OMNIgene™•ORAL and OMNIgene™•VAGINAL devices are validated tools for the collection and stabilization of nucleic acids from oral and vaginal microbiome samples

Anne Bouevitch, Evgueni Doukhanine and Brice LeFrançois
DNA Genotek, Ottawa, Ontario, Canada
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Introduction

In recent years, we have gained better understanding of the critical role the human microbiome plays in health and disease.[1-3] The oral and vaginal cavities are highly distinct ecological niches and, as such, harbour distinct microbial communities.[4] Beyond site-specific bacterial profiles, significant differences can exist between donors and their microbial community structures and individual profiles can change over time.[5,6] Having the appropriate tools to collect and stabilize human microbiome samples is critical for microbiome studies. These tools must be able to capture unique microbial profiles that represent the in vivo state as closely as possible. DNA Genotek has developed and validated 3 swab-based microbial collection devices for the study of the gum/plaque, tongue or vaginal microbiome. In this paper, we show that microbial nucleic acids (DNA and RNA) as well as microbial profiles of samples collected with OMNIgene™•ORAL or OMNIgene™•VAGINAL devices remain stable during shipping or storage at room temperature.

Materials and methods

Sample collection and processing

Donors collected oral and vaginal microbiome samples using OMNIgene devices (OMR-110, OMR-120 or OMR-130) following the instructions supplied with the products. When collecting paired samples (i.e., multiple tubes), donors were instructed to collect from the right and left gums (OMR-110) and the right and left sides of the tongue (OMR-120). For OMR-130, donors were to collect two vaginal samples. Upon collection, swabs were immediately placed into OMNIgene collection tubes, except for neutrality testing purposes where collected swabs were immersed in TES buffer (10 mM Tris-HCl pH7.5, 1 mM EDTA, 100 mM NaCl) and extracted immediately. Collected OMNIgene samples were extracted at baseline (T0), after storage at room temperature (23°C ± 3°C) or -80°C for 30 days or after 3 freeze-thaw cycles (20°C to +30°C, with a minimum of 3 hours at each temperature). Prior to nucleic acid extraction, samples were treated with Proteinase K for 1 hour at 50°C.

DNA and RNA extraction, quantification and qPCR

DNA was extracted from a 250 µL aliquot using the MasterPure™ Complete DNA and RNA Purification Kit (Lucigen®). Final DNA pellet was reconstituted in 50 µL H_2O. RNA was extracted from a 250 µL aliquot using the RNeasy® PowerMicrobiome® Kit (QIAGEN®) and eluted in 100 µL H_2O. DNA was quantified with the Quant-iT™ PicoGreen™ dsDNA Assay Kit (Thermo Fisher Scientific) and quality was assessed by running samples on a 0.8% agarose gel. RNA quality and concentration were determined by running samples on RNA 6000 Pico Chips on a 2100 Bioanalyzer system (Agilent Technologies). Relative bacterial DNA content in oral and vaginal microbiome samples was determined by qPCR using universal bacterial primers targeting the 16S rDNA gene (BacrRNA173-F 5'-ATTACCGCGGCTGCTGG; BacrRNA173-R 5'-CCTACGGGAGGCAGCAG 3'). Serial dilutions of *E. coli* gDNA were used to generate a standard curve and as a control (pure bacterial DNA).

Sequencing and analysis

Library preparation was conducted by amplification of the 16S V3-V4 hypervariable regions (Fwd: 5'- CCTACGGGNGGCWGCAG-3'; Rev: 5'-GACTACHVGGGTATCTAATCC-3'). For RNA profile analysis, an aliquot of total RNA (7 µL) was reverse transcribed into cDNA using random hexamers and MMLV-RT. 2.5 µL cDNA and 12.5 ng DNA were used as an input into the V3-V4 16S PCR reaction, respectively. Final libraries were pooled, and paired-end sequencing was performed using PE-300 V3 kit (2 x 300 bp) on a MiSeq® System (Illumina®).

For 16S V3-V4 hypervariable regions sequencing, the FLASH algorithm[7] was used for read merging and automated rejection of low-quality sequences; quality screening for length and ambiguous bases was performed in mothur.[8] A closed-reference taxonomic classification was performed where each sequence was aligned to the Greengenes version 13.8 reference database. Sequences were aligned at 97% sequence identity using the NINJA-OPS tool, version 1.2 (Knights Lab, unpublished data). A genus-level table was calculated by summarizing the absolute abundance before rarefaction. All samples were rarefied to an even sampling depth of 31,261 reads per sample after taxonomic classification at the operational taxonomic unit (OTU) level. Alpha diversity metrics (Shannon index (SI), Chao1) were obtained by measuring the proportion of each OTU relative to the total number of OTUs. Bray-Curtis dissimilarity was calculated on the rarefied OTU-level table with the beta_diversity.py workflow in QIIME 1.9.1.

For whole genome sequencing (WGS), samples were sequenced using Diversigen's proprietary BoosterShot™ pipeline on an Illumina NovaSeq platform, and downstream sequence processing and taxonomic annotation were performed within Diversigen's Core Analysis™ pipeline. Jensen-Shannon divergence was calculated on species-level taxonomic annotation after subsetting samples to a read depth of 14,000 reads. Principal coordinate analysis (PCoA) plots were generated in R using the phyloseq package. Taxonomic bar plots showing the percentage abundance at genus level were calculated from the total read depth per sample.

Results

Oral and vaginal microbiome samples have varying amounts of host DNA

OMNIgene•ORAL and OMNIgene•VAGINAL are swab-based collection devices that were specifically developed to collect gum/plaque (OMR-110), tongue (OMR-120) and vaginal (OMR-130) microbiome samples. Unlike stool microbial communities, oral and vaginal microbial communities are tightly associated with host epithelial tissue, and as such a significant amount of host material can be harvested during the collection process. We collected oral and vaginal microbiome samples from 10 healthy donors using OMR-110, OMR-120 or OMR-130 kits.

Total DNA was extracted and the relative abundance of bacterial DNA was determined using a qPCR assay targeting 16S rDNA. We found that tongue microbiome samples had a significantly higher proportion of bacterial DNA (44% on average) than did gum/plaque or vaginal samples (4% and 1.8% on average, respectively) (Figure 1). We determined that tongue samples were the most challenging sample to stabilize in the OMNIgene kits, as the relative abundance of bacteria was 10x-20x higher than in the other sample types. As a result, we used tongue microbiome samples as a paradigm in subsequent experiments (i.e., neutrality testing and 30-day room temperature stability).

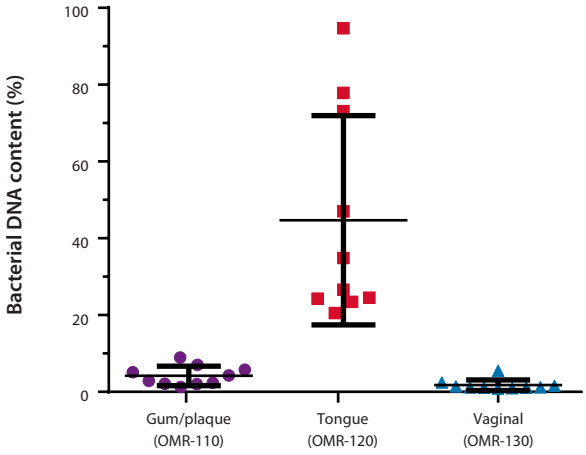


Figure 1: Tongue microbiome samples have higher relative bacterial content than gum/plaque or vaginal microbiome samples*. Oral (tongue vs. gum/plaque) and vaginal microbiome samples were collected by 10 donors in OMNIgene devices and DNA was extracted. The relative proportion of bacterial DNA was estimated based on 16S rDNA qPCR yields versus total concentration of DNA determined through PicoGreen. Average bacterial DNA content was 4% for gum, 44% for tongue and 1.8% for vaginal samples. (*** p < 0.001 One-way ANOVA)*

OMNIgene•ORAL devices are neutral and do not impact bacterial profiles upon sample collection

We first assessed if collection of a tongue microbiome sample in OMNIgene•ORAL preservation buffer would introduce a bias in the microbial profile. Paired tongue microbiome samples (left side vs. right side of the tongue) were collected by 8 donors with either OMNIgene•ORAL (OMR-120) or Puritan flocked swabs returned in 1 mL TES buffer (10 mM Tris-HCl pH7.5, 1 mM EDTA, 100 mM NaCl). DNA was extracted immediately after collection.

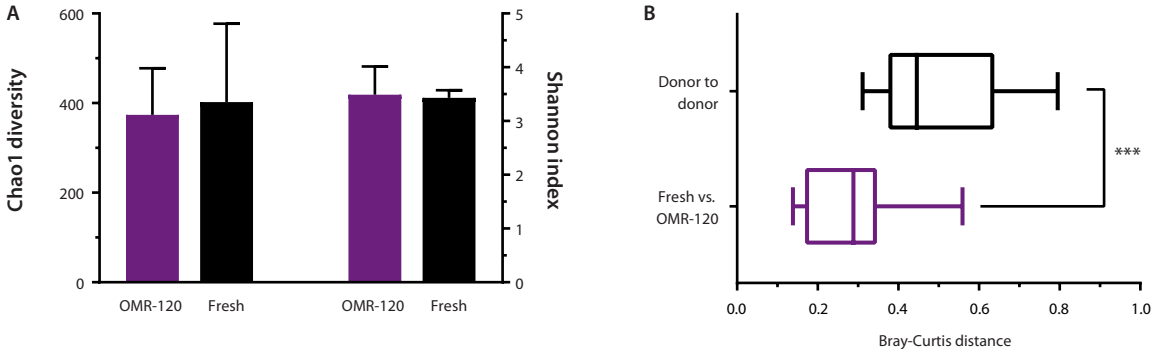


Figure 2: Sample collection in OMNIgene•ORAL is neutral and has no detectable impact on tongue DNA microbial profiles. *(A) Alpha diversity metrics (Chao1 and Shannon index) are similar in fresh tongue samples compared to a sample collected in OMNIgene•ORAL kits (OMR-120). n.s., non-significant Mann-Whitney test. (B) Beta diversity measure (Bray-Curtis dissimilarity) between paired tongue samples from the same donor (fresh vs. OMR-120) as compared to donor-to-donor dissimilarity. (*** $p < 0.001$ Mann-Whitney test). Paired tongue samples were collected (fresh vs. OMR-120) by 8 donors and DNA was extracted at baseline. Alpha and beta diversity metrics were generated following 16S sequencing (V3-V4).*

Bacterial profiles were generated by 16S amplicon sequencing and analyzed by donor. Alpha diversity metrics (Shannon index, Chao1) (Figure 2A) were similar for samples collected with OMNIgene•ORAL versus those in TES buffer (fresh), indicating that species richness and evenness were not affected by the use of preservation buffer in OMR-120. Similarly, Bray-Curtis distance between paired samples from the same donor (fresh vs. OMR-120) was low (median = 0.28) and was significantly lower than the dissimilarity seen when comparing samples from different donors (Figure 2B). This result demonstrates that collection in OMNIgene kits has no detectable impact on microbial profiles.

OMNIgene•ORAL devices preserve tongue and gum/plaque microbial nucleic acids and profiles

To assess nucleic acid stability during storage at room temperature or during shipping at ambient temperature, paired gum/plaque and tongue samples were collected by 20 healthy individuals with OMNIgene•ORAL devices (OMR-110 and OMR-120). DNA and RNA were extracted from each kit at baseline (T0) and following storage at room temperature/-80°C for 30 days or 3 freeze-thaw cycles (-20°C to +30°C). Freeze-thaw cycles were used to simulate harsh conditions that can be encountered during shipping at ambient temperature. DNA and RNA yields were highly donor- and site-dependent and were not impacted by storage and/or freeze-thaw cycles (Table 1).

Table 1: Average DNA and RNA yields for samples collected in OMNIgene•ORAL devices (OMR-110 and OMR-120). DNA and RNA were extracted from a 250 µL aliquot at baseline (T0), following storage at room temperature/-80°C for 30 days or for 3 freeze-thaw cycles. DNA was quantified with the PicoGreen assay while RNA concentration was determined by running the samples on the RNA PicoChip. Data are presented as mean ± standard deviation. Median yields are shown in brackets.

	Analyte	T0 (baseline)	3 freeze-thaw cycles	30 days at room temperature	30 days at -80°C
Gum/plaque (OMR-110)	**DNA**	1.6 ± 0.83 µg/kit (1.56 µg)	1.68 ± 0.80 µg/kit (1.70 µg)	N/A	2.74 ± 1.02 µg/kit (2.31 µg)
	RNA	577 ± 571 ng/kit (428 ng)	523 ± 713 ng/kit (258 ng)	N/A	N/A
Tongue (OMR-120)	**DNA**	2.54 ± 1.32 µg/kit (2.39 µg)	2.81 ± 1.36 µg/kit (2.66 µg)	3.52 ± 1.91 µg/kit (2.94 µg)	3.65 ± 1.71 µg/kit (3.82 µg)
	RNA	490 ± 376 ng/kit (440 ng)	929 ± 1062 ng/kit (566 ng)	581.7 ± 377 ng/kit (424 ng)	N/A

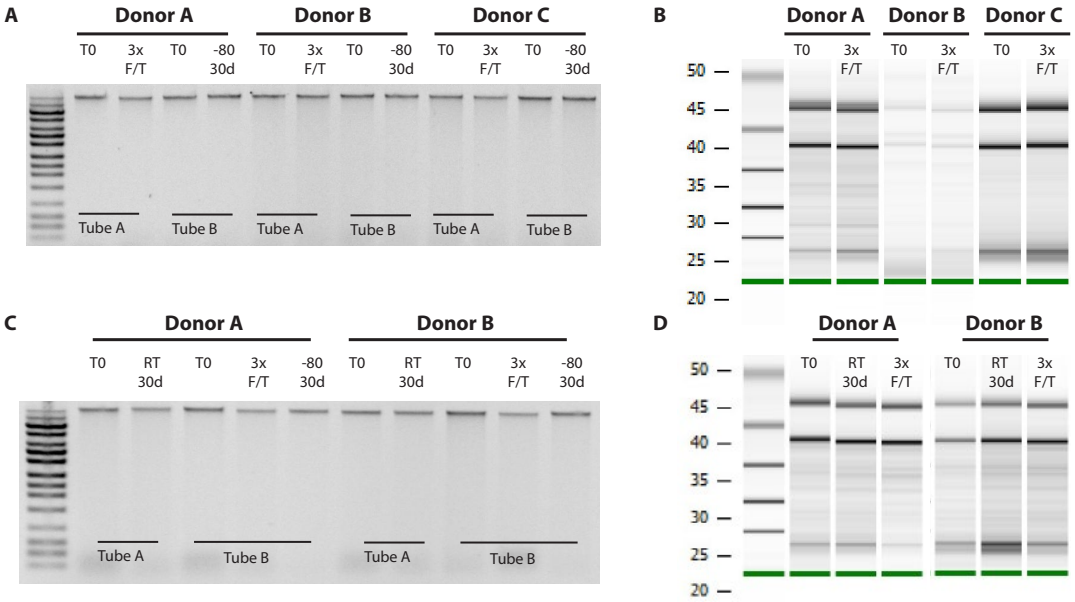


Figure 3: OMNIgene•ORAL devices maintain DNA and RNA quality of collected oral microbiome samples (gum/plaque and tongue) during extended storage at room temperature or following multiple freeze-thaw cycles*. (A and B) DNA and RNA quality of gum/plaque samples collected in OMNIgene•ORAL (OMR-110) and extracted at baseline (T0), after 3 freeze-thaw cycles (3x F/T) or storage at -80°C for 30 days (-80 30d). (C and D) DNA and RNA quality of tongue samples collected in OMNIgene•ORAL (OMR-120) and extracted at baseline (T0), after storage at room temperature for 30 days (RT 30d), after 3 freeze-thaw cycles (3x F/T) or storage at -80°C for 30 days (-80 30d). Samples from 2-3 representative donors are shown.*

For both OMNIgene•ORAL devices (OMR-110 and OMR-120), DNA integrity was maintained when samples were kept at room temperature for up to 30 days or subjected to 3 freeze-thaw cycles (Figure 3A and 3C). DNA quality was comparable to the quality of a sample stored at -80°C, a control storage condition for nucleic acids. Similarly, gum/plaque and tongue RNA quality was largely unaffected by storage at room temperature for up to 30 days or following multiple freeze-thaw cycles (Figure 3B and 3D). No significant differences were seen between samples stored 30 days at room temperature and those put through 3 freeze-thaw cycles.

To assess sample stability in collected devices, bacterial profiles across different post-collection time points were generated from the extracted nucleic acids using 16S amplicon sequencing targeting the V3-V4 region. Bray-Curtis dissimilarity between OMNIgene•ORAL (OMR-110) samples extracted at baseline (T0) and samples extracted after 3 freeze-thaw cycles was relatively small and comparable to the dissimilarity between samples extracted at baseline (T0) and samples stored at -80°C for 30 days (control) for both DNA and RNA (Figure 4A); in contrast, comparisons between biological replicates (left gum line vs. right gum line) or across donors showed greater distances (Figure 4A), as expected. Taxonomic bar plots (genus level) further demonstrate the stability of both DNA and RNA in OMNIgene•ORAL devices with highly consistent gum/plaque bacterial taxonomic profiles between samples extracted at baseline (T0), subjected to 3 freeze-thaw cycles or stored at -80°C for 30 days (Figure 4B and 4C). Similar DNA and RNA profile stability was seen for tongue microbiome samples, including following extended storage at room temperature (Figure 5). Our results demonstrate that OMNIgene•ORAL captures and maintains gum and tongue microbial profiles, during 30-day storage at room temperature or following multiple freeze-thaw cycles (Figure 4 and 5). Taken together, our data clearly indicates that OMNIgene•ORAL is a highly effective at-home collection tool for oral microbiome studies.

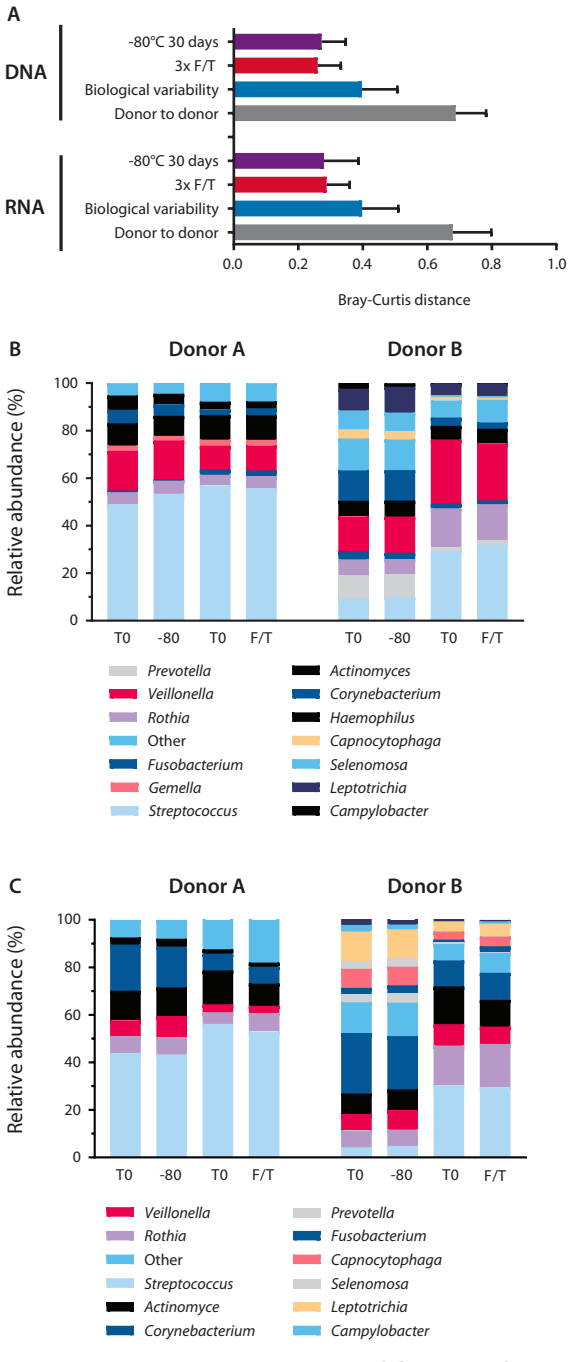


Figure 4: OMNIgene•ORAL (OMR-110) stabilizes gum/plaque microbial profiles through multiple freeze-thaw cycles. *(A) Bray-Curtis dissimilarity for DNA and RNA samples extracted following 3 freeze-thaw cycles or storage at -80°C for 30 days as compared to baseline (T0). Donor-to-donor dissimilarity and biological variability (difference between tube A and B at baseline) are also shown for reference. (* $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ Kruskal-Wallis test) (B and C) 16S taxonomic profiles (V3-V4 - genus level) for paired DNA (B) or RNA (C) samples collected in OMR-110 devices and extracted at baseline (T0) after 3 freeze-thaw cycles or storage at -80°C for 30 days. Bar plots from 2 representative donors are shown.*

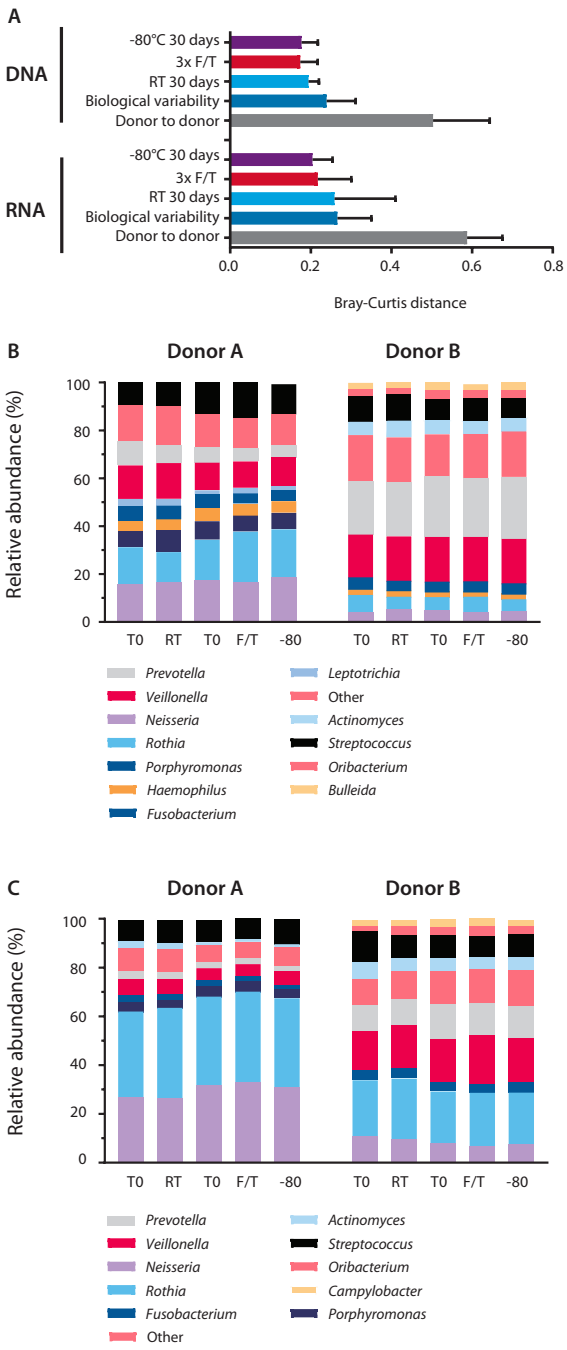


Figure 5: OMNIgene•ORAL (OMR-120) captures and stabilizes tongue microbial profiles during extended storage at room temperature or through multiple freeze-thaw cycles. *(A) Bray-Curtis dissimilarity for DNA and RNA samples extracted following 3 freeze-thaw cycles, storage at room temperature for 30 days or storage at -80°C for 30 days as compared to baseline (T0). Donor-to-donor dissimilarity and biological variability (difference between tube A and B at baseline) are also shown for reference. (* $p < 0.05$ and *** $p < 0.001$ Kruskal-Wallis test) (B and C) 16S taxonomic profiles (V3-V4 - genus level) for paired DNA (B) or RNA (C) samples collected in OMR-120 devices and extracted at baseline and after 3 freeze-thaw cycles or storage at -80°C and at room temperature for 30 days. Bar plots from 2 representative donors are*

Table 2: Average DNA and RNA yields for samples collected in OMNIgene•VAGINAL devices (OMR-130). DNA and RNA were extracted from a 250 µL aliquot at baseline (T0), following storage at -80°C for 30 days or for 3 freeze-thaw cycles. DNA was quantified with the PicoGreen assay while RNA concentration was determined by running the samples on the RNA PicoChip. Data are presented as mean ± standard deviation. Median yields are shown in brackets.

	Analyte	T0 (baseline)	3 freeze-thaw cycles	30 days at room temperature	30 days at -80°C
Vaginal (OMR-130)	**DNA**	15.1 ± 6.5 µg/kit (13.4 µg)	14.5 ± 7.1 µg/kit (15.3 µg)	N/A	17.3 ± 7.1 µg/kit (18.33 µg)
	RNA	942 ± 521 ng/kit (823 ng)	820 ± 400 ng/kit (668 ng)	N/A	N/A

shown.

OMNIgene•VAGINAL devices preserve vaginal microbial nucleic acids and profiles

Paired vaginal microbiome samples were collected by 20 healthy individuals with OMNIgene•VAGINAL (OMR-130) devices. Stability was assessed following multiple freeze-thaw cycles, a treatment known to be highly detrimental to nucleic acid stability. Briefly, DNA and RNA were extracted from each sample at baseline (T0) or following 3 freeze-thaw cycles (-20°C to +30°C with a minimum of 3 hours at each temperature). As a control, samples were also stored for 30 days at -80°C before DNA and RNA were extracted. Freeze-thaw cycles had no detectable impact on nucleic acid yields (Table 2) or on the quality/integrity of the extracted DNA and RNA (Figure 6A and 6B). High molecular weight DNA and high-quality total RNA (as demonstrated by the presence of intact 16S and 23S rRNA bands) were recovered for all donors and conditions.

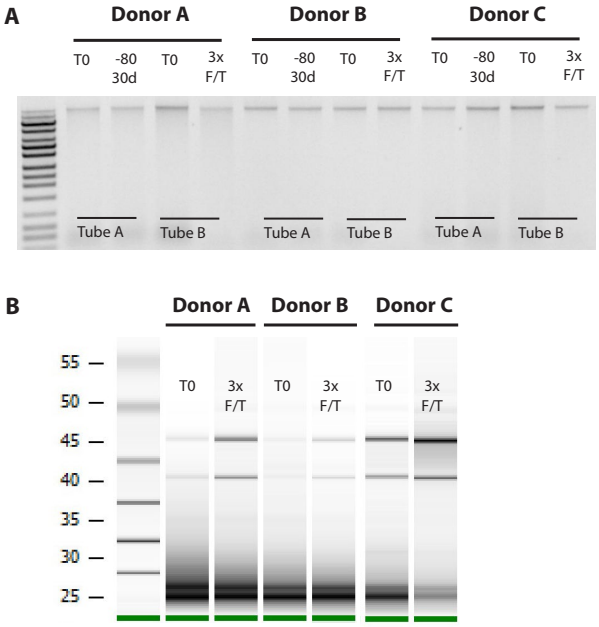
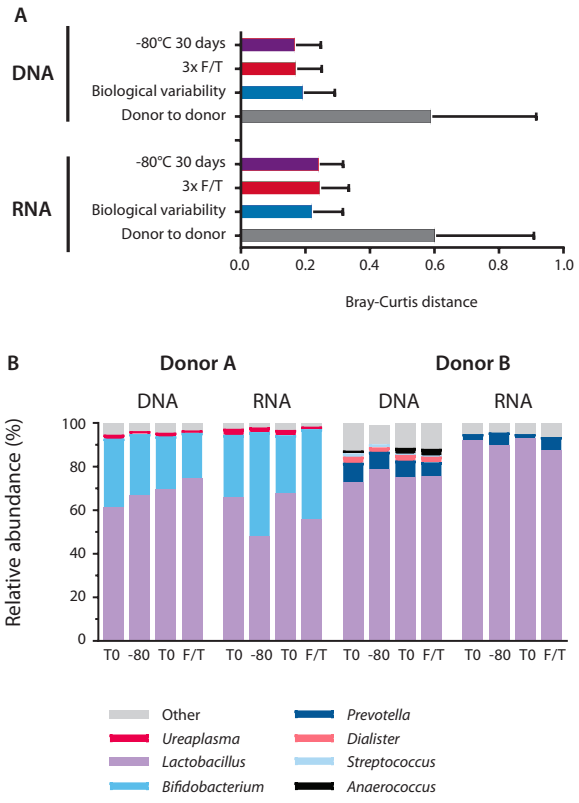


Figure 6: OMNIgene•VAGINAL devices maintain DNA and RNA quality of vaginal microbiome samples following multiple freeze-thaw cycles. *DNA (A) and RNA (B) quality of vaginal microbiome samples collected using OMNIgene•VAGINAL (OMR-130) devices and extracted at baseline (T0), after 3 freeze-thaw cycles (3x F/T) or storage at -80°C for 30 days (-80 30d). DNA and RNA samples from 3 representative donors are shown.*

To further assess profile stability, bacterial profiles were generated from the extracted nucleic acids using 16S amplicon sequencing, targeting the V3-V4 region. Bray-Curtis dissimilarity between DNA and RNA samples extracted at baseline (T0) and samples extracted after 3 freeze-thaw cycles was relatively small and comparable to the biological variability (2 samples collected side by side by the same donor in 2 different tubes). In contrast, Bray-Curtis dissimilarity was significantly higher when DNA and RNA profiles were compared across donors. This result indicates that each donor's unique microbial profile is captured and stabilized in OMNIgene•VAGINAL devices. Moreover, taxonomic profiles between samples extracted at baseline (T0) and those subjected to freeze-thaw cycles or to controls (-80°C for 30 days) were highly similar — this was observed for both DNA and RNA derived profiles (Figure 7B and 7C). Importantly, both *Lactobacillus*-dominated vaginal samples (Figure 7B) and non-*Lactobacillus*-dominated samples (Figure 7C) were preserved in OMNIgene•VAGINAL devices.



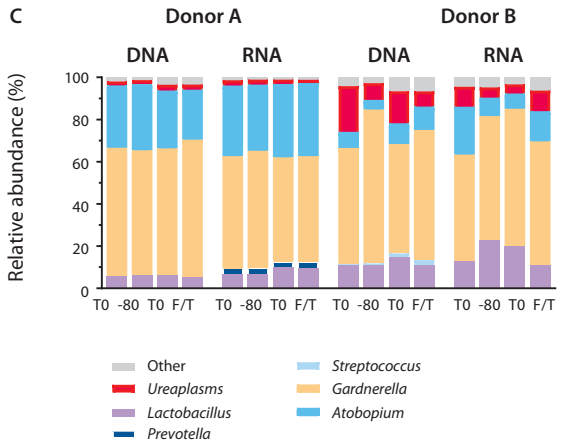


Figure 7: OMNIgene•VAGINAL (OMR-130) devices stabilize vaginal microbial profiles through multiple freeze-thaw cycles. *(A) Bray-Curtis dissimilarity for DNA and RNA samples extracted following 3 freeze-thaw cycles or storage at -80°C for 30 days as compared to baseline (T0). Donor-to-donor dissimilarity and biological variability (difference between paired replicate tube A and B at baseline) are also shown for reference. (*** $p < 0.001$ Kruskal-Wallis test) (B and C) 16S taxonomic profiles (V3-V4 - genus level) for Lactobacillus-dominated (B) and non-Lactobacillus-dominated (C) dominated samples collected in OMR-130 devices with DNA and RNA extracted at baseline (T0) after 3 freeze-thaw cycles or storage at -80°C for 30 days. Bar plots from 2 representative donors are shown.*

OMNIgene•ORAL and OMNIgene•VAGINAL devices stabilize bacterial profiles under real-life conditions

Lastly, we tested the performance of our OMNIgene devices in a real-life sample shipping and lab processing scenario. OMNIgene•ORAL (tongue and gum/plaque) and OMNIgene•VAGINAL samples were collected by 10-15 healthy individuals in Ottawa, Canada, and shipped at ambient temperature (no cold-chain transport) to Diversigen in Minneapolis, USA. Upon receipt, samples were stored for a few days at room temperature before DNA was extracted using DNeasy® PowerSoil® Pro HT Kit (QIAGEN®). Metagenomic sequencing of the samples was then performed with BoosterShot, and taxonomic profiles were generated with Diversigen's Core Analysis pipeline. Representative vaginal and oral profiles were obtained from these samples (Figure 8A and 8B). WGS data identified both *Lactobacillus* (donors 1 and 2 — Figure 8A) and non-*Lactobacillus* vaginal samples (donors 3 and 4 — Figure 8A). Oral samples, on the other hand, were dominated by *Streptococcus*,

Neisseira and *Rothia*, with discrete differences between tongue and gum/plaque samples. Principal coordinate analysis revealed that each site had a unique microbial profile (Figure 8C), echoing the results of the Human Microbiome Project.[4] Taken together, these data indicate that OMNIgene devices capture and maintain site- and donor-specific bacterial profiles from point of collection to point of extraction/processing.

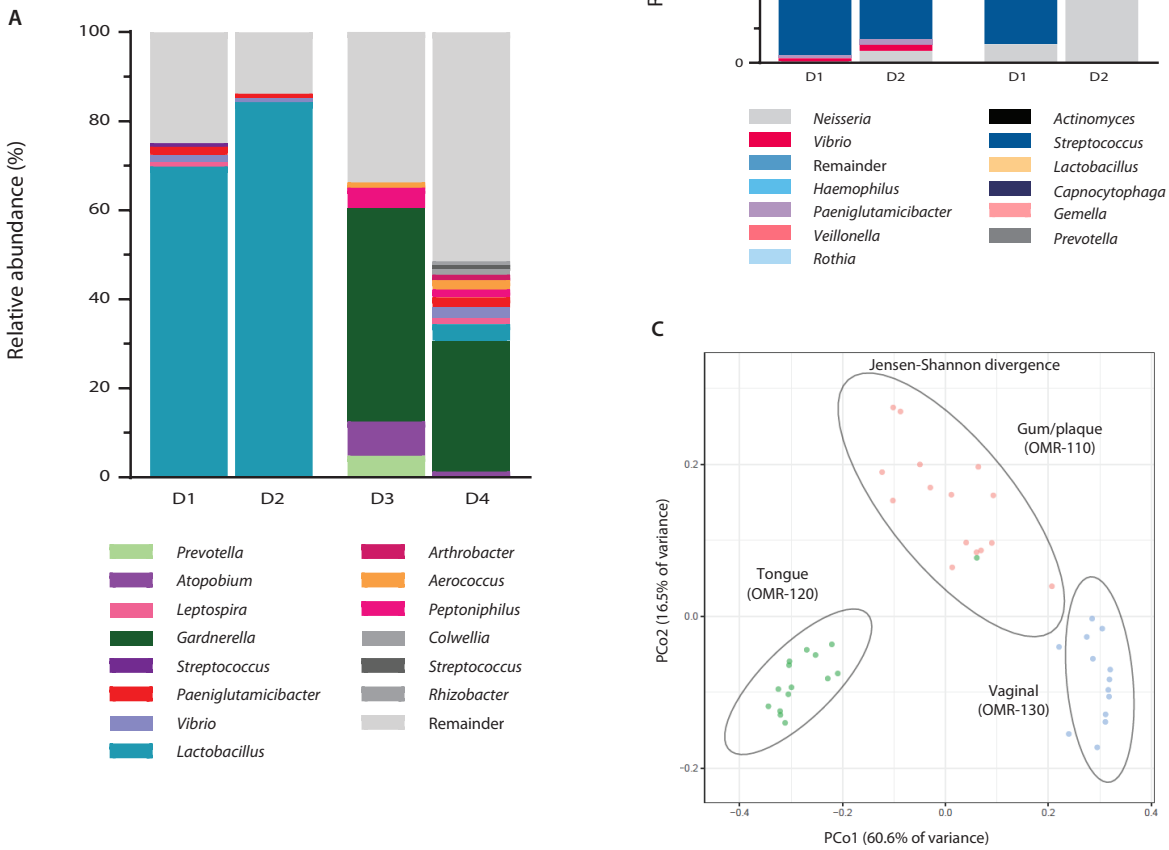


Figure 8: OMNIgene•ORAL and OMNIgene•VAGINAL devices capture and stabilize site-specific microbiome profiles at point of collection. *(A and B) Taxonomic profiles (genus level) of vaginal (A) or oral (B) microbiome samples collected in OMNIgene devices (OMR-110, 120 or 130) and sequenced by whole genome sequencing. (C) Principal coordinate analysis (PCoA) based on Jensen-Shannon divergence between oral and vaginal samples. Samples were collected in Ottawa (Ontario, Canada) and shipped at ambient temperature to Diversigen (Minnesota, USA), where they were extracted and processed. Samples were sequenced with BoosterShot and taxonomic assignment was performed with the Core Analysis™ workflow. Ellipses represent the 95% confidence interval for each sample type: gum/plaque (red), tongue (green) and vaginal (blue).*

Conclusions

OMNIgene•ORAL and OMNIgene•VAGINAL devices:

- utilize intuitive designs and instructions to enable highly compliant at-home collection of oral and vaginal microbiomes
- preserve high-quality microbial DNA and RNA during shipping at ambient temperature and extended storage at room temperature
- maintain neutral microbial profile of samples without introduction of any detectable bias
- maintain oral and vaginal DNA and RNA bacterial profile stability during shipping at ambient temperature and during storage at room temperature for up to 30 days
- are validated tools for microbiome studies with the ability to accurately capture and stabilize complex microbial profiles from point of collection onward

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