipeout buffer (Qiagen). These RNA samples were used to check for the 16S rRNA gene as internal standard (GenBank accession no. (L37597.1)) using 16s gene-forward (5-CGGTCCAGACTCCTACGGGAGGCAGCA3) and reverse (5GCGTGGACTACCAGGGTATCTAATCC-3), but without the reverse transcriptase step (no cDNA). The RT-qPCR reactions were carried out using EvaGreen PCR Master Mix (Qiagen, Germany) in the Realplex (Eppendorf, Germany). The final volume of the PCR mixture was 20 µl and the mixture contained 10 µl master mix, 0.1 µl each of the forward and reverse primers, 2 µl of total RNA and 7.8 µl RNase free water. The cycling conditions were as follows: 1 cycle at 95 °C for 5 min followed by 40 cycles at 95 °C for 10 s, 55 °C for 30 s, 72 °C for 10 s. According to the manufacturer's instructions, purified RNA was reverse transcribed into cDNA using QuantiTect® Reverse Transcription kit (Qiagen). To check whether the cDNA template was of satisfactory and quality, it was adjusted to a final concentration and subjected to amplified 16S rRNA gene using qRT-PCR with 10× serial dilutions.

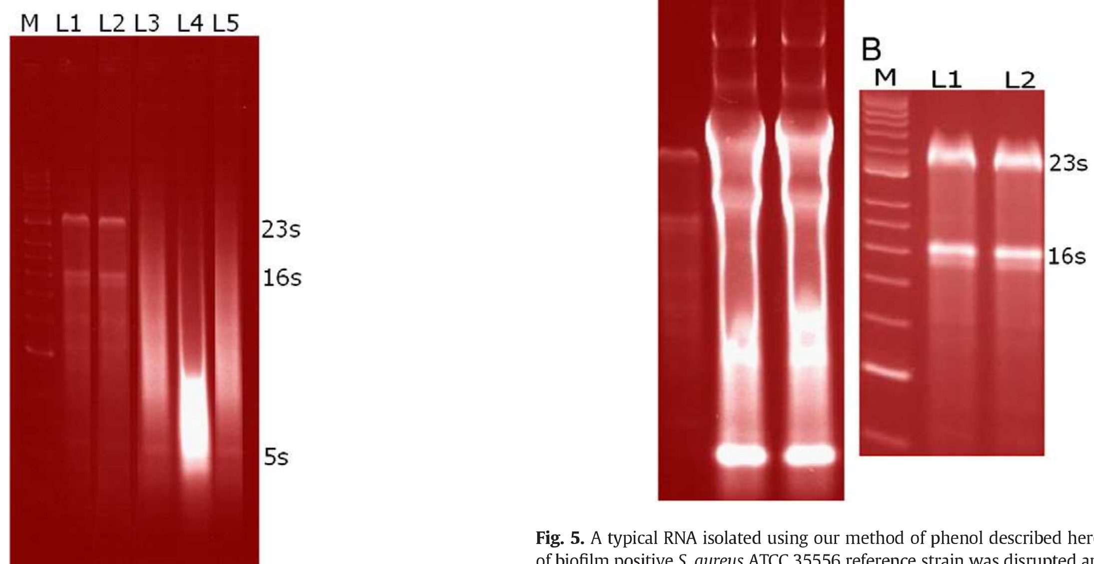

Fig. 3. Comparison of RNA extraction methods. 5 µl of total RNA from biofilm positive ATCC 35556 *S. aureus* reference strain was loaded on a 1.4% agarose gel and electrophoresed at 120 V for 35 min. L1, total RNA by the Trizol kit; L2, RNA by the MasterPure kit; L3, RNA by the RNeasy Mini kit; L4, RNA by the Nucleo Spin RNAII kit; L5, RNA by the InnuPREP kit.

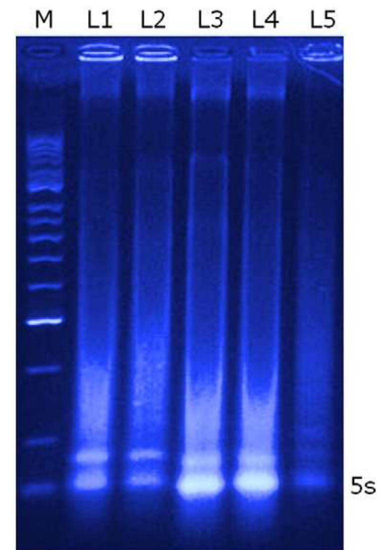

Fig. 4. 5S RNA integrity recovery from different bacteria positive biofilms by the boiling extraction method. The amount of RNA loaded in each lane was 5 µl. M, DNA ladder marker; L1, *S. epidermidis*; L2, Methicillin resistant *S. aureus* ATCC 700698; L3, Methicillin sensitive *S. aureus* ATCC 29278; L4, *Escherichia coli*; L5, *Pseudomonas aeruginosa*.

3. Results

Fig. 2 shows a heavy thick biofilm layer production by positive biofilm producer ATCC 35556 using scanning electron microscopy. The RNeasy Mini extraction kit, NucleoSpin RNAII kit and InnuPREP RNA Mini kit produced low level RNA recovery with an absence of integrity (Fig. 3, L3.4.5). Production of a high concentration of RNA recovery, with a poor integrity of degraded RNA, was achieved with the MasterPure and Trizol kits (Fig. 3, L1.2). The boiling method reported here yields good quantity and quality of RNA that is free of 16S and 23S rRNA, contained 5S RNA as shown in Fig. 4. A final alternative

Fig. 5. A typical RNA isolated using our method of phenol described here. The cell wall of biofilm positive *S. aureus* ATCC 35556 reference strain was disrupted and yielded RNA of sufficient quality and quantity using the phenol/isopropanol method (A, lane 1) and phenol/Qiagen kit (A, lane 2). B: refers to the RNA which was generated from diluted total RNA in the image of A to 1:10 in dH_2O, and then was loaded into 1.4% agarose gel and electrophoresed at 120 V for 35 min, yielded RNA of sufficient non-degraded 23S and 16S rRNA quality.

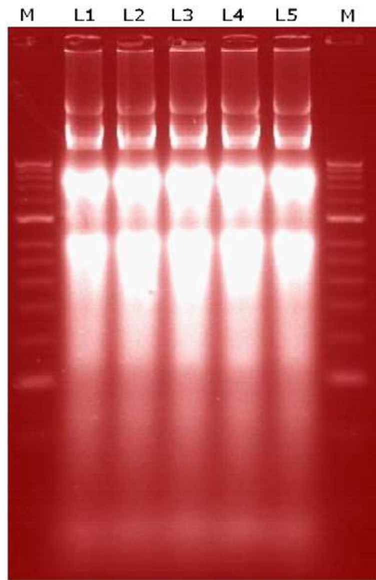

Fig. 6. RNA was successfully isolated from several different bacterial positive biofilms by the phenol/isopropanol extraction method. The amount of RNA loaded in each lane was 5 μl. M, DNA ladder marker; L1, *S. epidermidis*; L2, Methicillin resistant *S. aureus* ATCC 700698; L3, Methicillin sensitive *S. aureus* ATCC 29278; L4, *Escherichia coli*; L5, *Pseudomonas aeruginosa*.

simple method of phenol/isopropanol described in this study that was not kit based gave both high quantity and quality RNA from bacterial biofilm production compared to the commercial kits (Fig. 5, L1 and Table S1). Moreover, this method when used in combination with Qiagen kits (phenol/Qiagen) gave high integrity of RNA content and quantity (Fig. 5, L2). It also successfully isolated a typical RNA from different bacterial positive biofilms including *S. epidermidis*, MRSA ATCC 700698, MSSA ATCC 29278, *E. coli* and *P. aeruginosa* (Fig. 6).

For the purpose of verifying the integrity of DNA free RNA, the results indicated that the RNA extracted by five commercial extraction method, when non-treated and treated as many as 4 times with DNase and gDNA, the DNA was still present for the use of RNA in RT-qPCR experiments (Fig. 7), while the method with phenol/isopropanol, phenol/Qiagen and boiling showed a significant reduction of DNA that was treated once with DNase and gDNA buffer (Fig. 8). Fig. 9 shows an example of a real-time PCR 16S rRNA product curve that was successfully amplified using 10× serial dilutions of cDNA reverse-transcribed from the extracted RNA by method of phenol.

4. Discussion

The present study demonstrates that isolation of RNA from biofilm bacterial production requires a lysis step and that high quantity and quality RNA can be obtained using a simple phenol extraction method. Our work is part of growing evidence suggesting that RNA quality must first be evaluated before downstream applications are pursued. Methods for isolating intact RNA from biofilm are challenging. We examined RNeasy Mini Kit, NucleoSpin RNAII kit and InnuREP RNA Mini kit. Sample processing for these kits involves cell lysis and subsequently the recovery of RNA. The quantities of RNA obtained using these methods were low (Fig. 3, L3.4.5), probably because poor lysis of bacterial cells that were buried in dense studded structure due to the physiological characteristics of the biofilm, e.g. the constitution of the bacterial cell wall may impede cell lysis and the liberation of RNA from the cell (Ludwig and Schleifer, 2000). An increase in yield was achieved with lysis step inserted before proceeding through the use of Trizol and MasterPure RNA Purification methods. Although we saw an increase in yield, the RNA had poor integrity and was partially degraded (Fig. 3, L1.2). These methods did not enhance the quantity or quality of RNA isolation. We found that a biofilm bacterial lysis step was required for all methods to achieve larger quantities of RNA. However, high quality of RNA was achieved through boiling and simple phenol method described in the present study. The boiling method provided good quality RNA that was free from 16S and 23S rRNA (Fig. 4) and did not require further purification. The absence of rRNA in this preparation is probably due to heat degradation (Kidon et al., 2003).The phenol method has been used by many (VanKeulen et al., 2004; Chomczynski and Sacchi, 1987; Cook and Britt, 2007; Phongsisay et al., 2007). This method is not favored because of the complicated steps involved. In addition, more biochemical reagents are needed and it is also time-consuming. The phenol method proposed here involves only three steps for the isolation of RNA. We found that the integrity of RNA was high in comparison to other methods. It was optimized to produce intact RNA that includes 16S, 23S rRNA and 5S RNA. Furthermore, it is time saving. The presence of 16S and 23S rRNA intense bands indicates that the extracted

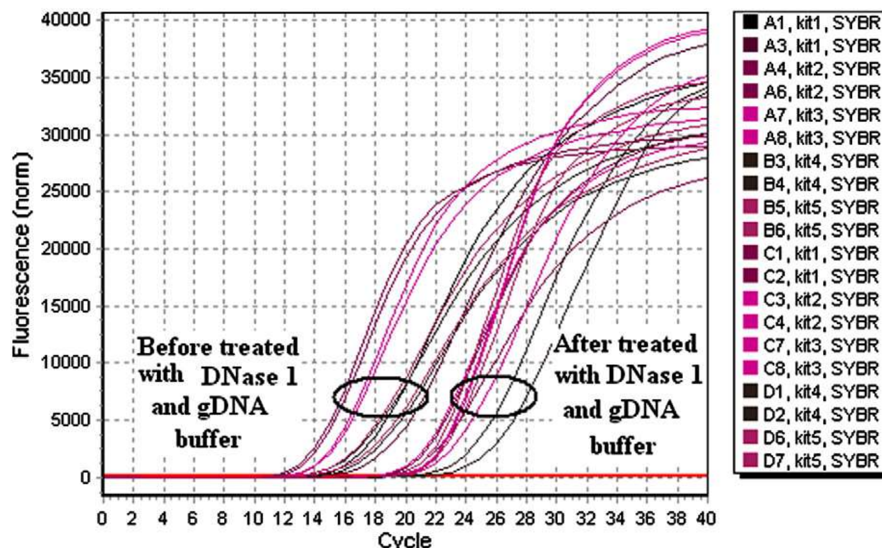

Fig. 7. Determine the purity of the RNA by RT-qPCR. Curves left and right show the amplification of 16sRNA in RNA samples prepared from five RNA extraction kits [Trizol kit (kit1) MasterPure kit (Kit2), RNeasy Mini Kit (KIT3), Nucleo Spin RNAi kit (KIT4) and InnuPREP kit (kit5)] that were not reversed into cDNA. The left curve was not treated with DNase 1 and gDNA buffer (Qiagen). The right curve was treated 4 times with DNase and buffer gDNA. The experiments were performed in duplicate.

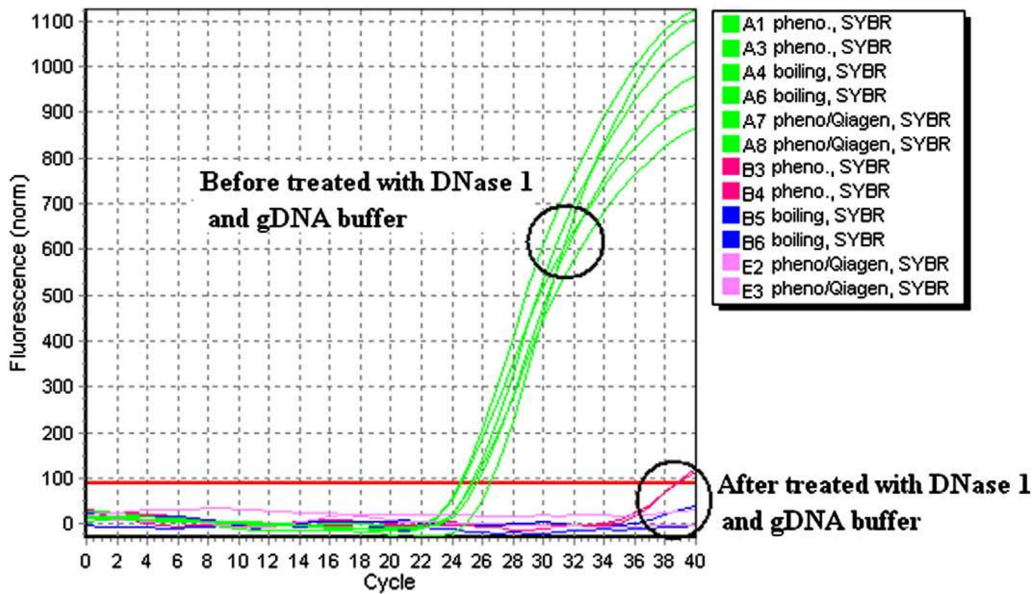

Fig. 8. Determine the purity of the RNA by RT-qPCR. Left curve shows the amplification of 16sRNA in RNA samples that are not reversed to cDNA and were prepared from the method of phenol/isopropanol, phenol/Qiagen and boiling. They were not treated with DNase 1 and Buffer gDNA. Right curve was treated once with DNase 1 and buffer gDNA and giving negative Ct values of amplification, indicating the level of contamination of genomic DNA was not deductible by 16sRNA. The experiments were performed in duplicate.

RNA is not degraded (VanKeulen et al., 2004). The RNA produced by the phenol method was not similar to that of commercial kits as it did not involving enzymatic disruption method (Fig. 5, L1). RNA extracted by this method before the isopropanol step could be mixed with 700 μl of lysis buffer RLT (Qiagen) and the RNA yield was significantly higher than the methods that used enzymatic lysis step (Fig. 5, L2). The phenol method is also applicable to other gram positive and gram negative bacteria (Fig. 6). A comparison of the concentration and purity ratios and the integrity of RNA obtained by different methods suggested comparable quality and yield (Table S1).

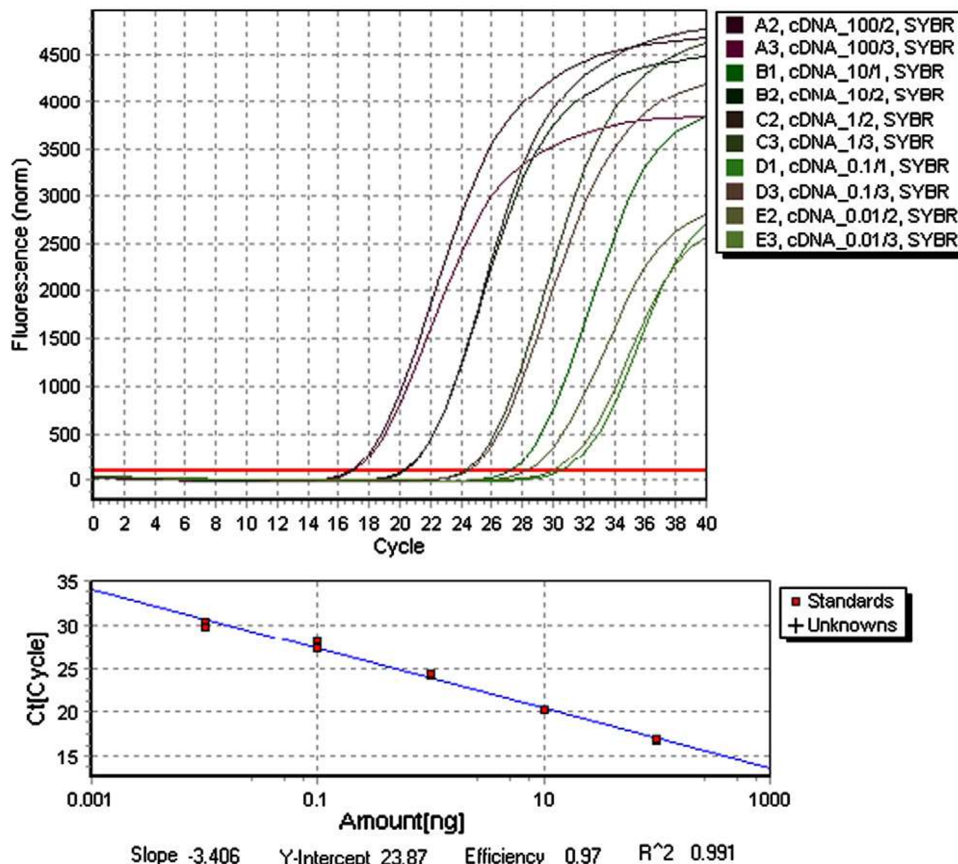

Fig. 9. Real-time PCR with serial dilutions of reverse-transcribed isolated RNA. The standard curve was generated from 10× serial dilutions of cDNA. RNA isolated using our method of cell wall disruption phenol yielded RNA of sufficient quality and quantity for satisfactory real-time PCR.

As a control prior for ensuring that DNA free RNA, RT-qPCR of 16sRNA was used to detect the presence of DNA in the RNA preparations produced by RNA extraction method reported here. The Ct values obtained from amplification of 16sRNA still had detectable genomic DNA contamination at levels too high in RNA extracted from five commercial kits before and even treated more than 4 times with DNase I and gDNA buffer, as a high DNA content of RNA samples is not reduced, it was interfering with the detection of 16sRNA (Fig. 7). While, the Ct values obtained from amplification of 16sRNA after one treatment with DNase and gDNA buffer completely reduced, giving a negative signal in RT-qPCR with the phenol/isopropanol or phenol/Qiagen RNA extraction and boiling method (Fig. 8), these methods gave a high concentration of RNA and low levels of DNA, leading to easy removal of DNA. Therefore, the phenol method may be more useful and appropriate for testing, such as Northern blot analysis, which requires large amounts of RNA. In addition, the purified RNA using the method of phenol descript here when reversed to cDNA template, the derived standard curve showed that the correlation coefficient, R2, is 0.99 and amplification efficiency, E, is 0.97 of the serial dilutions as calculated by Realplex software indicate that the template was of satisfactory quality and purity using RT-qPCR (Fig. 9).

5. Conclusion

The modified method of cell wall disruption for subsequent use in a silica spin column-based RNA isolation kit or the phenol/isopropanol method described is universally effective for RNA recovery from bacterial producing biofilms. It is cost effective compared to enzymatic digestion or mechanical disruption. This method also drastically reduces the amount of DNA contamination, resulting in less DNase treatment. It can be said that this method can be utilized as an alternative cell wall disruption method for isolating RNA from biofilm.

Supplementary materials related to this article can be found online at doi:10.1016/j.gene.2011.12.010.

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