OMNIgene®·GUT

OMNIgene®•GUT stabilizes the microbiome profile at ambient temperature for 60 days and during transport

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OMNIgene•GUT is an all-in-one system for the easy self-collection and stabilization of microbial DNA from feces/stool for gut microbiome profiling. OMNIgene•GUT is uniquely able to take a snapshot of the microbial profile at the moment of collection, and maintain it for 60 days at ambient temperature. In this paper, we demonstrate that profiles of gut microbiota collected in OMNIgene•GUT are stable, highly reproducible and represent the in vivo state.

Introduction

The study of the relationship between the microbiome and its human host in health and disease relies on identifying and monitoring the microbial communities over a period of time. Recent discoveries demonstrate the utility of these microbial profiles as potential biomarkers with prognostic and diagnostic value. It is becoming evident in the literature that due to the dynamic nature of the gut microbiome, repeated sampling of large populations over time is essential to the development of such biomarkers. These studies, known as Microbiome-Wide Association Studies (MWAS) are challenged by low donor compliance, unreliable self-collection of biological samples, high cost and cumbersome shipping and handling procedures. Current methods for stool sampling and microbiota analysis involve the transport of specimens at ambient temperature or 4°C. However, these methods have the potential to expose samples to temperatures incompatible with microbiome stabilization. Failure to properly stabilize the microbiome during sample collection, transport and processing risk obscuring the biological and clinical meaning of the microbiome profile. Consequently, proper pre-analytical procedures should be followed to ensure the best possible representation of the *in vivo* microbiome profile.

Materials and methods

Sample collection

OMNIgene•GUT kits were used by adult donors to self-collect fecal samples according to the standard instructions provided with the kit. 15 donors were asked to collect 3 OMNIgene•GUT samples from the same bulk fecal sample, subsequently these samples were split into two studies of 6 and 9 donors. First study involved 6 donors that were used for evaluation of neutrality and sources of variability. Each of 6 donors collected 3 OMNIgene•GUT samples from the same bulk fecal sample (n=18 total). Additionally, aliquots of fresh feces were collected from the same bulk fecal sample by each donor and transported in a styrofoam box with frozen cold packs as per Human Microbiome Project standard procedure[1]. In the second study collection was expanded to 9 donors which were used to establish effectiveness of OMNIgene•GUT in recovering high quality DNA and stabilizing the microbiome profile over time.

DNA extraction and sample storage

Baseline extractions were performed within 3 hours of collection. For baseline analysis, a 0.25 mL aliquot was taken from OMNIgene•GUT samples and extracted using the PowerFecal® DNA Isolation Kit (MO BIO Laboratories, Inc.)[2]. Each 0.25 mL sample contains approximately 50 mg feces and 200 µL stabilizing liquid. Equivalent amounts of feces (approximately 50 mg) were extracted from fresh samples. Remaining OMNIgene•GUT and fresh samples were aliquoted into cryovials. OMNIgene•GUT and unstabilized (without stabilization liquid) samples were stored at room temperature (23±3°C) for up to 60 days, exposed to simulated transport conditions (50°C for 3 days, 37°C for 3 days) or subjected to 6 cycles of freezing and thawing (each cycle consisted of a minimum of 3 hours at -20°C and a minimum of 3 hours at 30°C).

Additionally, an aliquot of fresh stool from each donor was stored at -80°C as a control. After the room temperature holds, simulated transport conditions, freeze-thaw cycling or -80°C storage, an aliquot was extracted from all samples.

DNA analysis

DNA concentration and yield were determined using the Quant-iT™ PicoGreen® reagent (Life Technologies). DNA integrity and stability over time was evaluated by running approximately 50 ng of purified DNA on a 0.8% agarose gel and staining with ethidium bromide. λ DNA-HindIII ladder was used to determine the size of purified DNA.

16S sequencing

16S rRNA sequencing library preparation, sequencing and bioinformatics were conducted by Diversigen, Microbiome Discovery Service. V4 hypervariable region paired-end amplicon sequencing was performed using the Illumina® MiSeq®. A subset of samples were sequenced through GenoFIND™ Genomic Services (DNA Genotek) using the Illumina® MiSeq® platform with V3-V4 hypervariable region paired-end amplicon sequencing. Using QIIME and custom scripts, sequences were quality filtered. Paired-end reads were assembled and compared to the Greengenes database, clustered at 96% by UCLUST. After data normalization, sample-to-sample distance was measured using weighted UniFrac and Bray-Curtis distance on Operational Taxonomic Unit (OTU) abundance data (utilizing taxon abundance differences across samples, employing a pair-wise normalization by dividing the sum of differences by the sum of all detected OTU abundances). In all Bray-Curtis measurements, a donor matched T0 sample that had been extracted shortly after collection was used as one side of the pair-wise comparison. Analysis of the Shannon Index (SI) for each stabilization method was performed by measuring the proportion of each OTU relative to the total number of OTUs, and then multiplied by the natural logarithm of this proportion. Summation of the resulting product across all OTUs produced the SI for each sample. Sample collection methods were compared using the Mann-Whitney test.

Results

OMNIgene•GUT stabilization liquid maintains microbiome profile neutrality at the point-of-collection

The study of the microbiome requires that the profile generated represents the *in vivo* microbial communities present in the donor; thus, the collection and stabilization method should not introduce changes to the microbiome.

The use of chemical stabilization buffers can potentially modify the microbial composition of the sample by accelerating growth of some microbes while allowing the decay of others. In ideal conditions, the stabilization liquid should be neutral (i.e., it should not introduce any bias to the microbiome). Comparison of the 16S rRNA microbiome profile from fresh and OMNIgene•GUT stabilized samples showed that the liquid solution maintains a neutral profile and does not introduce bias (Figure 1).

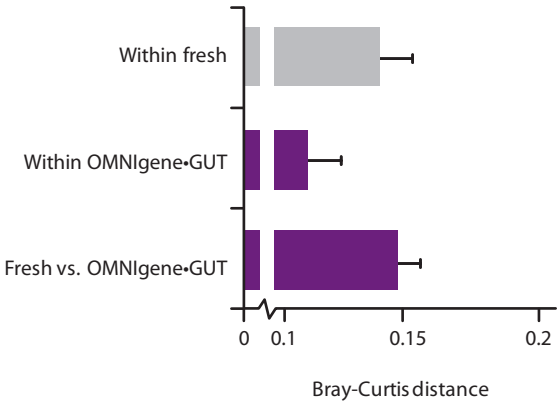

***Figure 1**: Bray-Curtis dissimilarity distances within and between fresh and OMNIgene•GUT collected samples. Mann-Whitney test showed comparable dissimilarity in all conditions, no statistical difference was observed.*

The study of relative OTU abundance by different statistical methods (e.g., Weighted UniFrac) provides a valuable description of the microbial community, however, it can obscure the understanding of the microbial community by minimizing the contribution of low abundance microbes. Proper study of the microbiome profile requires the preservation of the "richness" of the microbial communities. Richness is defined as the enumeration of microbial species or OTUs present in the sample

and is highly susceptible to environmental conditions, including changes in temperature, pH, oxygen concentration and chemical composition. These and other factors can induce bacterial growth or decay, thereby altering the number of OTUs detected in the sample[3].

Fresh and OMNIgene•GUT collected samples from 6 donors were extracted shortly after collection. The microbial OTUs identified in OMNIgene•GUT samples were compared with the OTUs present in corresponding fresh samples. Shannon Index (SI) for diversity was calculated by converting OTU abundance data into presence/absence calls. A Mann-Whitney test on the SI values showed no significant difference between OMNIgene•GUT and fresh samples, indicating that OMNIgene•GUT had no impact on the richness of the samples (Figure 2).

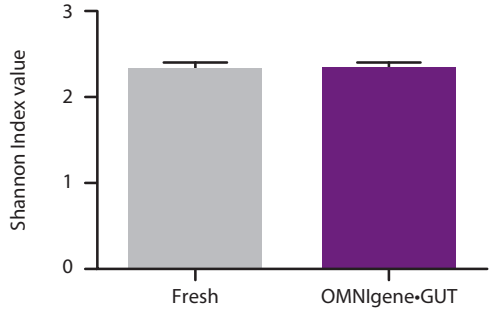

***Figure 2**: OMNIgene•GUT preserves sample richness. Richness was assessed by assigning presence/absence to individual OTUs and compared using Shannon-Index. Mann-Whitney test showed no significant differences between fresh and OMNIgene•GUT samples.*

Sources of variability in fecal sample collection

Bray-Curtis analysis showed systematic dissimilarity within replicates of fresh and OMNIgene•GUT samples. To understand the sources of such dissimilarity, we evaluated the variability introduced during collection and processing of fecal samples. Biological variability was assessed by generating microbiome profiles from 3 fresh and 3 OMNIgene•GUT samples collected from different sites within the same bulk sample. Technical variability was assessed using OMNIgene•GUT collected samples because this collection system provides homogenized liquid samples, reducing experimental errors during processing. We compared the profiles of replicate DNA extractions from the same tube (extraction variability) and replicate library preps from the same DNA (sequencing variability) (Figure 3). Bray Curtis dissimilarity distances were generated within the replicate groups.

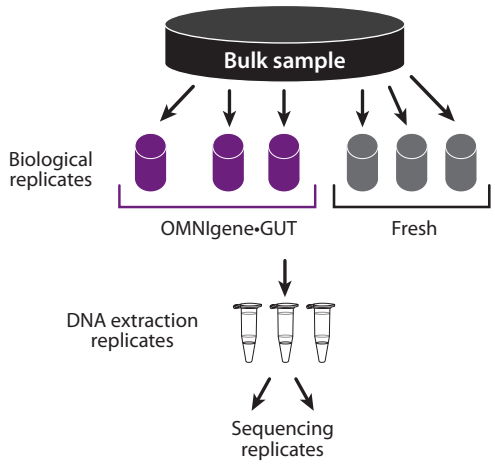

***Figure 3**: Sample collection scheme per donor. Each of 6 donors collected samples from 3 different sites of the bulk sample. Additionally, 3 DNA extraction replicates were performed from 1 of the OMNIgene•GUT samples (purple) and triplicate DNA aliquots from 1 extraction were sent for 16S rRNA sequencing.*

Similar variability was observed in biological replicates of fresh and OMNIgene•GUT collected samples (Bray-Curtis distances 0.14± 0.01 and 0.11± 0.01 respectively) (Figure 4). Our analysis showed that technical and biological variability introduce some dissimilarity to the 16S rRNA microbiome profile (Bray-Curtis distances biological variability 0.11; extraction variability 0.09 and sequencing variability 0.08). We concluded that the source of dissimilarity observed can be explained by the technical or biological variability, and that OMNIgene•GUT does not introduce any bias.

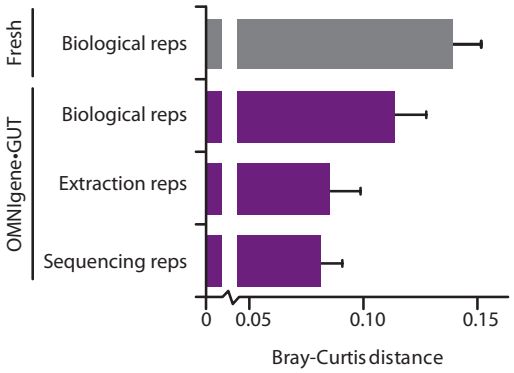

***Figure 4**: OMNIgene•GUT samples render highly reproducible microbiome profiles. Mann-Whitney tests on Bray-Curtis distances showed comparable dissimilarity in triplicate samples.*

OMNIgene•GUT preserves microbiome profiles for up to 60 days

In addition to maintaining profile neutrality, the accurate preservation of microbial community structure over time is a requirement for MWAS. We evaluated the capability of OMNIgene•GUT to stabilize samples during storage at 23°C for 60 days and exposure to simulated transport conditions (see next section).

OMNIgene•GUT stabilized samples were extracted at baseline (T0) and again after storage for 14 and 60 days at room temperature (23°C). Paired unstabilized samples were extracted at baseline (T0, fresh) and after 14 days at room temperature (23°C) or 14 days at -80°C (Figure 5). The similarity of the samples at baseline and after storage was evaluated using Bray-Curtis distances. Mann-Whitney analysis showed no significant difference in the Bray-Curtis distance for the OMNIgene•GUT samples stored at 23°C for 60 days when compared to unstabilized samples stored at -80°C for 14 days. In contrast, the Bray-Curtis distances for the unstabilized samples kept at 23°C for 14 days were significantly different when compared either to the OMNIgene•GUT(60 days) or -80°C storage.

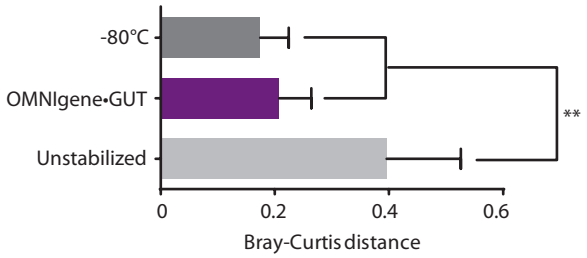

***Figure 5**: Bray-Curtis distance dissimilarity between unstabilized (14 days at 23°C), OMNIgene•GUT (60 days at 23°C) and -80°C (14 days at -80°C) samples when compared with fresh samples. Significant dissimilarity was assessed using Mann-Whitney (**P≤0.001).*

In order to understand the reproducibility among replicates, a cluster analysis of weighted UniFrac was performed using biological replicates of fresh (T0), unstabilized samples (T14 days at 23°C) and OMNIgene•GUT stabilized samples (T0 and T14 days at 23°C) (Figure 6). The fresh biological replicates and T0 and T14 OMNIgene•GUT biological replicates clustered tightly (96% similarity). The unstabilized biological replicates clustered together with a high separation from the fresh biological replicates (~63% similarity). Proper stabilization therefore has a large effect on profile clustering over time.

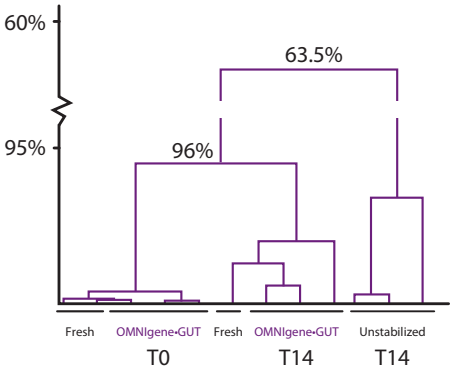

***Figure 6**: Dendrogram of microbiome weighted UniFrac % similarity of a representative donor. Extractions from 3 biological replicates were evaluated for each condition. Low % similarity to fresh sample indicates changes in the microbiome profile over time.*

OMNIgene•GUT effectively preserves the microbiome profile during transport

Samples are commonly exposed to undesirable conditions during transport from the point-of-collection to the processing laboratory. To simulate standard shipping conditions, unstabilized and OMNIgene•GUT stabilized samples were exposed to 50°C for 3 days, 37°C for 3 days or multiple freeze-thaw cycles. OMNIgene•GUT preserved high molecular weight DNA bands whereas unstabilized samples showed various degrees of degradation, particularly when exposed to 50°C or freeze-thaw cycles (Figure 7).

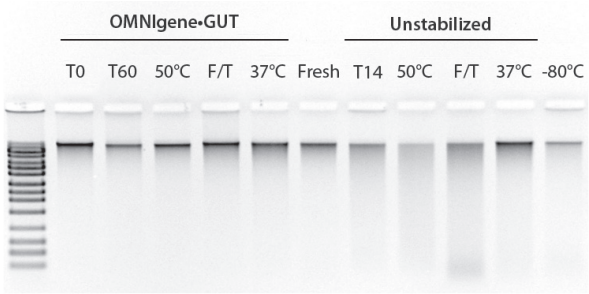

***Figure 7**: DNA integrity of OMNIgene•GUT samples subjected to simulated transport conditions. OMNIgene•GUT samples from a representative donor were stored at 23°C for 60 days (T60), 50°C for 3 days (50°C), 37°C for 3 days (37°C) or exposed to 6 freeze-thaw cycles (F/T). Unstabilized samples from the same donor were stored at 23°C for 14 days (T14), 1 day 50°C (50°C), 3 days at 37°C (37°C) and at -80°C for 14 days (-80°C).*

16S rRNA analysis confirmed that OMNIgene•GUT preserves the microbial community structure even at extreme temperatures. A Mann-Whitney test comparing Bray-Curtis distances of OMNIgene•GUT samples subjected to common shipping temperature and paired samples held at -80°C showed no significant differences. Conversely, unstabilized samples held at 37°C or subjected to freeze-thaw cycles showed significant differences when compared with samples held at -80°C (Figure 8).

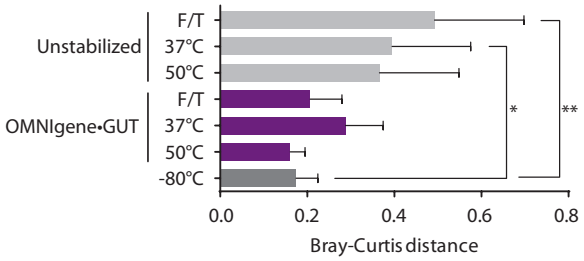

***Figure 8**: Bray-Curtis distance dissimilarity of OMNIgene•GUT samples exposed to simulated shipping conditions (as in Figure 7). Mann-Whitney test showed no differences between OMNIgene•GUT samples stored at various temperatures and unstabilized samples stored at -80°C. Significant dissimilarity was observed in unstabilized samples held at 37°C or subjected to freeze-thaw (F/T) conditions when compared to paired -80°C samples (*P≤0.05 and **P≤0.01, respectively).*

Conclusions

Stabilization, in the context of metagenomics, is a multi-dimensional attribute that encompasses:

- Neutrality (ability to capture unbiased profiles)
- Reproducibility (homogenous sample material from which, highly concordant aliquots can be taken), and
- DNA integrity (molecular weight), as measured over time.

Based on tightly controlled experiments and rigorous analysis, OMNIgene•GUT is the first and only device of its kind proven to effectively stabilize gut microbiota in human stool for 60 days at room temperature in addition to during real-life shipping and handling conditions and freeze-thaw cycling. This is of utmost importance to cost-effective scaling of MWAS, as well as for ensuring effective biomarker discovery and research translation.

References

1 Manual of Procedures - Human Microbiome Project (2010).
2 OMNIgene•GUT gut microbial DNA purification using MoBio PowerFecal DNA Isolation Kit. DNA Genotek. PD-PR-00434.
3 The Open Microbiology Journal, 3, 40-46 (2009).