Isolation and purification of total RNA from *Streptococcus mutans* in suspension cultures and biofilms

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Abstract: The presence of extracellular polysaccharides matrix makes extraction and purification of RNA from *Streptococcus mutans* within biofilms challenging. In this study, several approaches to purify RNA extracted from *S. mutans* in suspension cultures and biofilms were examined. The combination of sonication (3 pulses of 30 s at 7 W), suspension in NAES buffer (50 mM sodium acetate buffer, 10 mM EDTA and 1% SDS; pH 5.0) and homogenization-mechanical cells disruption in NAES-acid phenol:chloroform, yielded 9.04 mg (or 0.52 mg) of crude preparation of RNA per 100 mg of total cell (or biofilm) dry-weight. The crude RNA preparations were subjected to various DNAse I treatments. The combination of DNAse I in silica-gel based column followed by recombinant DNase I in solution provided the best genomic DNA removal, resulting in 4.35 mg (or 0.06 mg) of purified RNA per 100 mg of total cell (or biofilm) dry-weight. The cDNAs generated from the purified RNA sample were efficiently amplified using *gtfB S. mutans*-specific primers. The results showed a method that yields high-quality RNA from both planktonic cells and biofilms of *S. mutans* in sufficient quantity and quality for real-time RT-PCR analyses.

Descriptors: Dental plaque; *Streptococcus mutans*; Polymerase chain reaction; RNA; Polysaccharides.

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Introduction

Real-time reverse transcription-polymerase chain reaction (RT-PCR) is the most sensitive and precise method to measure low abundance gene expression in biological samples.[1] Determination of mRNA levels using real-time RT-PCR by monitoring formation of PCR products (amplicons) with the fluorescence dye SYBR Green I is being extensively used in biomedical sciences, including oral microbiology; SYBR Green I fluorescence dye binds specifically to the minor groove double-stranded DNA.[2]

Real-time RT-PCR in general is a reliable technique, but requires a reproducible and well-defined methodology for RNA extraction and purification for accurate determination of mRNA levels. Several methods of RNA extraction and purification have been reported for oral bacteria grown in planktonic state, including oral streptococci.[3-8] In contrast to planktonic cells, RNA isolation from microorganisms within biofilms is challenging because of the presence of an extracellular polysaccharide matrix, which may interfere with the extraction and purification of the nucleic acids, especially with genomic DNA removal.[9] RNA isolation and purification from polysaccharide-rich tissues, such as in plants and fruits, are difficult because these polymeric substances bind to nucleic acids, inhibit translational activity of RNA and interfere with DNAse activity.[10-12] Therefore, a RNA isolation and purification method that consistently yields high quality RNA from both planktonic cells and biofilms in sufficient quantity for functional genomic based experiments such as real-time PCR would be useful.

In this study, several methods of RNA purification were examined using *Streptococcus mutans* in suspension cultures and biofilms as source of RNA.

Materials and Methods

Preparation of planktonic cells and biofilms of *S. mutans* UA159

Planktonic cells of *Streptococcus mutans* UA159 (serotype c)[13] were grown in 10 ml of ultrafiltered (Prep/Scale, Millipore Co., Billerica, MA, USA) tryptone-yeast extract broth (2.5% tryptone and 1.5% yeast extract, pH 7.0) with 1% glucose.[14] Biofilms of *S. mutans* UA159 were formed on standard glass microscope slides (surface area of 37.5 cm^2; Micro slides, VWR Scientific Inc., West Chester, PA, USA) in batch cultures for 5 days as detailed elsewhere.[15,16] Bacterial suspensions (planktonic cells) were harvested at late-exponential phase ($OD_{600\ nm}$ 1.0), and the cell pellet (~ 4 mg dry weight) was kept in RNALater according to the manufacturer's protocols (Ambion, Inc., Austin, TX, USA). The biofilm (5 days-old, ~ 84 mg dry-weight) was removed from the glass slides using a sterile spatula, split in 3 samples (~28 mg dry weight each) and also kept in RNALater at 4°C overnight (~18 h).

Extraction of total RNA

Initially, the RNALater solution was carefully removed using an automatic pipette without disturbing the biofilm or cell pellet. The biofilm or cell pellet was washed three times with 7.0 mL of cold PBS. At the third washing step, the suspension was sonicated with three pulses of 30 s at 7 W with 1 min interval on ice (Branson Ultrasonics Co., Danbury, CT, USA). After the washing-sonication procedure the biofilm (or cell) suspension was centrifuged at 5,500 × *g*, 4°C, for 10 min and the pellets were subjected to RNA extraction according to published protocols,[4,8] with some modifications. Briefly, the pellets were re-suspended in 0.75 ml RNAse-free NAES buffer (50 mM sodium acetate buffer, 10 mM EDTA and 1% SDS, w/v, pH 5.0) and vortexed for 1 min. The same volume of acid phenol:chloroform (5:1, pH 4.5; Ambion, Inc., Austin, TX, USA) was added to the suspension, then vortexed and transferred to 2.0-ml screw-cap microcentrifuge tubes containing 0.8 g of glass beads (0.5-mm diameter; Biospec Products, Bartlesville, OK, USA). The cells were lysed in a Mini-Bead Beater homogenizer (Biospec Products) at 4°C for a total of 120 s (beat three times for 40 s with 1 min interval). The homogenized suspension was centrifuged at 10,000 × *g* for 5 min at 4°C, and the aqueous phase was collected and transferred to a microcentrifuge tube to which was added 0.75 ml of acid phenol:chloroform (5:1, pH 4.5). The tube was vortexed briefly, and centrifuged at 13,000 × *g* for 5 min at 4°C. The aqueous phase was collected and extracted with a 1:1 solution of chloroform:isoamylalcohol (24:1; Ambion,

Inc.) once. Total RNA was precipitated using a 1/10 volume of 3 M sodium acetate (pH 5) and 1 volume of isopropanol at –20°C for at least 30 min.

DNAse treatments

The RNA precipitates were recovered by centrifugation (13,000 × *g*, 4°C for 15 min) and the pellet washed with ice-cold 75% ethanol (v/v) three times followed by 99% ethanol; the crude RNA was resuspended in molecular grade water, and quantified spectrophotometrically (absorbance at 260 nm, A_{260}). Extracted crude RNA was treated enzymatically with DNAse I to remove contaminant genomic DNA. Two types of commercially available DNAse I treatments were used:

1. on column DNAse I (Qiagen RNeasy Mini kit, Qiagen Sciences, MD, USA)
2. protein engineered DNAse I in solution (Turbo DNAse; Ambion Inc.).

An aliquot of the extracted RNA (~ 50 μg) was treated with the DNAse I according to the manufacturer's protocols either alone or in combinations as follows:

1. Approach T: Ambion's Turbo DNAse I protocol (2 U of Turbo DNAse I/10 μg RNA) followed by Qiagen RNeasy MinElute (for DNAse I removal)
2. Approach Q: Qiagen's RNeasy on column DNAse I protocol (2.7 U of DNAse I/10 μg RNA)
3. T followed by Q
4. Q followed by T

Determination of RNA purity and integrity

The quality of the purified RNA was examined by:

1. 1.2% formaldehyde agarose gel electrophoresis and ethidium bromide staining;[17]
2. Agilent 2100 electrophoresis bioanalyzer (Agilent Technologies, Santa Clara, CA, USA);[18]
3. absorbance ratio A_{260}/A_{280} in Tris buffer 10 mM pH 7.5.[17]

Carbohydrate analysis

The total concentration of carbohydrates in crude RNA preparations was quantified by the phenol-sulfuric method using glucose as standard.[19,20] Since nucleic acids react in this method a correction was applied based on the concentration of carbohydrate expected in RNA.[10] The mathematical relationship between RNA concentration found at 260 nm and its carbohydrate concentration estimated by phenol-sulfuric method was determined using RNA standard (R6750, Sigma-Aldrich, St. Louis, MO, USA). Therefore, by difference it was possible to estimate the polysaccharide contamination found in the RNA.

Real-time reverse transcriptase PCR (RT-PCR) analysis

To check for genomic DNA contamination, 1 μg of purified RNA (without reverse transcriptase) from each of the DNAse treatment procedures were amplified by a MyiQ real-time PCR detection system with iQ SYBR Green supermix (Bio-Rad Laboratories, Inc., CA, USA) containing specific primer sets for *gtfB*.[6,14] In addition, cDNAs were synthesized from 1 μg of purified RNA samples using BioRad iScript cDNA synthesis kit (Bio-Rad Laboratories, Inc., CA, USA) which contains a MMLV RNase H+ reverse transcriptase and random hexamers. The resulting cDNA was used as template in the real-time PCR step; a reaction containing only the reagents (no template control) was also included.

Results

The yield and purity of the crude preparations of RNA extracted from planktonic cells and biofilms of *S. mutans* are shown in Table 1.

Table 2 shows the yield and indicators of the RNA purified after the DNAses treatments.

In general, the combination of DNase I treatments (T + Q and Q + T) provided better genomic DNA removal than did either treatment alone. Table 3 shows real-time RT-PCR data using purified

Table 1 - Yield and purity of the crude RNA.

Growth condition	Yield, mg/100 mg[a]	Ratio	
		A_{260}/A_{280} ηm	[b][Carbohydrate]/[RNA]
Planktonic cells	9.04 ± 0.89	2.15 ± 0.02	0.17 ± 0.03
Biofilm	0.52 ± 0.07	2.11 ± 0.01	0.30 ± 0.04

Values are means ± SD (n = 3). [a]Dry-weight; [b]RNA Sigma = 0.19.

Table 2 - RNA yield and estimation of genomic DNA removal after DNAse treatments.

DNAses Treatments	RNA yield mg /100 mg dry-weight		DNA Removal (%)		Ratio [Carbohydrate]/[RNA]*	
	Planktonic	Biofilm	Planktonic	Biofilm	Planktonic	Biofilm
[a]T	6.07 ± 0.35	0.14 ± 0.02	32.5 ± 3.9	72.5 ± 3.0	0.16 ± 0.05	0.19 ± 0.02
[b]T + Q	3.61 ± 0.84	0.08 ± 0.01	59.9 ± 9.4	84.6 ± 2.0	0.16 ± 0.06	0.20 ± 0.02
[c]Q	4.86 ± 0.57	0.10 ± 0.04	46.0 ± 6.4	81.7 ± 6.8	0.16 ± 0.01	0.23 ± 0.01
[d]Q + T	4.35 ± 0.32	0.06 ± 0.03	51.6 ± 3.5	87.6 ± 5.9	0.16 ± 0.02	0.18 ± 0.03

Values are means ± SD (n = 3). [a]Turbo DNAse treatment + RNeasy cleanup; [b]Turbo DNAse treatment + RNeasy protocol; [c]RNeasy protocol; [d]RNeasy protocol + Turbo DNAse treatment + RNeasy cleanup. *RNA Sigma = 0.19.

Table 3 - Real-time RT-PCR data (C_t values) using purified RNA as a template.

Treatments	*gtfB*	
	Planktonic	Biofilm
[a]T	25.7 ± 0.2	26.2 ± 0.3
[b]T + Q	29.2 ± 2.9	30.2 ± 0.3
[c]Q	24.2 ± 0.9	22.8 ± 0.6
[d]Q + T	33.0 ± 1.0	33.1 ± 0.9

Values are means ± SD (n = 3). [a]Turbo DNAse treatment + RNeasy cleanup. [b]Turbo DNAse treatment + RNeasy protocol. [c]RNeasy protocol. [d]RNeasy protocol + Turbo DNAse treatment + RNeasy cleanup.

RNA (using different approaches) as a template. Clearly, RNA samples subjected to combination of DNAse I treatments provided much higher C_t values than a single DNAse I treatment, which agrees well with the data shown in Table 2. Moreover, the approach Q + T resulted in RNA samples with the highest C_t values (≥ 33) indicating negligible amounts of genomic DNA; the no-template control showed C_t values ≥ 34.

Figure 1 shows the integrity of RNA extracted and purified according to different approaches.

Discussion

The procedure for RNA extraction described in this study yielded about 9.04 (or 0.52 mg) of crude RNA extract per 100 mg of cell (or biofilm) dry weight (Table 1). The A_{260}/A_{280} ratios were above 2.1 irrespective of whether the RNA is extracted from planktonic cells or biofilms, suggesting little or no protein contamination. In contrast, the ratio [carbohydrate] / [RNA] of crude RNA from planktonic cells was 0.17 whereas the same ratio for the RNA from biofilms was 0.30, indicating presence of contaminant polysaccharides since the ratio for pure RNA (Sigma) is 0.19. Polysaccharides have been shown to interfere with cDNA synthesis, RT-PCR and hybridization in northern analyses,[10,21,22] and should therefore be eliminated during the RNA purification process.

For successful isolation of intact RNA, it is also important to remove as much contaminant genomic DNA as possible in an RNA preparation,[1,23] which is a challenge procedure particularly for biofilms.[9] Thus, each of the RNA extracts was subjected to various approaches of DNAse treatments to examine their effects on RNA yield and genomic DNA removal. The yield after DNAse I treatments was 3.61 to 6.07 mg of RNA/100 mg of cell dry-weight and 0.06 to 0.14 mg of RNA/100 mg of biofilm dry-weight, showing an estimated 32.5 to 59.9% and 72.5 to 87.6% of genomic DNA removal (Table 2). The ratio [carbohydrate]/[RNA] for the RNA from biofilms was between 0.18 and 0.23, indicating that most if not all of the contaminant polysaccharide was removed (Table 2). In addition, the integrity of RNA was examined by denaturating 1.2% agarose gel and lab-on-chip capillary electrophoresis[18] as shown in Figure 1. For all samples of RNA, the agarose gel and the micro-fluidic capillary electrophoresis showed sharp and distinct 23S and 16S ribosomal RNA bands with minimal degradation. The Bioanalyzer 2100 also provides the RNA integrity number (RIN), a new tool for RNA quality assessment.[23] RIN higher than 5 is considered a good total RNA quality for downstream applications, such

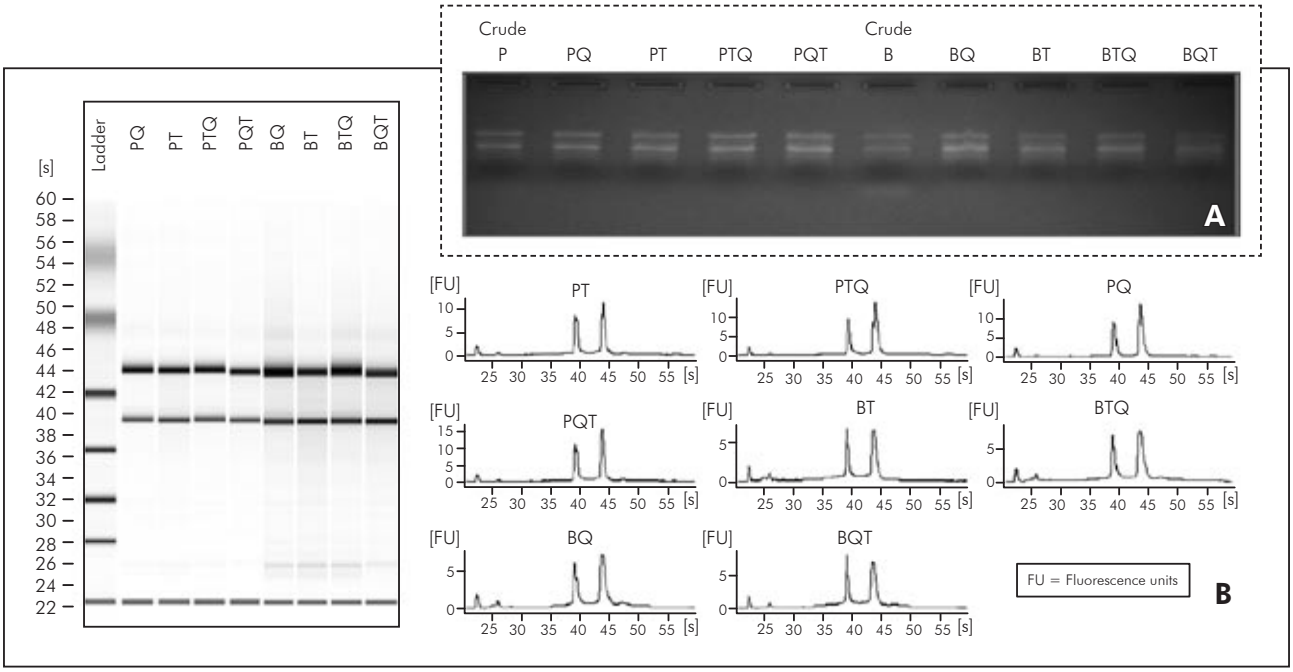

Figure 1 - Analyses of the RNA integrity of the purified samples by denaturating 1.2% agarose gel (A) and lab-on-chip capillary electrophoresis (B). Crude P – crude RNA extracted from planktonic cells of *S. mutans*; PQ – RNA from *S. mutans* cells purified according to approach Q, PT – RNA purified according to approach T; PTQ – RNA purified according to approach TQ; PQT – RNA purified according to approach QT. Crude B – crude RNA from *S. mutans* biofilms; BQ – RNA from *S. mutans* biofilms purified according to approach Q; BT – RNA purified according to approach T; BTQ – RNA purified according to approach TQ; BQT – RNA purified according to approach QT.

a qRT-PCR.[23] In the present study, the RIN in all of our purified samples was higher than 8.3.

Furthermore, the quality of RNA preparations was analyzed using real-time RT-PCR, SYBR Green I and primer-sets for *gtfB*; *gtfB* was selected because it is a critical virulence gene associated with extracellular polysaccharide synthesis,[24] and is commonly used for *S. mutans* detection in clinical samples using qRT-PCR based experiments.[25] First, the purified RNA samples (1 μg) were used as templates in PCR reaction to examine for the presence of residual genomic DNA by monitoring of the increasing fluorescence intensity after each PCR cycle; a greater amount of DNA results in lower C_t value, as a result of requiring less PCR cycles for the fluorescence emission intensity to reach the threshold. The various DNAse treatments examined in this study resulted in purified RNA samples with distinct levels of genomic DNA (Table 3). Clearly, the type, number and sequence of DNAse treatments are critical for DNA removal. The RNA treated with Qiagen's RNeasy on column DNAse I approach (Q) was the less effective method; the Ambion's Turbo DNAse I approach (T) was more effective than approach Q likely due to the greater catalytic efficiency of its recombinant DNAse I than the wild-type DNAse I used in Q.[9,26] The combination of two DNAse I treatments was more efficient at removing the coextracted DNA from the RNA fraction than one treatment, especially for biofilms. However, Q followed by T (QT) was superior than T followed by Q (TQ) based on PCR data (Table 3). The RNA extracts purified using the approach QT showed detectable fluorescence signal only after 33 cycles when using the primer sets for *gtfB* indicating only trace amounts of DNA; the C_t values are 6.9-10.3 times higher than those obtained with RNA using approach Q or T, and 2.9-3.8 times higher than the values from RNA purified with the TQ approach. Since the quantity of DNA (or cDNA) doubles every cycle during the exponential phase, a difference of only 3 cycles would represent as much as 8 times more (or less)

DNA template. Thus, it is evident that QT is more effective in removing genomic DNA than other approaches. It is still unclear how exactly the sequence of the DNAse I treatments affects the genomic DNA removal. We are currently investigating the putative mechanisms involved in DNA removal by the combination of DNAse treatments.

Lastly, cDNA pools were synthesized from 1 µg of purified RNA using random hexamers. The amplification (with *gtfB* primers) of either two- or five-fold serial dilution of cDNAs from RNA purified according to protocol QT provided correlation coefficient of 0.98-0.99 and slope between –3.289 and –3.365 (98.2 to 101.4% amplification efficiency), which is within the range of acceptable slope (–3.0 to –3.5) indicating little or no PCR inhibitors. The no-template control showed negligible amplification (≥ 34 cycles).

Conclusions

In conclusion, a method (QT approach) that yields high-quality RNA from both planktonic cells and especially biofilms of *S. mutans* in sufficient quantity for real-time RT-PCR analyses was described in this study.

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