APPLICATION NOTE

MagMAX Microbiome Ultra Nucleic Acid Isolation Kit

Metagenomic analysis of the human microbiome with a new MagMAX kit automated on a KingFisher platform

Key message

- The Applied Biosystems™ MagMAX™ Microbiome Ultra Nucleic Acid Isolation Kit isolates high-quality, inhibitor-free DNA and RNA from human stool and other sample types
- The quality of the nucleic acid is suitable for metagenomics sequencing and other techniques that are critical for developing a comprehensive profile of the genes in a given sample
- The use of Thermo Scientific™ KingFisher™ instruments with the MagMAX microbiome kit can reduce overall processing time for a faster workflow

Introduction

The human microbiome has emerged as a key player in the diagnosis and treatment of a number of diseases. Proper identification of the microbes that constitute the microbiome and the calculation of their abundance are both major challenges, overcome in part by the advent of metagenomics sequencing technology, which provides a comprehensive profile of all of the genes in a given sample.

The Human Microbiome Project (HMP) has demonstrated that each body habitat harbors different dominant signature taxa. Comparison of metagenomic data generated for diseased and healthy subjects identified a number of bacteria linked to the onset of, or protection from, many diseases [1].

The gut contains the bulk of microbiota associated with the human body and has been one of its most thoroughly examined ecosystems [2]. Fecal samples are commonly used for these analyses because they are easy to obtain and contain a large amount of biomass. Body fluids like saliva and urine are less studied, but each contains unique bacterial communities.

The isolation of microbial DNA is a key prerequisite for determining the microbiome profile of each body habitat, but challenges remain in obtaining sufficient DNA quantities for accurate genomic inference of microbiome composition, and facilitating comparability of findings across sample types. Here we demonstrate the capability of the MagMAX Microbiome Ultra Nucleic Acid Isolation Kit to isolate the total nucleic acid from feces, urine, and saliva. Microbial diversity of each sample type was studied in depth by metagenomics sequencing. The study highlights the potential for utilization of the microbiome as a disease marker.

Materials and methods

Human fecal, saliva, and urine samples were obtained from two healthy donors, and total nucleic acid isolations were performed in duplicate on the day of collection using the MagMAX Microbiome Ultra Nucleic Acid Isolation Kit. Inputs of 100 mg of feces and 400 µL of saliva and urine were used. Total nucleic acid was isolated in an automated fashion using the Thermo Scientific™ KingFisher™ Flex Magnetic Particle Processor with 96 Deep-Well Head. Yields were measured on the Thermo Scientific™ NanoDrop™ 2000 Spectrophotometer. Purity and quality of the fecal total nucleic acid preparations were assessed by measuring absorbance (A_{260}/A_{280}) on the spectrophotometer and subsequently by performing 1% agarose gel electrophoresis.

Next, qPCR analysis was performed in triplicate for each sample using Applied Biosystems™ TaqMan® Assays with Applied Biosystems™ TaqMan® Fast Advanced Master Mix under fast cycling conditions. Each reaction contained 20% of nucleic acid preparation by volume.

Metagenomics processing

Total nucleic acid samples isolated from feces, saliva, and urine were sent to Zymo Research (Irvine, CA) for metagenomics sequencing using the ZymoBIOMICS™ Shotgun Metagenomic Sequencing Service.

Sequencing libraries were prepared with the KAPA™ HyperPlus Kit using 100 ng of DNA as input, according to the manufacturer's protocol. The libraries used internal 8 bp barcodes and Illumina™ TruSeq™ adapters. All libraries were quantified with the Agilent™ TapeStation™ system and pooled evenly. The final pool was quantified using qPCR and sequenced on an Illumina™ HiSeq™ system.

Raw sequence reads were trimmed to remove low-quality fractions and adapters with Trimmomatic-0.33 [3]. Quality trimming was done using a sliding window of 6 bp and a quality cutoff of 20, and reads consisting of less than 70 bp were removed. The microbial composition was profiled using MetaPhlAn2 [4] and visualized using KronaTools [5]. Species-level abundance information was extracted from MetaPhlAn2 outputs and further analyzed:

- To create microbial composition bar plots and to perform beta-diversity analysis (based on Bray-Curtis dissimilarity) using QIIME [6]
- To create a taxon abundance heat map with hierarchical clustering (based on Bray-Curtis dissimilarity) with an in-house Python™ script
- For biomarker discovery with LEfSe [7] with default settings (p>0.05 and LDA effect size >2)

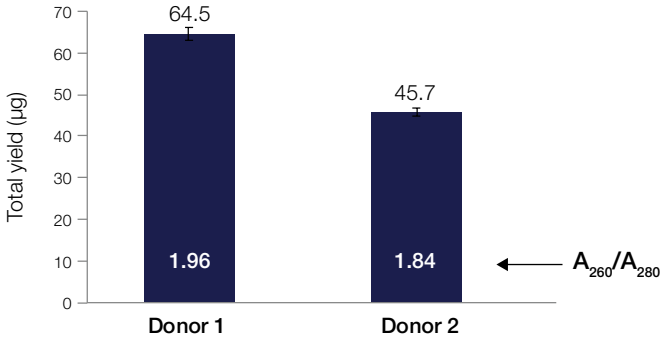

Figure 1. Total nucleic acid yields from 100 mg fecal samples. Samples from two donors were processed using the MagMAX Microbiome kit. The KingFisher Flex Magnetic Particle Processor with 96 Deep-Well Head was used for isolation, with an up-front bead beating step. A_{260}/A_{280} values are indicated.

Results

Total nucleic acid—yield, purity, and quality

High-throughput isolation using the MagMAX Microbiome Ultra Nucleic Acid Isolation Kit allows for faster processing of samples due to the innovative magnetic bead–based chemistry and up-front bead beating step, which enables purification of up to 96 samples at a time with the Thermo Scientific™ KingFisher™ Flex or Duo Prime instruments. For fecal samples, high total nucleic acid yields (45–64 µg; Figure 1) were obtained from 100 mg of sample input. For saliva, the total nucleic acid yield ranged from 1.5 to 2.5 µg from 400 µL of sample input (data not shown). For urine, the total nucleic acid yield ranged from 0.2 to 0.3 µg from 400 µL of sample input (data not shown). All total nucleic acid isolated from all sample types displayed A_{260}/A_{280} ratios of >1.7, demonstrating the high purity of the preparations. Agarose gel analysis of the fecal total nucleic acid demonstrated the high quality of recovered microbial DNA and RNA (Figure 2).

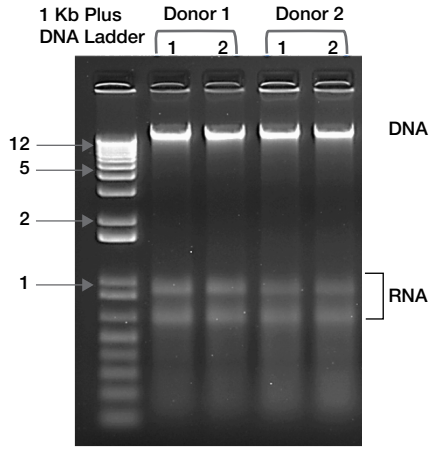

Figure 2. Quality of total microbial nucleic acid purified from feces. For each donor, duplicate 1 µg samples of total nucleic acid were run on a 1% agarose gel. Clear bands of both genomic DNA and RNA can be seen. The Invitrogen™ 1 Kb Plus DNA Ladder was used for size determination. Note that since this is not a denaturing gel, apparent sizes of the RNA differ from actual sizes.

TaqMan qPCR Assays were performed for Firmicutes, Bacteroidetes, and *E. coli* using total nucleic acid isolated from fecal, saliva, and urine samples. All of these bacteria are routinely found in the human body, though *E. coli* levels are typically low. By probing for these two phyla and *E. coli*, a broad range of coverage was tested. As expected, fecal samples contained high levels of Firmicutes and Bacteroidetes (Figure 3A), whereas urine and saliva samples contained high levels of Firmicutes (Figures 3B and C). *E. coli* abundance was lower, but in the detectable range, for all sample types tested (Figures 3A, B, and C). A TaqMan Assay with Applied Biosystems™ Xeno™ DNA and RNA controls showed no inhibition from the total nucleic acid preparations (data not shown), confirming that the MagMAX Microbiome Ultra Nucleic Acid Isolation Kit enables isolation of high-quality, inhibitor-free nucleic acid, even from the most challenging samples.

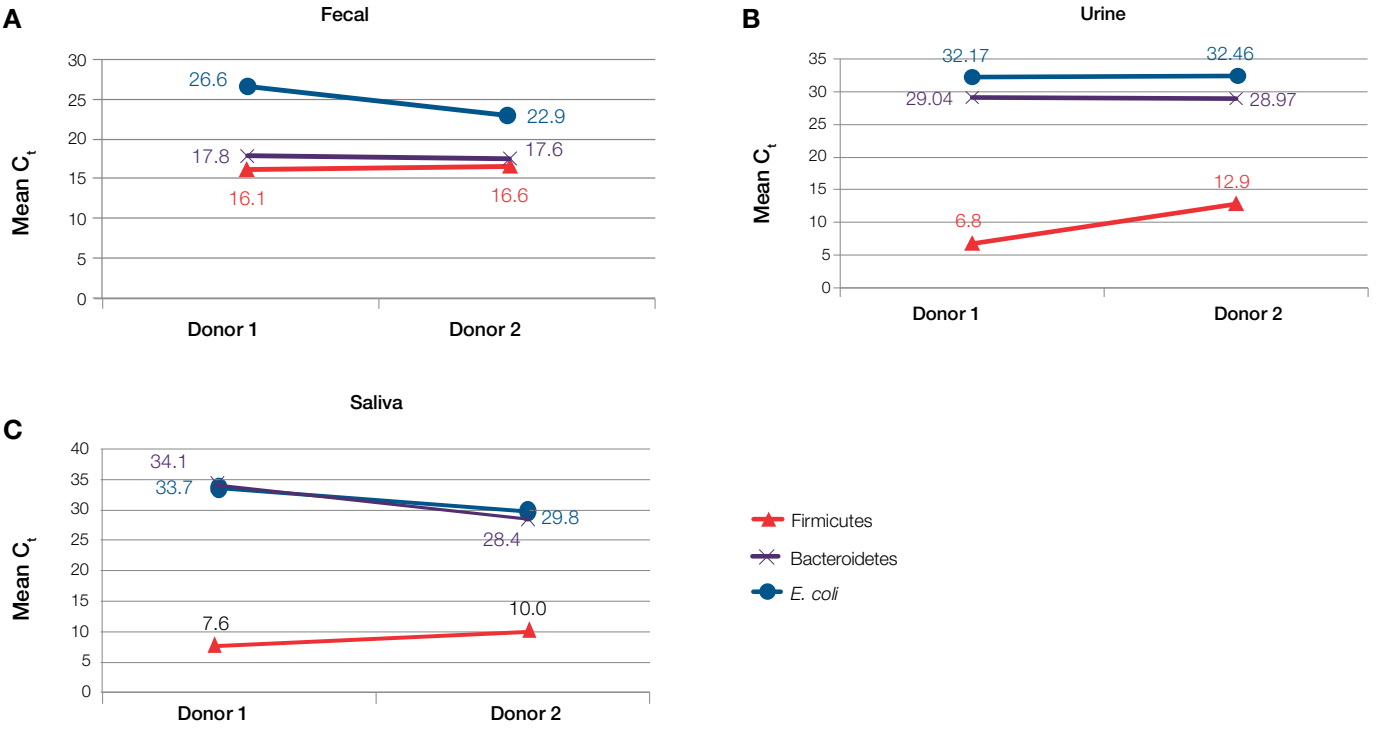

Figure 3. qPCR analysis of DNA purified from (A) fecal, (B) urine, and (C) saliva samples of two donors, with the MagMAX Microbiome Ultra Nucleic Acid Isolation Kit. TaqMan Assays were utilized for one category of gram-positive (Firmicutes) and two categories of gram-negative (Bacteroidetes and *E. coli*) bacteria. The total nucleic acid samples were diluted 1:100 for the Firmicutes and Bacteroidetes assays, whereas the input was not diluted for the *E. coli* assay. TaqMan Fast Advanced Master Mix was used under fast cycling conditions.

Shotgun metagenomics sequencing of total nucleic acid isolated from feces, urine, and saliva

Total nucleic acid from fecal, urine, and saliva samples from two donors was isolated and sequenced; Figure 4 shows the metagenomics sequencing data as abundance heat maps for all fecal, urine, and saliva samples. The abundance heat maps shown here do not represent the full microbiome profile for each sample type. The species-level whole-genome profiles are shown in Figure 5. Note that the MagMAX Microbiome Ultra Nucleic Acid Isolation Kit can isolate viral total nucleic acid in addition to bacterial (shown in urine and saliva profiles in Figure 4). Metagenomics profiles for feces and saliva have only *Prevotella* species as a common bacterial target, whereas urine and saliva metagenomics profiles share the same viral targets. Metagenomics sequencing of fecal nucleic acid from donor 1 (zr2132.1) shows high abundance of *Prevotella*, *Faecalibacterium*, *Roseburia*, *Bacteroides*, *Alistipes*, and others, and from donor 2 (zr2132.2) shows high abundance of several *Bacteroides*, *Faecalibacterium*, *Bifidobacterium*, *Parabacteroides*, and others. All samples were mapped to a human database using the program Centrifuge (data not shown), and the data demonstrated that the MagMAX Microbiome Ultra Nucleic Acid Isolation Kit enriches for isolation of bacterial targets up to 99%, and human targets were detected at a very low level (0.06–0.45%) in the fecal samples.

A whole-metagenome profile at the species level shows multiple bacterial and viral targets detected in the profile that are specific to a given sample type. Fecal samples from both donors show high amounts of Bacteroidetes, Firmicutes, Actinobacteria, and Proteobacteria. The fecal sample from donor 1 shows *Prevotella copri*, several *Bacteroides* species, *Rothia, Faecalibacterium, Sutterella,* and other species as highly abundant, whereas the fecal sample from donor 2 shows high abundance of *Bacteroides, Bifidobacterium, Faecalibacterium, Rothia, Sutterella,* and other species. Urine samples from both donors have high abundance of *Lactobacillus, Streptococcus, Bacteroides, Ruminococcus,* and *Bifidobacterium, Pseudomonas* phages, and other species. Saliva samples from both donors show high abundance of *Streptococcus mitis,* other *Streptococcus* species, *Granulicatella, Neisseria, Prevotella,* and other species (Figure 5).

Conclusion

We have developed an automated, faster workflow for isolation of high-quality, inhibitor-free DNA and RNA from human stool samples and other sample types, using the new MagMAX Microbiome Ultra Nucleic Acid Isolation kit. Whole-genome sequencing of samples from two donors showed the total profile of microorganisms in feces, urine, and saliva. The workflow that we developed to harness the power of microbiome research enables fast generation of metagenomic data for bacterial communities residing within human body, which can be used as diagnostic biomarkers for certain diseases, and potentially pave the path for future microbiome therapeutics.

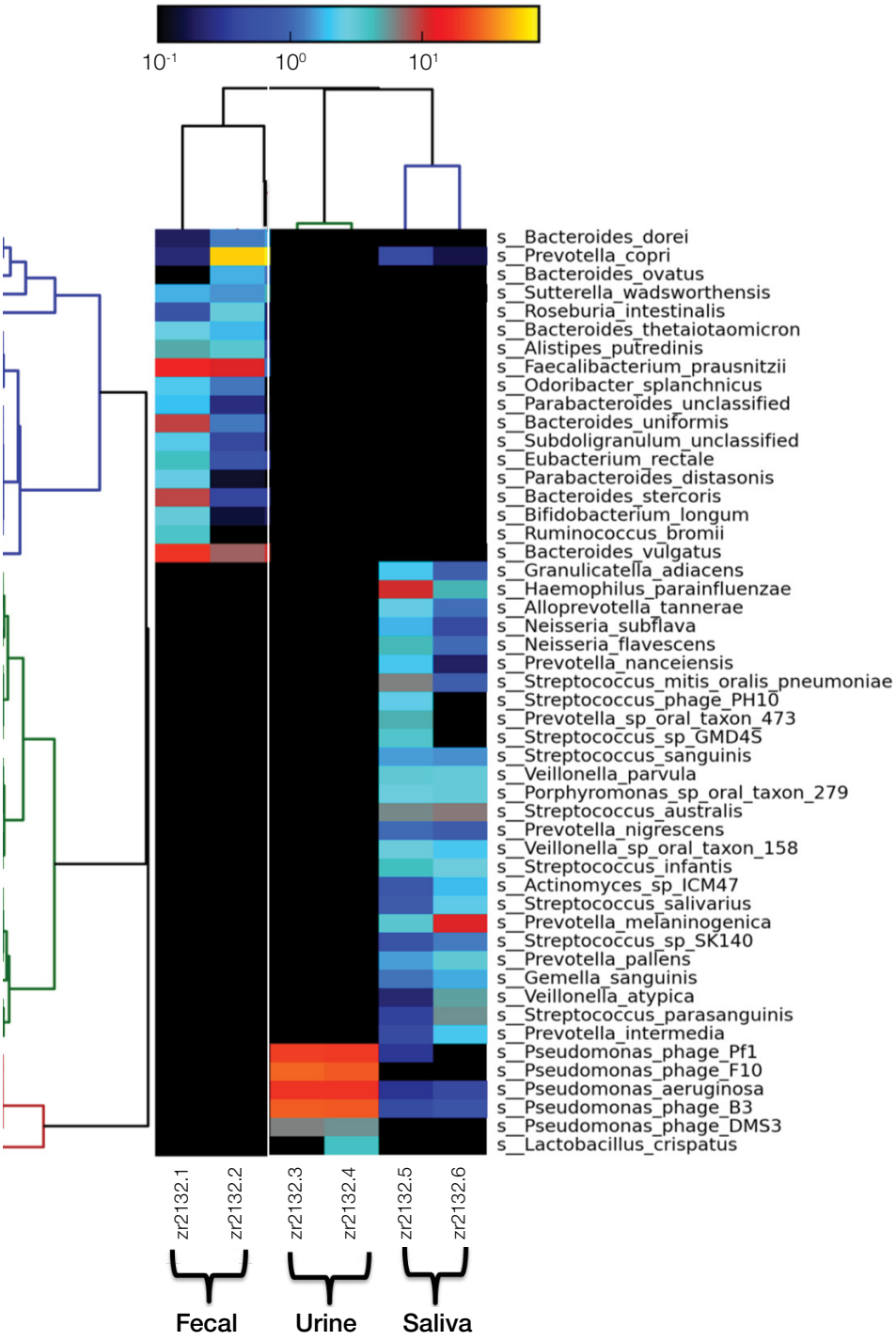

Figure 4. Shotgun metagenomics sequencing abundance heat maps for fecal, urine, and saliva sample isolations.

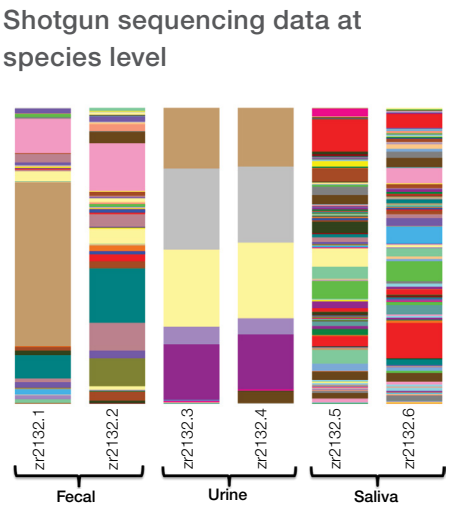

Figure 5. Identification of whole-metagenome profiles for fecal, urine, and saliva samples using shotgun metagenomics sequencing. Each color represents a different operational taxonomic unit (OTU) identified per sample.

Shotgun sequencing data at species level

Top 10 abundant species from zr2132.1 (donor 1, feces)
Prevotella copri
Faecalibacterium prausnitzii
Bacteroides vulgatus
Alistipes putredinis
Barnesiella intestinihominis
Roseburia intestinalis
Bacteroides thetaiotaomicron
Bacteroides ovatus
Sutterella wadsworthensis
Bacteroides caccae

Top 10 abundant species from zr2132.2 (donor 2, feces)
Bacteroides vulgatus
Faecalibacterium prausnitzii
Bacteroides uniformis
Bacteroides stercoris
Alistipes putredinis
Eubacterium rectale
Ruminococcus bromii
Bifidobacterium longum
Bacteroides thetaiotaomicron
Parabacteroides distasonis

Top 10 abundant species from zr2132.3 (donor 1, urine)
Pseudomonas phage F10
Pseudomonas phage B3
Pseudomonas phage Pf1
Pseudomonas aeruginosa
Pseudomonas phage DMS3
Halomonas stevensii
Unclassified *Halomonas*
Human adenovirus B
Unclassified *Alcaligenes*
Varibaculum cambriense

Top 10 abundant species from zr2132.4 (donor 2, urine)
Pseudomonas phage F10
Pseudomonas phage B3
Pseudomonas phage Pf1
Pseudomonas aeruginosa
Pseudomonas phage DMS3
Lactobacillus crispatus
Halomonas stevensii
Unclassified *Alcaligenes*
Prevotella bivia
Haemophilus parainfluenzae

Top 10 abundant species from zr2132.5 (donor 1, saliva)
Haemophilus parainfluenzae
Streptococcus mitis, oralis, and *pneumoniae*
Streptococcus australis
Prevotella spp. oral taxon 473
Neisseria flavescens
Streptococcus infantis
Streptococcus sp. GMD4S
Prevotella melaninogenica
Veillonella parvula
Veillonella sp. oral taxon 158

Top 10 abundant species from zr2132.6 (donor 2, saliva)
Prevotella melaninogenica
Streptococcus australis
Streptococcus parasanguinis
Veillonella atypica
Haemophilus parainfluenzae
Prevotella pallens
Veillonella parvula
Porphyromonas sp. oral taxon 279
Streptococcus infantis
Streptococcus salivarius

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