Comparison of Stool DNA Preservation Methods and their Impact on Gut Microbiome Diversity

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INTRODUCTION

Appropriate preservation and storage of stool samples is crucial in maintaining DNA fidelity and microbial community composition for downstream applications and analysis, including next-generation sequencing (NGS) and microbiome characterization. Post-collection bias may be introduced in cases where sample material is inadequately preserved, highlighting the importance of preservation in study design. Currently, rapid freezing to −80°C is considered best-practice for preserving stool samples; however, this modality may be unfeasible when sample collection takes place in non-laboratory settings, such as in participants' homes.

This application note investigates differences in microbiota authenticity, DNA concentration, and DNA fidelity when stool is preserved by rapid freezing to −80°C, using Norgen Biotek's Stool Preservative, or using TE buffer. Following storage for 1, 3, and 6 days, stool DNA was isolated from two samples for each preservative condition and several parameters were assessed, including DNA concentration, 260/230 ratio, and 260/280 ratio. Next-generation sequencing of the fecal microbiome was also performed. Norgen Biotek's Stool Preservative was associated with the greatest DNA concentration, and the least divergence in microbiota diversity and composition throughout the study. Similarly, rapid freezing was associated with minimal divergence in microbiota diversity and composition. However, samples preserved using TE buffer showed substantial divergence in microbiota diversity.

Therefore, this application note illustrates the utility of Norgen Biotek's Stool Preservative for accurate, stable characterization of the gut microbiome, and shows that this preservative can be used in cases where freezing and cold-chain transport is unavailable.

MATERIALS AND METHODS

Stool sample collection and processing

Stool samples were collected from two healthy donors and three 200 mg aliquots from each donor were immediately applied to each different storage condition; ambient temperature, -80˚C, TE buffer (pH 8.0) and Norgen's Stool Nucleic Acid Collection and Preservation Tubes (Cat. 45630, 45660) containing Norgen's Stool Preservative. The aliquots were used in order to minimize the potential of inter-subject variation.

Stool DNA extraction and quantification

DNA extraction was performed using Norgen's Stool DNA Isolation Kit (Cat. 27600). Inputs of 200 µL were used from either the TE buffer/stool mixture or Norgen's Stool Preservative/stool mixture and 200 mg from fresh or frozen stool samples for Day 0, Day 1, Day 3 & Day 6. Stool samples were bead homogenized after adding 800 µL of Lysis Buffer L for preserved samples or 1 mL for fresh and frozen samples. Next, the samples were centrifuged and 100 µL of Binding Buffer I was added to the clean supernatant and incubated on ice for 10 minutes. Equal amounts of 70% Ethanol were then added to the clean supernatant from Binding Buffer I lysate after centrifugation. The protocol was then followed as written to complete the DNA isolation. For visual inspection of genomic DNA, 10 µL of the elution was loaded onto a 1.2 % agarose TAE gel and run for 30 minutes at 150 V alongside Norgen's HighRanger 1 kb DNA ladder (Cat. 11900). Gel photos were taken using an AlphaImager™ IS-2200 (Alpha Innotech). The purified DNA from each of the four conditions was quantified with the A260, A280 A260/A230 and A260/A280 ratios using the NanoDrop™ 2000 Spectrophotometer.

16S rRNA gene sequencing

The V3-V4 hypervariable region of the bacterial 16S rRNA gene was amplified from 12.5 ng of stool DNA quantified by PicoGreen. The amplicons were then cleaned, Dual-indexed with the Nextera XT Index Kit (Illumina Inc., San

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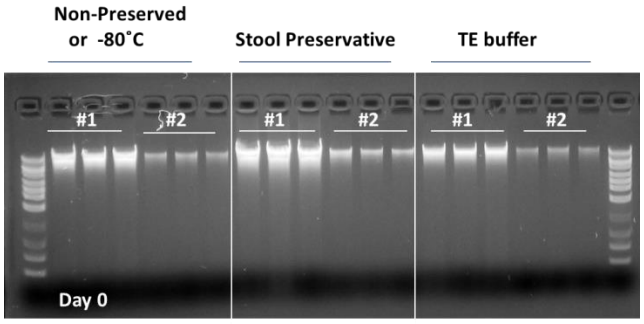
Diego, CA, USA) according to the manufacturer's instructions and sequenced according to the Illumina MiSeq 16S Metagenomic Sequencing Library Preparation protocol (http://support.illumina.com/downloads/16s_metagenomic_sequencing_library_preparation.html).

The final library was paired-end sequenced at 2 × 300 bp using a MiSeq Reagent Kit v3 on the Illumina MiSeq platform. Sequencing was performed at Norgen Biotek. For Bioinformatic analysis, the sequencing data was analyzed using the Illumina 16S metagenomics app.

RESULTS AND DISCUSSION

Stool samples were collected from two healthy donors, yet the DNA yield was notably different, thus indicating the diversity of microorganisms between individuals. In general, Donor Sample #1 demonstrated higher DNA yields than Donor Sample #2 regardless of the preservation method used.

The DNA yield was substantially reduced within 24 hours from non-preserved samples after storage at room temperature. In contrast, samples preserved in Norgen's Stool Preservative or stored at -80˚C showed a consistency in DNA yield up to 6 days (Figure 1, 2). Interestingly, the DNA yield from samples stored in TE buffer seemed consistent on the gel over time (Figure 1), but Figure 2 showed more detailed changes with DNA yield decreasing in Sample #1 and increasing in Sample #2 during the storage period. This fluctuation of DNA yield may have been influenced by the change of microorganisms' growing condition (aerobic or anaerobic) during the storage (Figure 2).



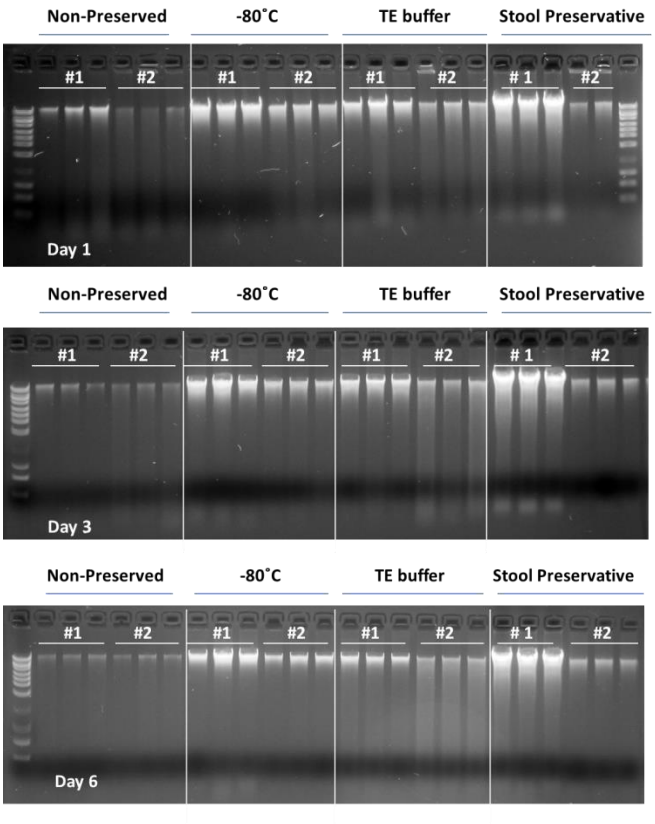


Figure 1. Isolation of DNA from Stool Preserved Using Different Preservative Methods for Day 0, 1, 3 and 6. DNA was isolated using Norgen's Stool DNA Isolation kit (Cat. 27600) and 10 µL of elution was loaded on 1.2% 1x TAE Agarose gel.

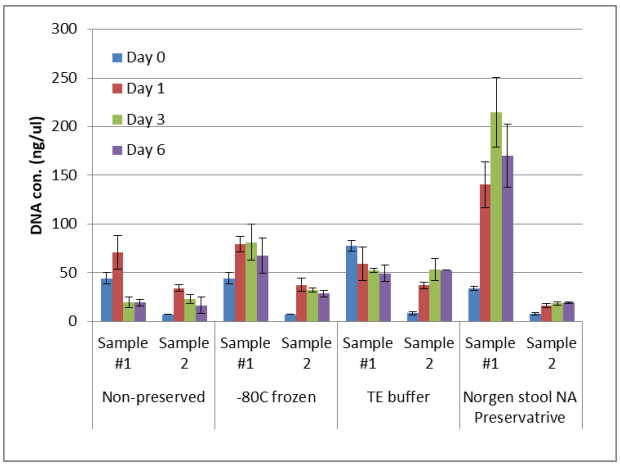


Figure 2. DNA Concentration of Samples Isolated from Stool Preserved Using Different Preservative Methods Over Six Days.

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In terms of DNA quality (260/230 and 260/280), the non-preserved fresh sample had a decrease in the ratio over time from both Sample #1 and #2 (Figure 3 and 4). The change of 260/230 ratio was quite noticable in the non-preserved samples, as well as the -80°C storage condition, while samples preserved in Norgen's Stool Preservative showed minimal variation in 260/280 and 260/230 ratio over the 6 days (Figure 3 and 4).

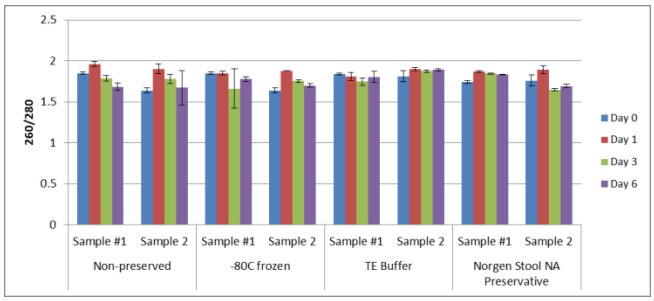

Figure 3. 260/280 Ratio of Samples Isolated from Stool Using Different Preservative Methods Over Six Days. The 260/280 ratio of the -80˚C frozen samples and the samples preserved in Norgen's Preservative demonstrated values closest to 1.8 over the six day period. The non-preserved samples were associated with a continual decline in the 260/280 ratio, indicative of lesser DNA quality.

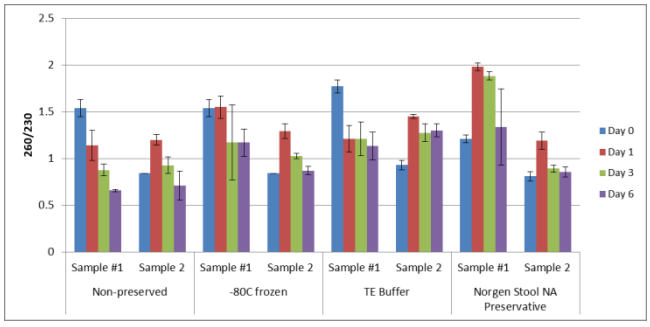

Figure 4. 260/230 Ratio of Samples Isolated from Stool Using Different Preservative Methods Over Six Days. The non-preserved samples were associated with a continual decline in the 260/230 ratio, indicative of contamination and lesser DNA quality. A similar decline was observed for the TE-preserved samples. The Norgen-preserved and rapid freezing samples were associated with 260/230 ratios closest to 1.8 over the six day period, indicative of minimal salt contamination.

The effect of storage conditions on faecal microbiota was assessed based on the relative abundance of bacterial phyla. The results indicated a substantial divergence in the non-preserved sample by Day 1, with an increase in Thermi, a decrease in Tenericutes, and a decrease in Nitrospirae. Gradual divergence was also observed in TE-preserved samples, with a decrease in Tenericutes and an increase in Thermi. Norgen's Stool Preservative and rapid freezing at -80˚C were associated with the least divergence in microbiota diversity and composition throughout the study, with the abundance of Thermi and Tenericutes varying minimally over time (Figure 5). Therefore, Norgen's Stool Preservative and rapid freezing provided the most accurate, representative measurements of bacterial phyla abundance.

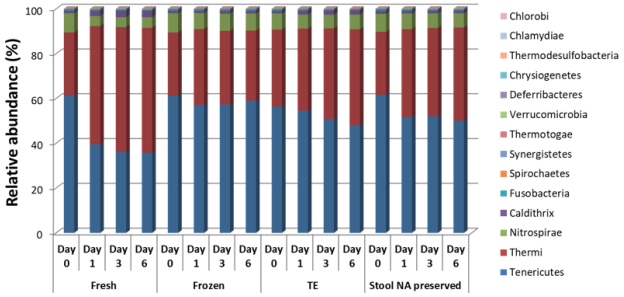

Figure 5. Relative Abundance of Bacterial Phyla in Stool DNA Preserved Using Different Methods. Day 0 is reflective of the microbial community composition at the time of collection; measurements thereafter reflect divergence from this baseline composition.

CONCLUSIONS

1. **Norgen's Stool Preservative is recommended for optimal DNA fidelity and concentration** — Quality and quantity of DNA rapidly decrease unless frozen at -80°C or preserved in Norgen Biotek's Stool Preservative.
2. **Norgen's Stool Preservative is associated with the least divergence in microbiome** — Norgen's Stool Preservative and rapid freezing were associated with the least divergence in microbiota diversity and composition throughout the study.

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