Effective Feces Aliquotting Workflow Supports Increased Microbiome Research

Joel Dore, Florence Levenez, Sean Kennedy, Nicolas Pons, Emmanuelle Le Chatelier, Edi Prifti, Larry Chin, Todd Basque, Joseph Fraone

INTRODUCTION

Global study of the human microbiome has been accelerating due to the growing understanding of how various microbes affect our health and play a role with various diseases. Fecal samples, the primary biosample of relevance in many microbiome studies, are proving to be rich sources of data key to understanding the relationship between the microbial community that lives within the human gut and a broad variety of health and disease indications, such as diabetes, obesity, celiac disease, and autism.

Efficient metagenomic analysis of human gut microbiota depends on the successful integration of multiple scientific techniques and technologies, such as nucleic acid extractions, whole genome shotgun sequencing, and bio-informatics and statistical analysis. Though costs for such techniques continue to decrease on an individual basis, the collective cost and effort for such workflows remain a significant factor in performing large and sustained efforts capable of leveraging the vast amount of data contained in the microbiome. Fecal samples are inherently difficult to work with in a systematic way due to safety and hygienic concerns. Moreover, the varied semi-solid nature and heterogeneous composition of fecal samples further complicates workflows by disqualifying them from standard high-throughput processing techniques such as automated liquid handling. Such challenges in sample handling can increase pre-analytical variability when working with large numbers of patient samples, leading to significantly biased data that misrepresents the in vivo microbial profile of individuals and patient populations.

INRA's Metagenopolis initiative, which is dedicated to establishing the impact of the gut mictobiota on health and disease, has employed frozen aliquotting technology as a means to standardize the processing of fecal samples in order to mitigate operational challenges and pre-analytical variability. The successful integration of frozen aliquotting into the Metagenopolis workflow was validated following thorough evaluation of several frozen aliquotting methodologies emphasizing as qualification criteria both the technique's reproducibility and comparability of quantitative metagenomic profiles with standard techniques previously employed.

METHODOLOGY

Table 1: Control and Frozen Aliquotting Condition Parameters

	Control	Frozen Aliquotting Conditions		
		Condition 1	*Condition 2*	*Condition 3*
Sample Preparation	Raw	Raw	Standardized	Standardized
Pre-analytical Storage Conditions	Stored at -80ºC	Stored at -80ºC	Stored at ambient temperature for 1 week	Stored at -80ºC for 1 week
Aliquotting Methodology	Manual	Frozen aliquotting on the CXT750	Frozen aliquotting on the CXT750	Frozen aliquotting on the CXT750

Sample Preparation, Storage, and Shipping.

Eleven volunteers provided fecal samples that were sampled by one standard (control) method and three experimental frozen aliquotting methodologies prior to performing DNA extractions (see Table 1). All sample collection and preparation activities were coordinated and performed by Metagenopolis. The control samples and Condition 1 samples were made up of individual fecal samples packed into discrete 2ml cryogenic vials prior to freezing. These samples were not subjected to any stabilization or normalization procedures and therefore are described as being raw. For Conditions 2 and 3, samples were stabilized with RNA Later solution and homogenized before being dispensed into 2ml cryogenic vials. This resulted in the generation of parent samples with a normalized matrix composition and volume. For Condition 2 samples, the normalized parent sample was left for 1 week at ambient temperature before freezing and storing at -80ºC. Condition 3 samples were immediately frozen and stored at -80ºC after normalization.

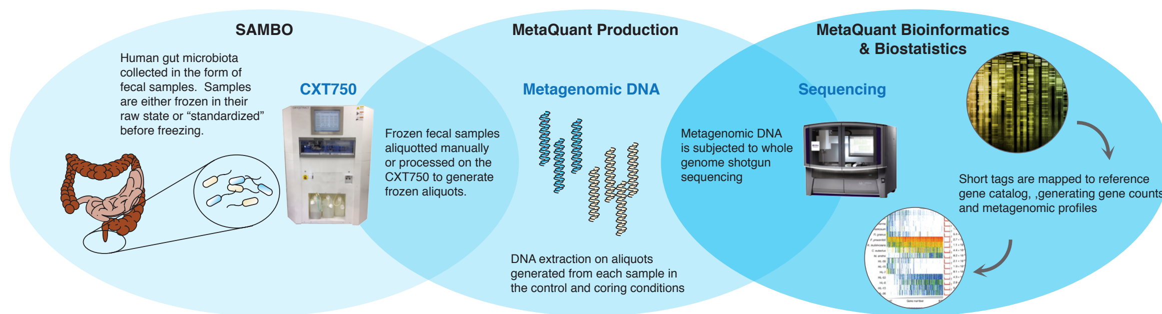
Sample Aliquotting

Control samples were manually aliquotted using a standard laboratory practices at Metagenopolis. For Conditions 1, 2 and 3, samples were shipped on dry ice to Cryoxtract Instruments for processing by automated frozen aliquotting using the CXT750 Frozen Sample Aliquotter. During frozen aliquotting, fecal samples and the resulting fecal cores were maintained at -80ºC. All parent samples and their respective frozen cores were immediately transferred back to -80ºC storage after the frozen aliquotting procedures were completed. All samples were shipped back to Metagenopolis for microbial DNA extraction and metagenomic analysis.

Quantitative Metagenomic Anaysis and Comparison

Aliquots generated from the control samples and frozen aliquotting conditions were delivered to Metagenopolis's MetaQuant platform in order to generate metagenomic profiles for comparison of control and test conditions. Specifically, DNA extraction was performed on the manual aliquots and cores produced from frozen aliquotting. After DNA extraction, the metagenomic DNA was subjected to whole genome shotgun sequencing using the SoLiD sequencer platform from Life Technologies. Short sequence tags (50 million per sample) were mapped onto a 3.3 million-gene catalog (Qin Nature 2010[1]) allowing for the generation of metagenomic profiles for each sample in the control and frozen aliquotting conditions. Figure 1 generalizes the strategy applied in determining the metagenomic profile of an individual's gut microbiota as well as the workflow followed in this evaluation. The resulting metagenomic profile for each sample processed in the control groups were statistically compared to their corresponding samples in the frozen aliquotting Conditions 1, 2, and 3.

Figure 1: Generalized Fecal Microbiome Analysis Workflow



RESULTS

Figure 2 shows the statistical comparison of each metagenomic profile generated for each subject and condition (control and frozen aliquotting conditions) in a colorized Spearman matrix chart. Specifically, the map displays the correlation of relative gene abundance for each sample and condition tested as referenced against the 3.3 million-gene catalog. Below the matrix chart, the Spearman correlation value for each conditional comparison is displayed. All three conditions resulted in correlation values greater than 0.8. This suggests that the resulting metagenomic profiles observed for samples subjected to frozen aliquotting had a high degree of correlation to the metegenomic profiles of those subjected to standard practices (manual frozen aliquots). However, amongst the frozen aliquotting conditions tested, the best performing condition was observed to be Condition 3 (Spearman correlation value = 0.95), which combined the use of stabilizing reagent, sample homogenization, and quick transfer to frozen storage at -80ºC. Conditions 1 and 2 were observed to result in Spearman correlation values of 0.89 and 0.92, respectively.

In addition to performing the best based on metagenomic profile, Condition 3 also holds additional benefits in comparison to Condition 1 in relation to sample homogenization. The standardization of fecal sample consistency and volume helps to ensure that the automated frozen aliquotting process produces uniformly sized cores on a consistent basis that can, in turn, be directly used in DNA extraction procedures.

CONCLUSIONS

Metagenomic research of the human gut microbiome requires the integration of many technological and scientific practices. The efficient collection and handling of fecal samples in order to mitigate pre-analytical variability should be a primary consideration since it conditions the quality of the overall analytical pipeline. Frozen aliquotting has been demonstrated to be a viable technology that is deployable during the early stages of a metagenomic workflow for fecal samples. The CXT750 Frozen Sample Aliquotter has been successfully implemented in Metagenopolis's SAMBO platform for sample collection and management in support of the establishment of a 1,000,000 plus fecal samples biobank. Integration of frozen aliquotting technology was supported by a rigorous evaluation of the technology using quantitative metagenomic analysis techniques to confirm the performance of the CXT750 instrument against formerly standardized fecal handling protocols.
For Metagenopolis, frozen aliquotting is playing a key role in the standardization of fecal sample quality control and storage by performing aliquotting procedures not possible by other automated techniques, such as liquid handling. This, in turn, mitigates pre-analytical variability and helps to ensure the generation of high-quality scientific data. Further tests will be implemented to fully automate all initial steps from sample reception to high-quality DNA preparation for metagenomic profiling.

INTEGRATION OF FROZEN ALIQUOTTING INTO SAMBO

SAMBO is the sample management and biobanking platform developed by Metagenopolis exclusively for the maintenance, tracking, and distribution of intestinal samples. The primary focus of SAMBO is the establishment of a national fecal biobank capable of storing and distributing over 1,000,000 fecal samples in support of gut microbiota research. Standardization of fecal sample collection, management, and distribution is critical to the SAMBO platform in regards to maximizing throughput while limiting pre-analytical variability for downstream analysis. Based on metagenopolis's evaluation of frozen aliquotting, the recent integration of two CXT750 Frozen Sample Aliquotters plays a central role in the standardization process.

Figure 3: Fecal Sample Collection and Standardization

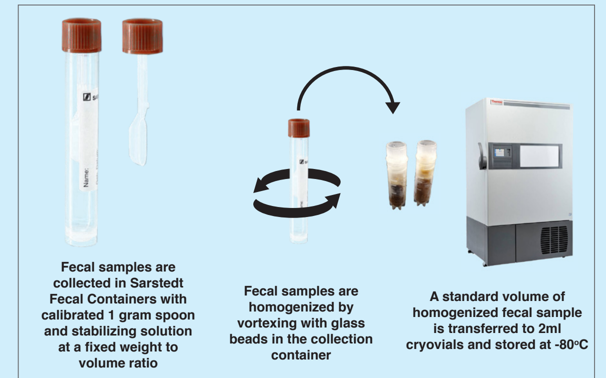
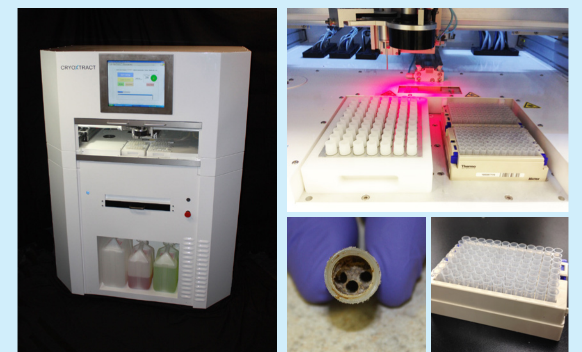


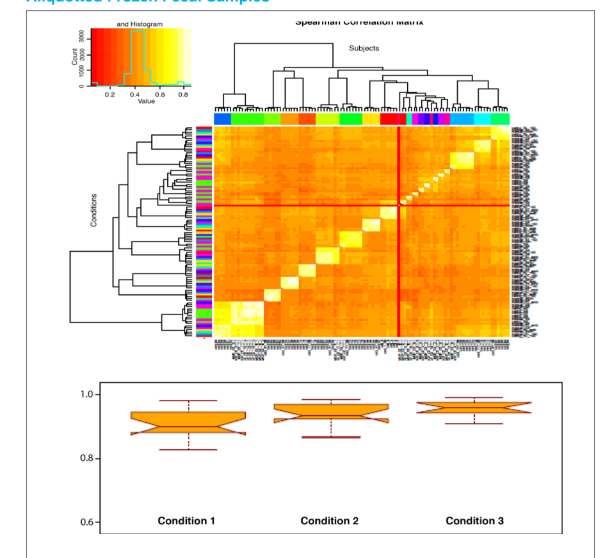
Figure 3 illustrates the workflow employed and the integration of frozen aliquotting in the SAMBO platform. Standard operating procedures proposed for auto-collection of samples utilize the addition of stabilizing solution to fecal samples as a means of preserving nucleic acids during collection and transfer of samples to Metagenopolis's biobank facility. Upon receipt, fecal samples containing the stabilizing solution are standardized by undergoing homogenization and transfer into two 2ml cryogenic vials at a fixed volume per sample. The standardized samples are then frozen and batch stored at -80ºC in preparation for quality control screening and long-term high-density frozen storage.

Figure 4: CXT750 Configuration for SAMBO Integration



The CXT750 Frozen Sample Aliquotter plays a key role in the quality control screen and high-density storage of fecal samples employed in the SAMBO platform. Up to 48 standardized samples, representing 24 patient samples, are frozen aliquotted on a single CXT750 instrument. For each subject (represented by two standardized samples), four frozen aliquots (2 cores per aliquot) are generated for high-density storage and one frozen aliquot for QC screening. By generating quality control aliquots and data in concert with sample transfer into high-density storage, pre-analytical variability is minimized and sample integrity maximized for every sample requested and distributed.

Figure 2: Metagenomic Comparison of Frozen Aliquotted and Manually Aliquotted Frozen Fecal Samples



[1]Qin J, Li R, Raes J. A Human Gut Microbial Gene Catalog Established by Metagenomic Sequencing. Nature. Mar 4, 2010; 464(7285): 59-65. doi: 10.1038 / Nature 08821