Purification and Detection of Bacterial RNA from Preserved Stool Samples

E. SONKE, BMSC[1] , W. KIM, PH.D[1] , AND Y. HAJ-AHMAD, PH.D[1,2]
[1]NORGEN BIOTEK CORPORATION, THOROLD, ONTARIO, CANADA
[2]CENTRE FOR BIOTECHNOLOGY. BROCK UNIVERSITY, ST. CATHARINES, ONTARIO, CANADA

INTRODUCTION

Stool is an excellent sample source for diagnosing cases of bacterial gastroenteritis, and analysis of gut microbiota is becoming increasingly important in diagnosing cases of irritable bowel syndrome[1,2]. Detection of 16S rRNA is commonly used as a means of detecting these forms of pathogenic bacteria[1,2]. The isolation of high quality RNA from stool is not without its problems however. The presence of humic acid compounds, fats and other bio by products in stool makes the isolation of quality nucleic acid samples that are free of PCR inhibitors very challenging[3]. Furthermore, the presence of RNases and DNases in stool poses a logistical problem in the form of nucleic acid degradation that occurs during sample collection and transport[4]. Current techniques which do not make use of preservative require that stool be collected into vials, transported on ice and then frozen at -20°C when received by the diagnostic testing facility. The addition of preservative to the collection vials eliminates the need to immediately process or freeze the stool samples and allows the samples to be shipped at ambient temperature.

Norgen Biotek Corp. has developed a Stool Nucleic Acid Preservative which allows for the long-term preservation of stool samples at ambient room temperature, making this buffer ideal for stool storage and shipping. This buffer is available as a product on its own, and the preserved stool samples are compatible for use with Norgen's various stool purification kits, including our Stool Total RNA Purification Kit. The Stool Nucleic Acid Preservative is an aqueous storage buffer designed to prevent the growth of contaminating bacteria and virus while preserving the DNA and RNA of pathogenic microorganisms within the sample. The preservative also inactivates pathogenic viruses while stabilizing their DNA and RNA which allows for safer handling and transport of samples. This application note illustrates that bacterial RNA subsequently isolated from the preserved stool samples is of a high quality and can be used directly in sensitive downstream assays such as realtime RT-PCR to monitor the RNA expression.

METHODS AND MATERIALS

RNA Isolation

A single stool sample was collected from a donor using Norgen's Stool Nucleic Acid Collection and Transport Tubes (Cat# 45620, 45650) according to the accompanying protocol. Various volumes of preserved sample (0.1 mL, 0.2 mL, 0.4 mL and 0.6 mL) were then processed immediately using Norgen's Stool Total RNA Purification Kit (Cat. 49400, 49500) using a 1 mL total volume of preserved stool and lysis buffer. Preserved samples were compared against 200 mg fresh stool samples not mixed with preservative. All samples were homogenized at 4.0 m/s for 20 seconds using the FastPrep®-24 homogenizer (MP Biomedicals). All samples were then processed following the recommended kit protocol. All samples were done in triplicate.

Gel electrophoresis

For visual analysis, 10 µL of RNA from the final 50 µL RNA elution was mixed with 10 µL 2x RNA loading dye and denatured at 70°C for 1 minute before loading onto a 1.2% 1x MOPS agarose gel. Samples were run for 25 minutes at 150 V. The gel photo was taken using an AlphaImagerTM IS-2200 (Alpha Innotech).

RT-PCR Amplification

The purified RNA was then used as the template in both real-time PCR and real-time RT-PCR reactions. In both sets of reactions, primers that recognize both 16S rRNA and 16S rDNA from prokaryotes were used for amplification at a final concentration of 0.25 µM, and 2 µL of RNA elution was added as template. SYBR Green was used for detection in both sets of reactions. Recombinant Taq Polymerase was incorporated into both the PCR and the RT-PCR reactions and SuperScript III reverse transcriptase was incorporated into only the one step real-time RT-PCR reactions. One step RT-qPCR; 50°C for 30 minutes for reverse transcription, 95°C for 3 minutes for denaturation, and 45 cycles of 95°C for 15 seconds for denaturation, 60°C for 30 seconds for annealing and 72°C for 45 seconds for extension. Meltingcurve analysis was also performed. The reaction was run on an iCycler iQ real-time system (Bio-Rad).

RESULTS AND DISCUSSION

Norgen's Stool Nucleic Acid Preservative allows for the preservation of stool RNA at room temperature for 7 days prior to RNA isolation. This application note illustrates that bacterial RNA subsequently isolated from the preserved stool samples is of a high quality and can be used directly in sensitive downstream assays such as realtime RT-PCR to monitor RNA expression. In addition, we also investigated the ideal amount of preserved stool to be used when isolating RNA with Norgen's Stool Total RNA Purification Kit. In determining the ideal amount of preserved stool to use, RNA quantity and quality were compared by visualizing on a 1%, 1x MOPS agarose gel and by spectrophotometric analysis. Real-time PCR analysis of 16S rDNA and rRNA (bacterial gene) was also used to determine the optimal tradeoff for RNA expression.

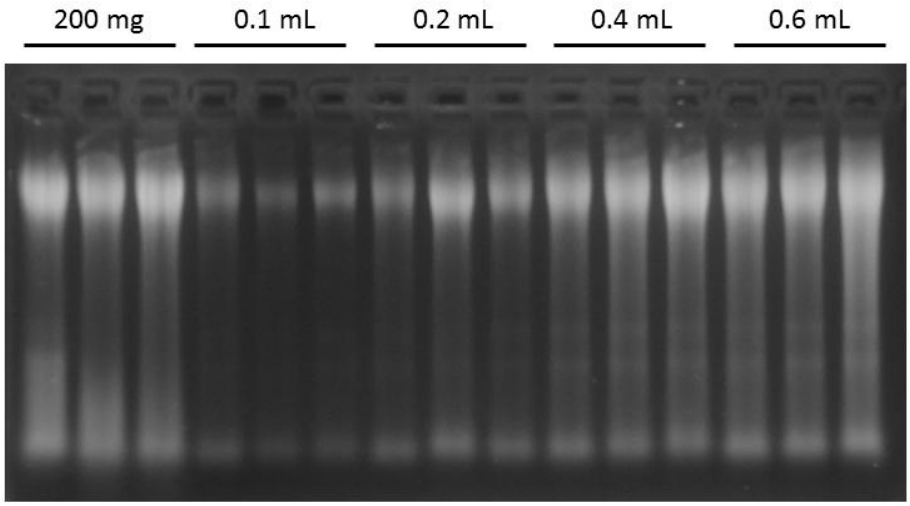

Figure 1. RNA isolated from various volumes of preserved stool samples compared to RNA isolated from 200 mg fresh stool.

RNA Quantity

RNA quantity was assessed using both gel electrophoresis **(Figure 1)** and spectrophotometry **(Figure 2B)**. Electrophoresis and spectrophotometry both revealed that extraction from 0.4 mL and 0.6 mL of preserved stool yielded an amount of RNA that was comparable to extraction from 200 mg fresh stool **(Figure 1, 2B)**. However, Figure 1 shows that extraction from fresh stool yielded more low-molecular weight RNA, which is indicative of degradation, than was observed with extraction from the preserved samples.

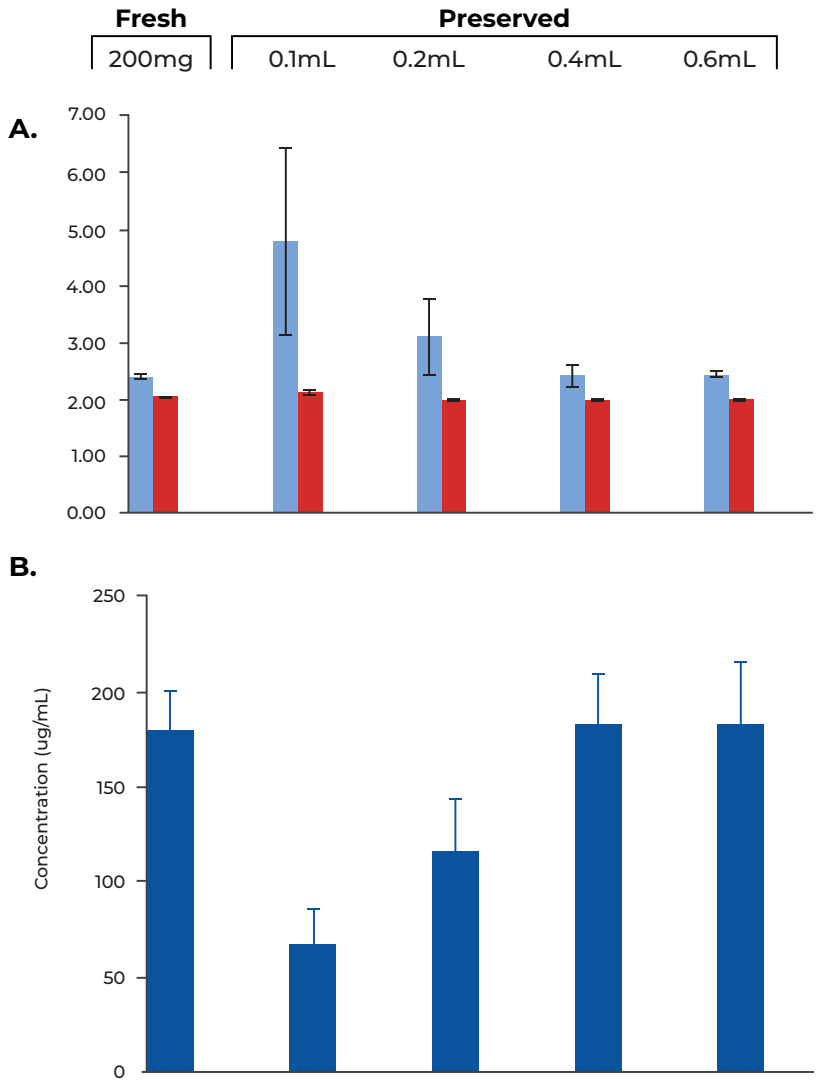

Figure 2. 260/230 ratios (A, blue), 260/280 ratios (A, red) and concentrations (B) of RNA obtained by spectrophotometric analysis of 25x diluted RNA samples obtained from fresh and preserved stool samples of various amounts. Error bars represent standard deviation of the mean (n = 3). Measurements made using Fisher Scientific's Ultrospec 2100 Pro.

RNA Quality

RNA quality was assessed by both spectrophotometry **(Figure 2A)** and by ΔCt analysis of 16S RT-PCR/PCR **(Figure 3 and Table 1)**. When comparing the RNA quality ratios calculated by the spectrophotometer (260/230 and 260/280), it is clear that all of the preserved samples are of a similar quality, if not higher quality, than the frozen samples **(Figure 2A)**. The lower standard deviation of the 0.4 mL and 0.6 mL preserved samples makes them more attractive in terms of generating reproducible data and is likely due to more accurate pipetting of the preserved stool.

Analysis of the RT-PCR data for 16S rRNA reveals that increasing the volume of preserved sample from 0.1 mL to 0.6 mL does not come at the cost of reduced PCR efficiency **(Table 1)**. This highlight's the ability of Norgen's Stool Total RNA Purification Kit to remove PCR inhibitors like humic acid.

We also observed that the low ΔCt was indicated from 0.4 mL and 0.6 mL sample input **(Table 1)**. This is simply due to the high DNA background (since there was no DNase I treatment) from the increased sample input but the real RNA amount is expected to be increased as shown on the gel **(Figure 1)**. Therefore it suggested that for certain application, DNase I treatment is recommended.

Stool	PCR (Squares)			RT-PCR (Circles)			ΔCt		
	Ct	AVG	SD	Ct	AVG	SD	Ct	AVG	SD
Frozen (Red)	15.9 15.4 16.6	15.6	0.3	11.5 11.7 12.1	11.8	0.3	4.4 3.7 3.5	3.9	0.5
0.1 mL preserved (light Green)	17.3 16.9 16.2	16.8	0.6	13.7 13.8 13.1	13.5	0.4	3.6 3.1 3.1	3.3	0.3
0.2 mL preserved (dark green)	15.7 15.8 16	15.8	0.2	13.3 12.9 12.8	13.0	0.3	2.4 2.9 3.2	2.8	0.4
0.4 mL preserved (blue)	15.2 15.7 15	15.3	0.4	13.4 13.6 13.6	13.5	0.1	1.8 2.1 1.4	1.8	0.4
0.6 mL preserved (pink)	15.3 15.4 15.8	15.5	0.3	13.2 13.8 13.6	13.5	0.3	2.1 1.6 2.2	2.0	0.3
NTC (Black)	32.8 31.6	32.2	0.8	22.6 22.9	22.8	0.2	10.2 8.7	9.5	1.1

Table 1. Prokaryotic RNA detection (16S rRNA) in frozen and preserved stool samples by One step real-time PCR analysis using Norgen's Stool Total RNA Purification Kit.

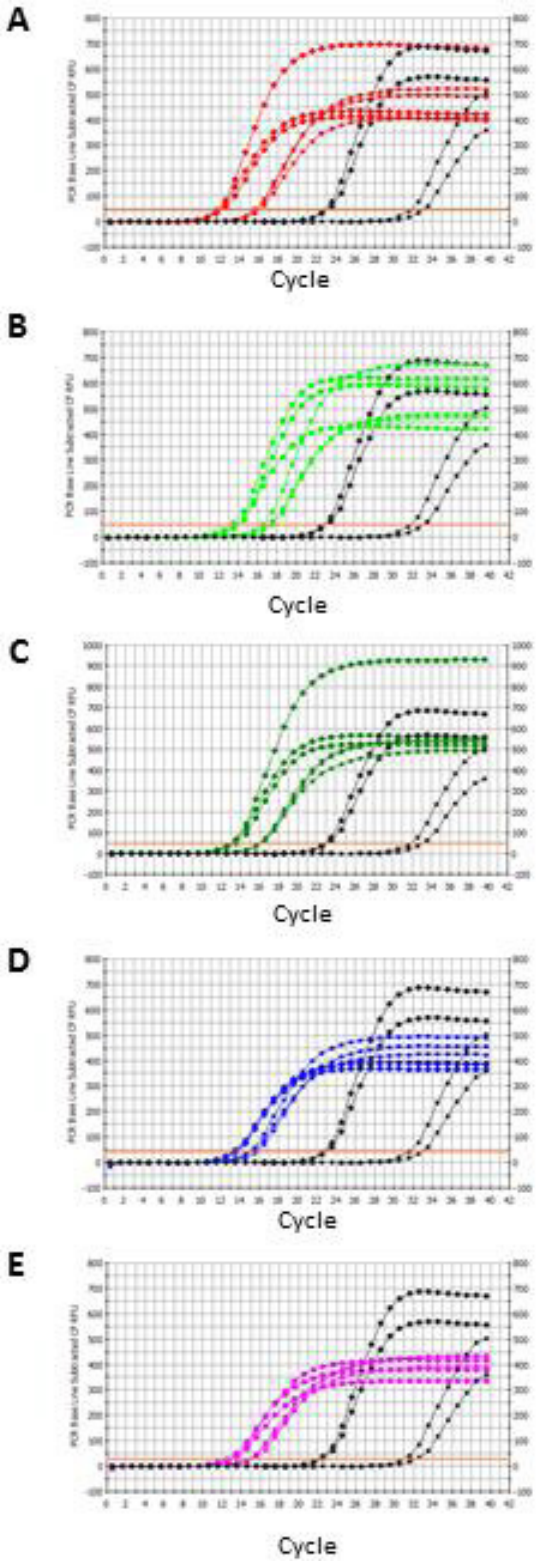

Figure 3. Bacterial 16S rDNA detection by PCR (squares) and 16S rRNA detection by RT-PCR (circles). Total RNA was isolated from fresh samples (A; red) and preserved samples that were 0.1 mL (B; light green), 0.2 mL (C; dark green), 0.4 mL (D; blue) and 0.6 mL (E; pink) in volume.

CONCLUSION

In evaluating bacterial RNA yield from various amounts of preserved stool samples in comparison to RNA yield from 200 mg of fresh stool, the results indicate that 0.4-0.6 mL of preserved sample yields a comparable amount of RNA. Furthermore, the RNA isolated from 0.4-0.6 mL preserved sample is of a similar quality to the fresh sample as indicated by 260/230 and 260/280 ratios. Finally, ΔCt analysis of 16S rDNA and rRNA detection revealed that preserved samples in the range of 0.4-0.6 mL should be used in Norgen's Stool Total RNA Purification Kit with DNase I treatment for optimal RT-PCR detection of prokaryotic RNA.

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