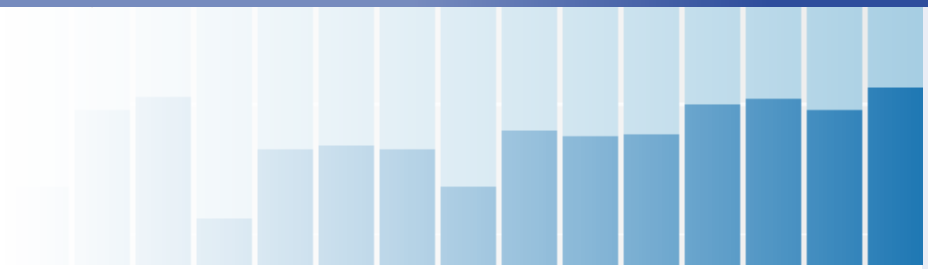
Gut Microbiome Diversity:
Comparison of Stool DNA Preservation Methods

Application Note 90



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Keywords

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+ Preservative
+ Stool
+ Storage
+ Method

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 each of the following conditions; rapid freezing to –80°C, Norgen Biotek's Stool Preservative, TE buffer, and storing samples with no added preservative. Following storage for 1, 3, and 6 days, stool DNA was isolated from two samples from each preservative condition and several parameters were assessed, including DNA concentration, 260/280 ratio, and 260/230 ratio. Next-generation sequencing of the fecal microbiome was also performed. Stool Preservative was associated with the least divergence in microbiota diversity and composition throughout the study. Rapid freezing, samples preserved in TE buffer, and samples absent of a preservation method were associated with noticeable divergence in microbiota diversity and composition over a 6-day period.

Therefore, this application note illustrates the utility of Norgen'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 Stool Preservative. The aliquots were used in order to minimize the potential of inter-subject variation.

Stool and 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 Stool Preservative/stool mixture and 200 mg from fresh or frozen stool samples for Day 0, Day 1, Day 3 and 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.

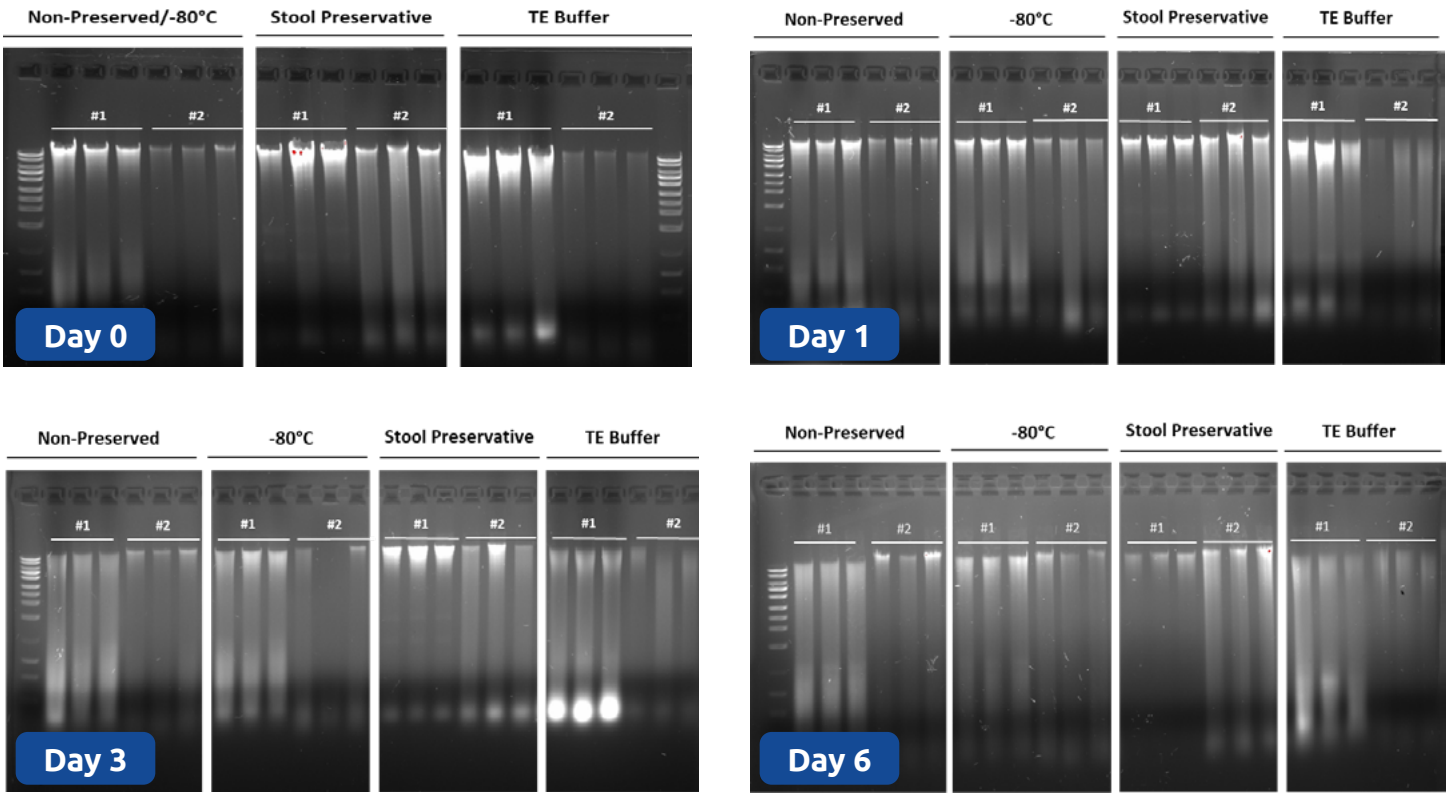

Figure 1. Isolation of DNA from stool preserved using different preservation methods for Day 0, 1, 3 and 6. DNA was isolated using Norgen's Stool DNA Isolation kit (Cat. 27600) and 10 µL of elution was loaded on 1.2% 1x TAE agarose gel.

16S rRNA Gene Sequencing

The V3-V4 hypervariable region of the bacterial 16S rRNA gene was amplified from 50 ng of stool DNA quantified by Nanodrop. Library preparation was performed using Norgen's 16S V3-V4 Library Preparation Kit for Illumina (Cat. 70400, 70410, 70420, 70430, 70440). One modification to the protocol was made during the PCR Clean-Up 2 step. Instead of using a bead cleanup followed by a qPCR quantification, the libraries were cleaned and normalized using Norgen's NGS Normalization 96-Well Kit (Cat. 61900). 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 16S metagenomics workflow with Mothur.

RESULTS AND DISCUSSION

Stool samples were collected from two healthy donors, yet the DNA yield was notably different depending on the preservation method used, thus indicating the diversity of microorganisms between individuals. Samples preserved in Norgen's Stool Preservative showed a fairly consistent DNA yield up to 6 days compared to the other preservative methods tested (**Figure 1**, **2**). Interestingly, the DNA yield from sample 1 stored in room temperature and TE buffer seemed to increase in concentration over time. This fluctuation of DNA yield may have been influenced by the change of microorganisms' growing condition (aerobic or anaerobic) during the storage (**Figure 2**).

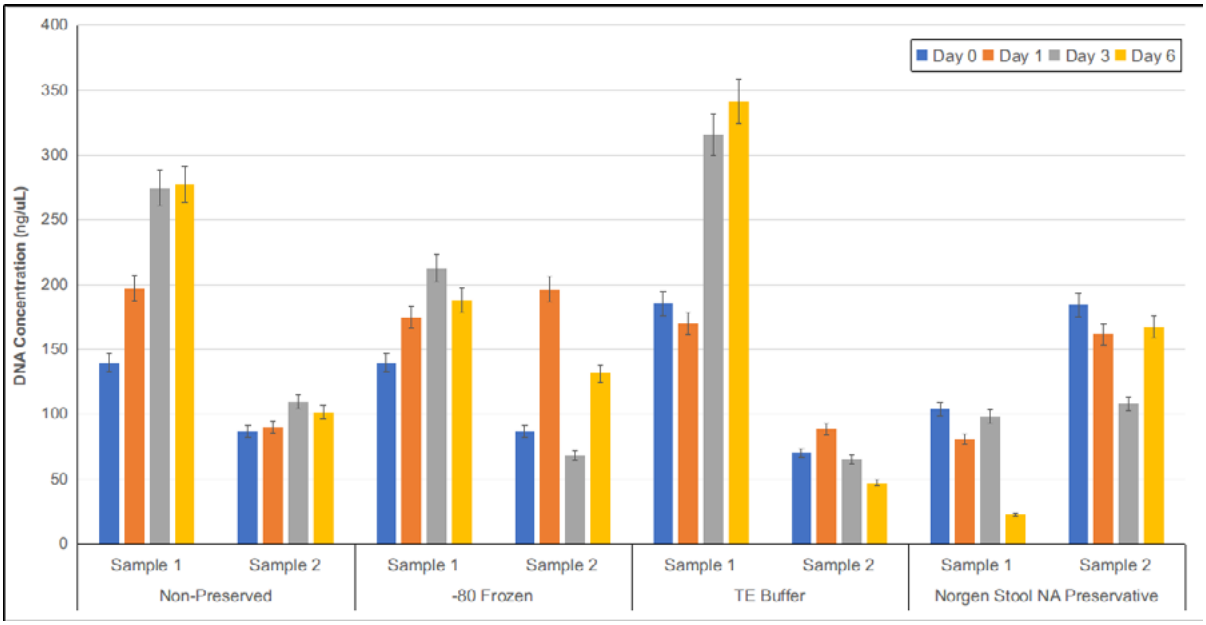

Figure 2. DNA concentration of samples isolated from stool preserved using different preservative methods over a six-day period. Data shown represents the average from three technical replicates per sample and condition tested.

In terms of DNA quality (260/280 and 260/230), the change in ratio was more noticeable in three conditions including non-preserved samples, -80°C storage, as well as TE buffer preservation, while samples preserved in Stool Preservative showed minimal variation in 260/280 and 260/230 ratio over the 6 days (**Figure 3**, **4**). The average values of the 260/280 ratio were well within the expected range (1.8-2) and did not differ significantly between the preservation methods, however samples preserved with Stool Preservative displayed the highest measure of DNA purity (**Figure 3**). The use of Norgen's Stool Preservative was associated with 260/280 and 260/230 ratios closest to 1.8, representative of good quality DNA and minimal contamination compared to other conditions.

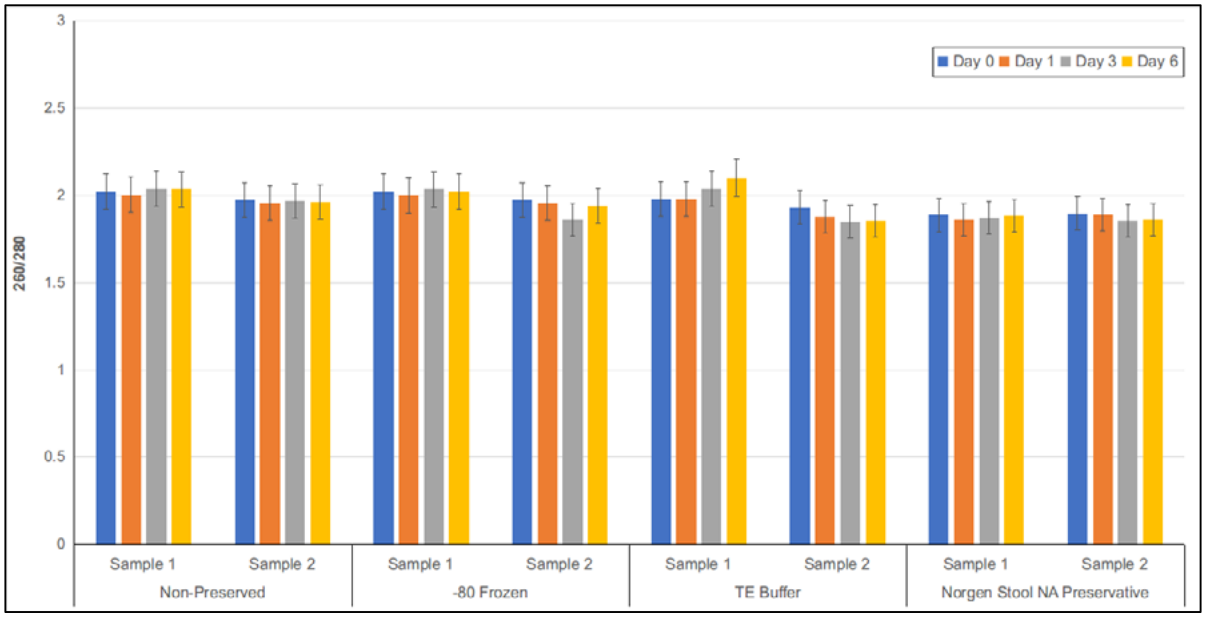

Figure 3. 260/280 ratio of samples isolated from stool using different preservative methods over six days. The 260/280 ratio of the samples preserved in Norgen's Stool Preservative demonstrated values closest to 1.8 over the six-day period. Samples preserved by other means were associated with a continual fluctuation in

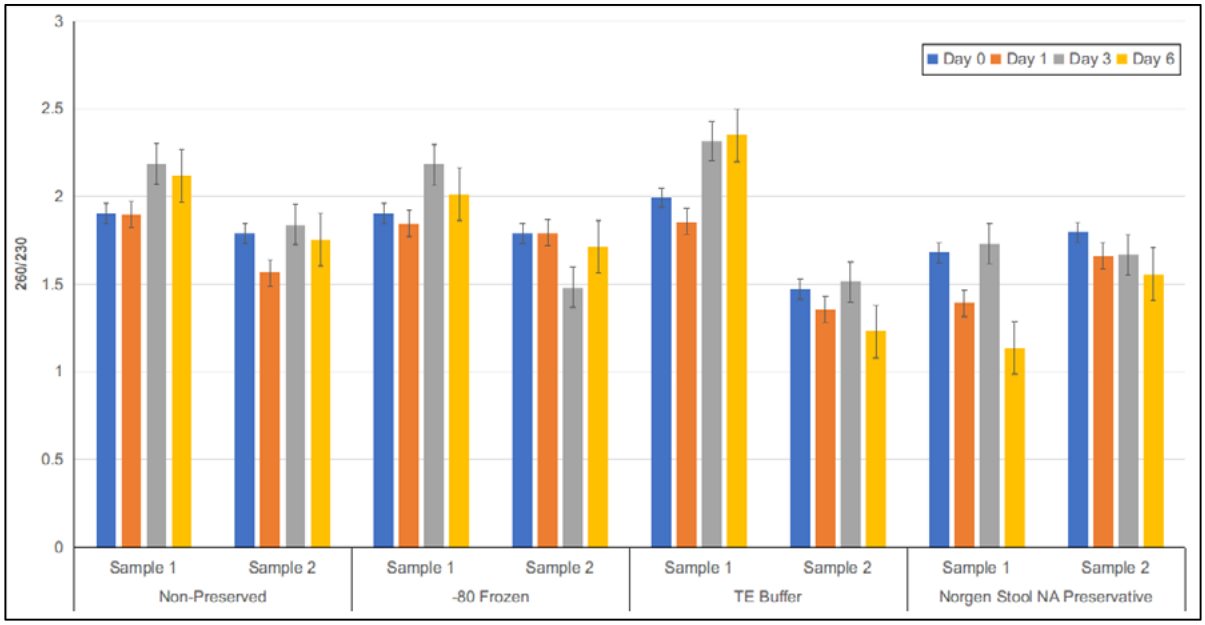

Figure 4. 260/230 ratio of samples isolated from stool using different preservative methods over six days. The Norgen-preserved samples were associated with 260/230 ratios closest to 1.8 over the six-day period, indicative of minimal salt contamination. Data shown represents the average from three technical replicates per sample and condition tested.

The impact of preservation methods on fecal microbiota composition was evaluated by measuring the changes in alpha diversity over different conditions and time periods. Alpha diversity metrics were assessed by the Shannon index, which captures the richness and diversity of species within a given sample. Non-preserved samples as well as samples preserved by rapid freezing and TE buffer showed the largest amount of variation in alpha diversity values. Interestingly, all preservative conditions except for Norgen's Stool Preservative resulted in higher measures of alpha diversity from day 0 to day 6, which suggests the possibility of microbial community changes caused by the storage method utilized (**Figure 5**). Therefore, stool samples preserved with Stool Preservative demonstrated the most consistent bacterial diversity and microbial profiles over the course of this study.

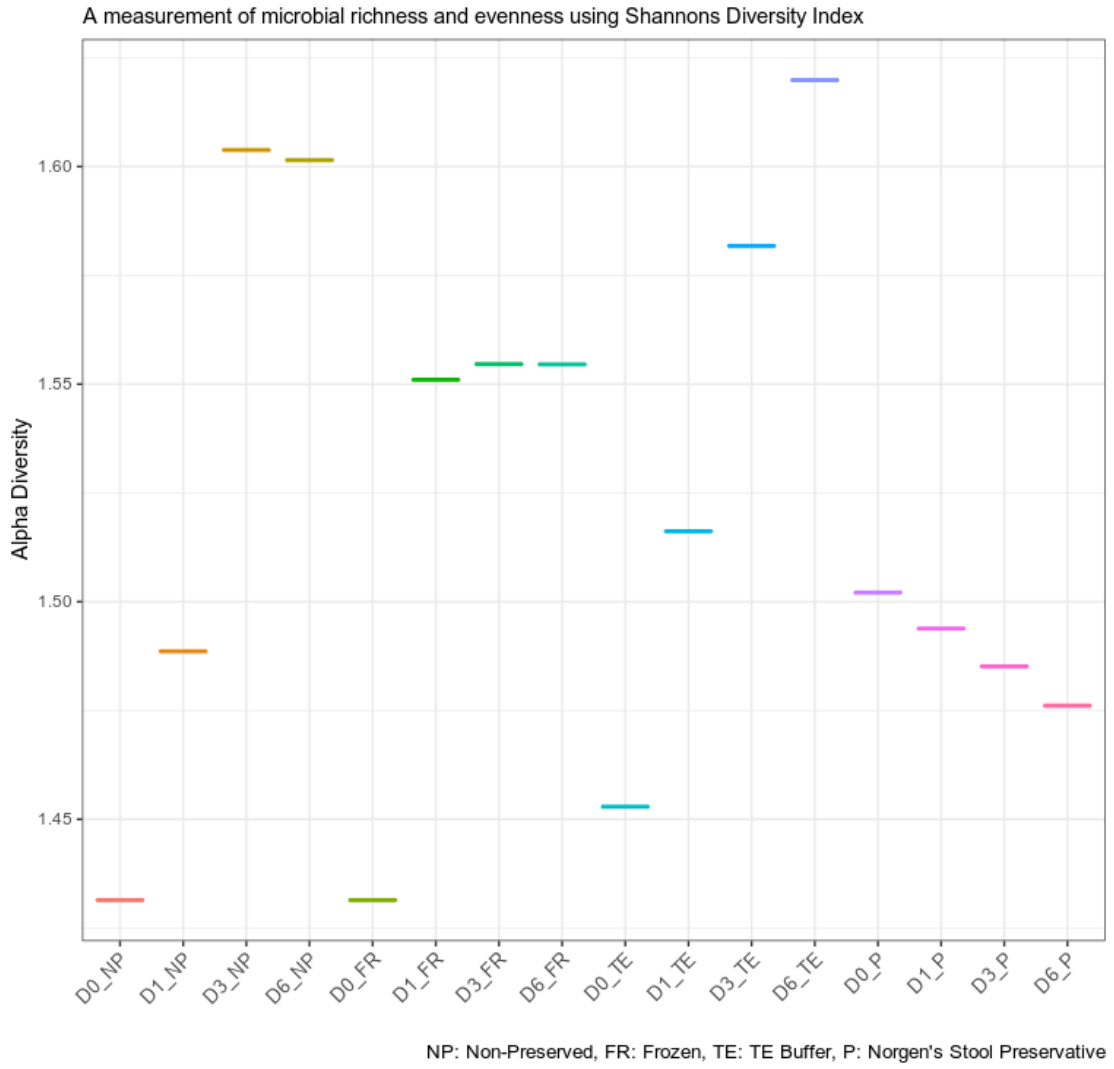

Figure 5. Overview of microbial richness and evenness of all samples isolated from stool using different preservative methods over six days. Day 0 (D0) is reflective of the microbial community composition at the time of collection; measurements thereafter reflect divergence from this baseline composition. Alpha diversity was measured using the Shannon Diversity index. Samples isolated using Norgen's Stool Preservative were characterized by a consistent species diversity over time, indicative of a more stable and uniform microbiome profile. Analysis was completed using the average from three technical replicates per condition tested.

The effect of storage conditions on fecal microbiota was also assessed based on the relative abundance of bacterial genera and phyla. At both the genus and phylum level, the microbial composition of stool samples preserved by rapid freezing, TE buffer, and samples absent of a preservation method displayed an increase in some bacterial species after storage, including *Bacteroides* and *Faecalibacterium* (**Figure 6**, **7**). Stool Preservative was associated with the least divergence in microbiota diversity and composition throughout the study, with the abundance of all bacterial species varying minimally over time (**Figure 6**, **7**). Therefore, Norgen's Stool Preservative provided the most accurate, representative measurements of bacterial genera and phyla abundance.

CONCLUSIONS

1. Norgen's Stool Preservative is recommended for optimal DNA fidelity and DNA purity. Quality of DNA varies and gradually decreases unless preserved in Stool Preservative

2. Stool Preservative is associated with the least divergence in microbiome profiles. Microbiota diversity and composition of samples are more stabilized and consistent at both the genus and phylum level when preserved using Norgen's Stool Preservative.

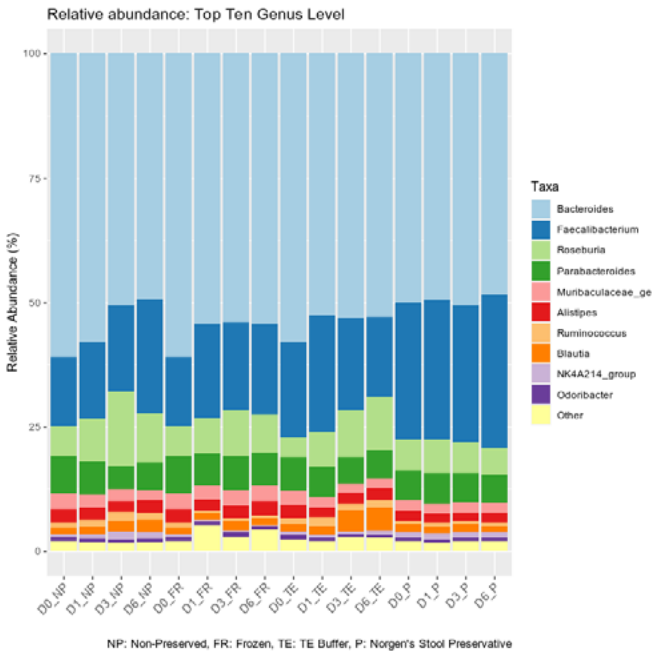

Figure 6. Relative abundance of top ten bacterial genera in stool DNA preserved using different methods. Day 0 (D0) is reflective of the microbial community composition at the time of collection; measurements thereafter reflect divergence from this baseline composition. Analysis was completed using the average from three technical replicates per condition tested.

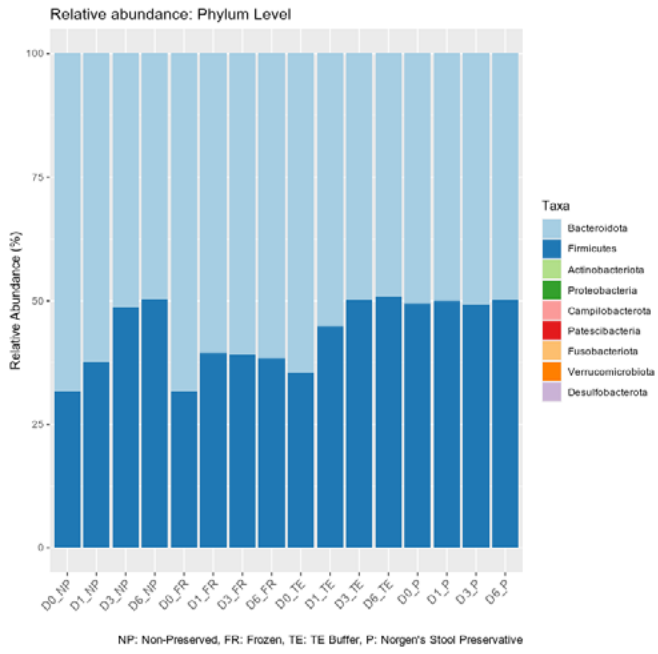

Figure 7. Relative abundance of bacterial phyla in stool DNA preserved using different methods. Day 0 (D0) is reflective of the microbial community composition at the time of collection; measurements thereafter reflect divergence from this baseline composition. Analysis was completed using the average from three technical replicates per condition tested.

Related Products	Research Use	CE Marked
Stool Nucleic Acid Collection and Preservation Tubes	45630, 45660	-
Stool DNA Isolation Kit	27600, 65600	-
HighRanger 1kb DNA Ladder	11900	-
16S V3-V4 Library Preparation Kit for Illumina	70400	-
NGS Normalization 96-Well Kit	61900	-

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