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A Biotechnical Newsletter

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Microbiomics
SPECIAL EDITION

What **story** does your **Microbiome** tell?

The Community Calls for Microbiomics Standards!

The Microbiomics community has raised concerns regarding poor data quality and reproducibility across labs due to the lack of standard reference materials and guidelines for quality microbiome measurements. When we asked Dr. Lynn Schriml, an Associate Professor from the University of Maryland and the President of the Genomic Standards Consortium, about the importance of standards for Microbiomics, she stated that "The implementation of rigorous metadata standards facilitates opportunities for discovery and the transcendence of knowledge by addressing data harmonization challenges posed by the vast variety of biomedical data resources." Moving forward, the health of the field depends on such standards to ensure microbiome measurements are accurate and reproducible. See below the feedback below from others regarding these widespread challenges.


Sinha et al. Genome Biology (2015) 16:276
DOI 10.1186/s13059-015-0841-8

Genome Biology

OPEN LETTER Open Access

The microbiome quality control project: baseline study design and future directions

Rashmi Sinha[1*], Christian C. Abnet[1], Owen White[2], Rob Knight[3] and Curtis Huttenhower[4]

"Studies have been difficult to reproduce across investigations"


nature microbiology

CONSENSUS STATEMENT

PUBLISHED: 11 JANUARY 2016 | ARTICLE NUMBER: 15015 | DOI: 10.1038/NMICROBIOL.2015.15

An assessment of US microbiome research

Elizabeth Stulberg[1*], Deborah Fravel[2], Lita M. Proctor[3], David M. Murray[4], Jonathan LoTempio[3], Linda Chrisey[5], Jay Garland[6], Kelly Goodwin[7,8], Joseph Graber[9], M. Camille Harris[10], Scott Jackson[11], Michael Mishkind[12], D. Marshall Porterfield[13] and Angela Records[14]

"Addressing the sources of variation in microbiota profiling is critical for optimizing protocols… Unfortunately, **variation at each step in the pipeline is enormous from** physical **specimen collection** and **processing** to **computational quantification of microbial communities.**"

A National Institute of Standards and Technology (NIST) scientist reported that such standard protocols are needed because 'the interlab comparability of measurements on microbiomes is generally poor. Biases exist along every step of the measurement process, from sample collection, extraction techniques, measurement technology employed (e.g. NGS, mass spec, NMR), and, finally, to data analysis and interpretation. There is a need for the adoption of reference materials, reference data, and reference protocols in order to identify and eliminate measurement bias.'

An assessment of US microbiome research

Elizabeth Stulberg[1*], Deborah Fravel[2], Lita M. Proctor[3], David M. Murray[4], Jonathan LoTempio[3], Linda Chrisey[5], Jay Garland[6], Kelly Goodwin[7,8], Joseph Graber[9], M. Camille Harris[10], Scott Jackson[11], Michael Mishkind[12], D. Marshall Porterfield[13] and Angela Records[14]

For Rashmi Singha, an epidemiologist at the National Cancer Institute, **the lack of reproducibility between studies was frustrating**. "To me it seemed like cowboy country. It needed to have some kind of order."

Rob Knight, "All sorts of unlikely things are possible, and finding out which one is true is difficult."

"I asked two different companies to analyze my gut microbiome. American Gut (left) gave nearly opposite results to those from uBiome (right) with respect to the major phyla of bacteria in a duplicate sample."

The figure was adapted from: "Here's the Poop on Getting Your Gut Microbiome Analyzed." Science News. 2014

"...the Federal Government should support the development of... protocol standards and reference materials to allow comparison of experiments..."

Fast-Track Action Committee on Mapping the Microbiome
National Science and Technology Council of the White House, 2015

"Critical to the utility of mNGS approach for infectious disease diagnosis will be clinical validation of the test in a CLIA-certified laboratory and eventual FDA regulatory approval. Key challenges that will thus need to be addressed include (1) generation of accurate reference materials and controls."

Charles Chiu, M.D./Ph.D. – University of California, San Francisco School of Medicine
Associate Professor, Laboratory Medicine and Medicine / Infectious Diseases
Director, UCSF-Abbott Viral Diagnostics and Discovery Center
Associate Director, UCSF Clinical Microbiology Laboratory

"Mixed sample reference material represents a significant advancement for the diagnostics community through enhancing the ability of timely proficiency testing of NGS-based diagnostics. Development and validation of genomic DNA reference materials can be timely and cost prohibitive for some laboratories. The stakeholders also proposed that this proficiency material can serve as a quality control option for an NGS-based diagnostic assay and it ensures that testing in the laboratory is being conducted in a reproducible and reliable manner."

NISTFDA Workshop: Standards for Pathogen Detection via NextGeneration Sequencing
Organizers: Scott Jackson[1], Heike Sichtig[2], Brittany Goldberg[2], Chelsie Geyer[2], Jason Kralj[1]
1. National Institute of Standards and Technology, Gaithersburg, MD USA
2. Food and Drug Administration, Silver Spring, MD USA

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About the Cover

The cover of this Microbiomics Peanuts Newsletter is a reflection of the unique stories told by our microbiome based on our environmental inputs, genetics, and food we eat. Understanding the causative factors driving the changes in the microbial community will be critical for navigating this new frontier of Microbiology. Dr. Jonathan Eisen describes this best:

"We have a poor understanding of how the environment shapes the microbial community, of how we get microbes into our gut from our food, our buildings, our dogs, and our friends, and the total systems-level approach to the microbial community will be very important. After a year when a baby is starting to be colonized by everything in its environment, why do some things take hold and some don't? What shapes why there are changes in the microbial community over time or over space or in response to diet? I think discovering those dynamics will be interesting, and understanding the inputs and outputs will be incredibly important."

Improving the Accuracy and Reproducibility of Microbiome Measurements Across Labs

Shuiquan Tang, Ryan Kemp, Elinne Becket, Larry Jia, Marc Van Eden, Standa Forman, Eric Kircher, John Sherman, Luigi Basillio, Michelle Thai, Steven Wong, and Mikayla Mager
from Zymo Research Corporation

Poor Data Reproducibility in Microbiome Measurements

Microbes influence almost every aspect of human health and our living environment. The advent of Next-Generation sequencing (NGS) technologies has enabled researchers to study microbes as communities rather than individual organisms, thus revolutionizing our understanding of the relationships between microbiota and human health, and between microbiota and the environment.

The field of microbiomics has developed at a break-neck pace in the past several years. While researchers have made countless new discoveries in that timespan, the fidelity of methods and protocols used in this field have never been fully assessed. The problem of poor data reproducibility of microbiome research across labs and the difficulties of addressing the enormous variations present in every step of the complex microbiomics workflow have been noted by many in the community and were recently characterized by Sinha et al. in the baseline study of the Microbiome Quality Control Project (MBQC)[1].

To demonstrate the severity of this issue, two striking examples are described below. The first example is a story published in 2014 Science News[2] comparing two well-known gut microbiome profiling organizations, American Gut and uBiome. The author provided the exact same human stool sample to the two organizations and yet the interpretation from the two organizations was dramatically different (Figure 1). While both profiles agree that *Bacteroidetes* and *Firmicutes* are in dominance, the profile from American Gut shows there are ~35% of *Firmicutes* and ~60% of *Bacteroidetes* whereas the profile from uBiome shows almost the opposite. Unfortunately, based on the data, no conclusions can be made about which measurement is more accurate because the actual composition of the fecal sample is unknown.

Another example comes from the comparison between the two best-known human microbiome profiling efforts, Human Microbiome Project (HMP) and Metagenomics of Human Intestinal Tract (MetaHIT). Figure 2 shows the average microbial composition (phylum level) of human gut microbiota determined by HMP and MetaHIT. Again, both agree that *Bacteroidetes* and *Firmicutes* are in dominance, but the HMP profile indicates the increased presence of *Bacteroidetes*, 73.9% as compared to 45.6% in the MetaHIT profile. Is the difference observed capturing a biological change? Since HMP samples were mostly collected from the US population and the MetaHIT samples were mostly collected from the European population, it is possible that there are some biologically relevant differences. However, it is more likely that the strong disparity observed in Figure 2 is caused by technical differences in sample processing. This hypothesis is supported by a study[3] showing that the HMP DNA extraction protocol yields a higher relative abundance of *Bacteroidetes* as compared to the MetaHIT DNA extraction protocol.

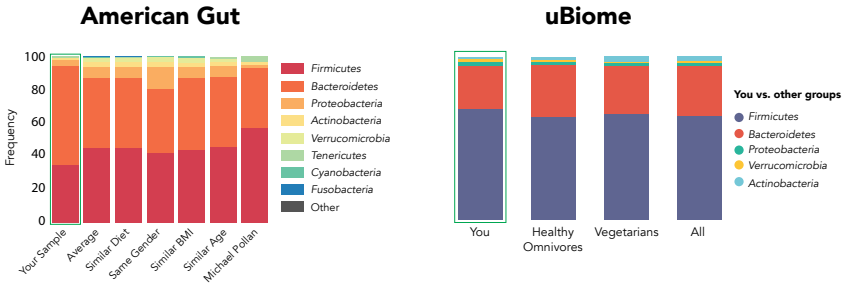

Figure 1. Inconsistent interpretation of the microbial composition of one stool sample by American Gut and uBiome. The figure was adapted from: "Here's the Poop on Getting Your Gut Microbiome Analyzed." *Science News*. 2014.

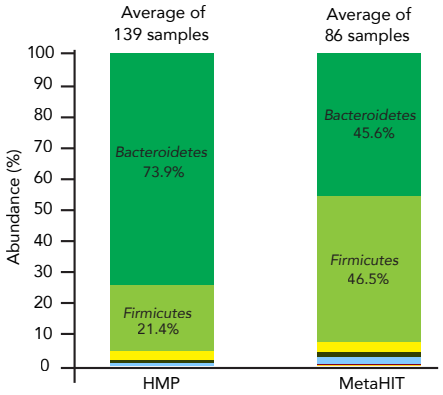

Figure 2. Inconsistent interpretation of the microbial composition of human gut microbiota by Human Microbiome Project (HMP) and the Metagenomes of Human Intestinal Track (MetaHIT). This figure was summarized from some analysis data downloaded from the website of metaphlan2, http://huttenhower.sph.harvard.edu/sites/default/files/gut.HMP+MH.healthy.txt.

With mounting evidence of systemic biases plaguing the field of microbiomics the need for more accurate microbiome measurements have become apparent. Organizations such as the National Institute of Standards and Technology (NIST) are in the process of creating reference materials for microbiome measurements and hosting workshops to inform the community. Additionally, the Microbiome Quality Control Project (MBQC, www.mbqc.org) and the International Human Microbiome Standards (IHMS, www.microbiome-standards.org) have also been established to improve the quality of microbiomic studies. The general consensus is that there is an urgent need for microbiome/microbiota reference materials to assess the performance of different microbiome measurements. To meet this demand, Zymo Research released a microbiome reference material called ZymoBIOMICS® Microbial Community Standard in 2016. It is the first readily-available, commercialized microbiome reference material.

Where do Bias and Errors Arise?

NGS microbiomics workflows contain multiple steps and are often complicated by biases and errors arising at every step. Figure 3 summarizes common challenges associated with each step of the microbiomic workflow. At the first step, sample collection and preservation, undesired microbial growth or decay, and nucleic acid degradation can introduce bias. To maintain sample integrity, researchers have been using a variety of methods to preserve microbial samples; however, many of them have been shown to be problematic[3-9]. Freezing is considered the gold standard, but even freezing can potentially suffer from bias caused by freeze-thaw cycles[10,11]. Next, DNA/RNA extraction can be biased because of uneven microbial cell lysis. Numerous studies have outlined the variations in microbial composition profiling caused by the use of different DNA extraction methods[5, 12-16]. The library preparation process in 16S sequencing is solely based on PCR amplification, which is prone to bias due to factors such as PCR chimera[17-19], primer degeneracy[20], amplicon size variations and GC content variation. Also, the library preparation process for shotgun sequencing frequently involves enzymatic reactions (e.g. fragmentation, end-repair, ligation, PCR, and tagmentation), and bias in sequencing results caused by the use of different library preparation methods has been reported[21]. Different sequencing platforms can also introduce specific bias. Pyrosequencing and IonTorrent sequencing have problems in estimating the length of homopolynucleotides[22]. Illumina sequencing may have bias related to GC content[23]. Third-generation sequencing techniques (e.g. PacBio and Nanopore) tend to have higher error rate[24, 25] and are still not as cost effective as NGS techniques. Lastly, bioinformatics analysis also suffers from bias. The choice of operational taxonomy unit (OTU) clustering algorithms and differences in sequencing depth of samples can introduce bias in OTU clustering and diversity analysis[26]; therefore, sequencing depth normalization before these analyses may be necessary[27]. Moreover, reference databases for the analysis of 16S or shotgun data suffer from the problems of incompleteness, erroneous sequences (e.g. chimeric 16S sequences[28]), mis-annotations and uneven microbial representation. The array of variations associated with each step of a microbiomics workflow explains why reproducibility and data quality are major concerns for the field[1].

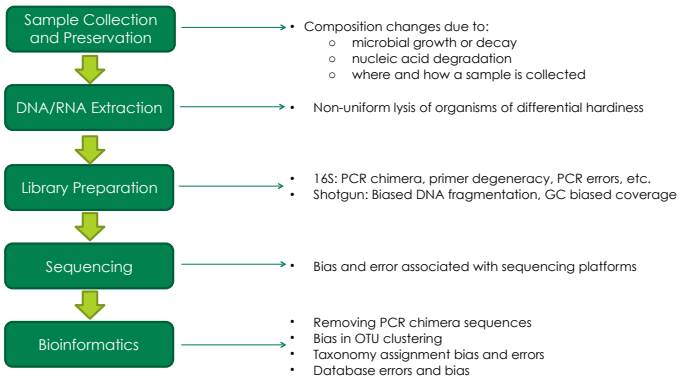

Figure 3. Sources of potential error or bias throughout the entire microbiomics workflow.

Microbiomics Poses Additional Methodology Requirements

The ultimate goal of a microbiome measurement is to reveal the real composition of a microbial community. This sets the ultimate goals for the design of microbiomics workflows to be unbiased and of low-bioburden. Unfortunately, most of the tools and methods currently employed in the field were developed prior to the establishment of the microbiomics field; therefore, their design did not consider these new requirements of microbiomics research. For example, previously typical considerations of microbial DNA extraction normally include high yield, clean DNA (260/280, 260/230 ratio), removal of PCR inhibitors, streamlined workflow, etc. Although these considerations are still useful, the demands of microbiomics research exceeds these criteria. For example, microbial DNA extraction for microbiome measurements needs to be unbiased in order to present the real microbial composition and also needs to be low bioburden to reduce false positives or background noise, which is of critical importance to the study of low biomass samples. To meet the new standards of microbiome measurements, the development of new tools is required. Zymo Research's mission in this area is to develop simple and streamlined microbiomics products that are validated for great accuracy and reproducibility across labs. With this in mind we have summarized what features an ideal microbiomics workflow should contain (using 16S rRNA sequencing studies for example).

Key Considerations for a 16S Microbiomics Workflow

- Sufficient quality controls with reliable reference materials
- A non-refrigerated and unbiased way to collect and preserve microbial DNA
- A DNA extraction process that overcomes cell lysis bias and minimizes reagent contaminations.
- A 16S library preparation process that minimizes PCR-associated bias and reagent contaminations.
- Cost-effective NGS sequencing
- A bioinformatics pipeline that is able to differentiate single nucleotide variations, eliminate PCR chimeric sequences, and error-resistant species-level taxonomy assignment.
- A well-curated 16S reference database

ZymoBIOMICS® Portfolio – Eliminating Bias and Uncovering the Truth

The ZymoBIOMICS® portfolio is Zymo Research's answer to the call for more reliable microbiome measurements. The ZymoBIOMICS® portfolio consists of multiple products that address key challenges in microbiomics workflow including microbial community standards, sample collection devices, DNA/RNA isolation kits, and library preparation kits. We also offer our entire workflow as a microbiomics sequencing service for customers that prefer to outsource technical processing.

Reliable Reference Materials

Reference materials are necessary for reliable microbiomics measurements and can also serve as positive and/or quality controls for routine workflows. It is a good practice for every microbiomics analysis run to contain at least two controls: a positive control with cellular microbial standards and a negative control (e.g. blank process controls during DNA isolation). The need for reference materials in the field is substantial and there are potentially innumerable criteria for what constitutes an excellent reference material, as it is difficult to create reference materials that can fit every researcher's interest. However, we believe that an effective reference material should have an accurately defined composition, contain extremely low contamination, and address major technical challenges in the workflow in order to mitigate the major contributors of bias and errors. With these considerations in mind, we have developed the ZymoBIOMICS® Microbial Community Standard, the first commercially-available microbiome reference material. The power of reference materials stems from the confidence of their accurately pre-defined composition. The microbial composition of ZymoBIOMICS® Microbial Community Standard was characterized and cross-validated with several measurements, including cell counting by hemocytometer, fluorescence microscopy-based digital cell counting equipment, total DNA quantification, and NGS shotgun metagenomic sequencing. We also certify all of our microbial standards to have <0.01% microbial contamination (by DNA abundance). In addition, the ZymoBIOMICS® Microbial Community Standard is specifically designed to overcome two common technical challenges in microbiomics workflows: (1) bias in DNA extraction caused by uneven microbial cell lysis and (2) bias in library preparation and sequencing caused by GC content variations. With this in mind, the standard was designed to consist of 10 different strains representing different cell wall recalcitrance (e.g. Gram-positive bacteria, Gram-negative bacteria, and yeast), different cell sizes (small bacteria vs. large yeast), and a wide range of GC content (15%-85%). You can learn more about the ZymoBIOMICS® Microbial Community Standard on page 11.

Hassle-free Sample Collection

Active microbial samples can alter their composition easily in response to changes in the environment. Therefore, all microbial samples require preservation methods if subsequent processing does not happen immediately. For this purpose, most researchers have been relying on freezing or refrigeration, which unfortunately is too inconvenient or costly to implement in many circumstances, e.g. collecting and transporting thousands of samples from individual homes and in the wild. This challenge leads to a need for convenient cold-free methods for microbial sample collection, preservation, and transportation.

Zymo's unique stabilization reagent, DNA/RNA Shield™, addresses this problem directly. DNA/RNA Shield is designed to preserve both DNA and RNA profiles of microbial samples at ambient temperature for up to one month, making it ideal for transportation of samples. Figure 4, gives a demonstration

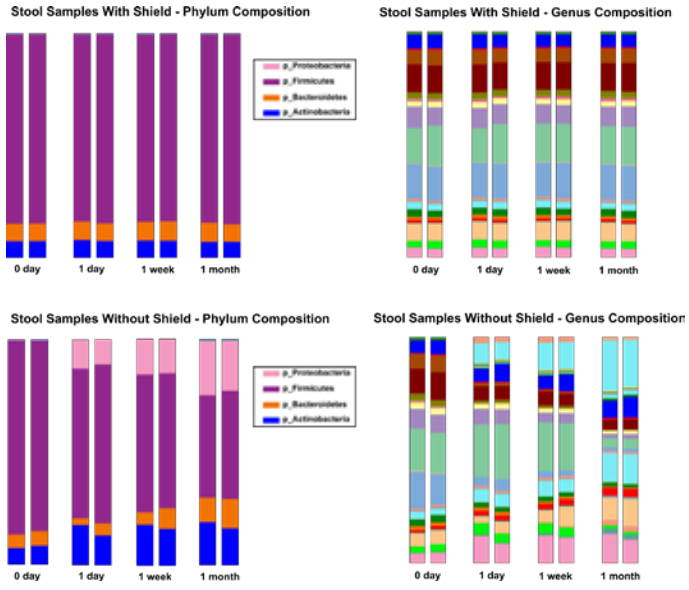

Figure 4. The development of microbial composition of a stool sample when saved at ambient temperature without DNA/RNA Shield (a) versus with DNA/RNA Shield™ (b). DNA was extracted with ZymoBIOMICS® DNA Miniprep Kit and then subjected to 16S targeted sequencing.

of its performance using a fecal sample. Zymo's DNA/RNA Shield™ comes in a variety of formats specifically for microbiomics research, including swab collection tubes, which are extremely easy to use; one simply needs to add sample and mix. DNA/RNA Shield™ reagent also inactivates potential pathogenic microorganisms and viruses within a sample, which aids in eliminating biosafety concerns for transportation or subsequent extraction steps, which is important when dealing with unknown samples. Additionally samples preserved in DNA/RNA Shield™ can be used directly with all commercially available DNA/RNA purification systems, with no need for removal of reagent from sample, thereby eliminating any bias that may come from removal of supernatant containing microbe DNA in solution. For more information on DNA/RNA Shield™ see page 30.

"Unbiased" and Low Bioburden DNA Extraction

There are several reports in the literature citing variations in microbial composition profiling caused by the use of different DNA extraction methods[5, 12-16]. A striking example was given at the beginning of this article regarding the different interpretations of a single fecal sample comparing the HMP to the MetaHIT project. The problem of poor data reproducibility across labs is likely attributed to the use of biased DNA extraction techniques. Most DNA extraction methods used in the field were developed before the field of Microbiomics blossomed. As such, the requirements that define a quality DNA purification system have extended beyond simply yielding pure DNA free of PCR inhibitors and have moved to new microbiomics-specific requirements. With identification and abundance being the most important factors in a microbiomics measurement, lysis efficiency and bioburden/background contamination should be major considerations when using a DNA isolation system. Problems with these two factors can completely distort the truth.

The ZymoBIOMICS® DNA Miniprep was built specifically for microbiome research and was designed with these new requirements in mind. After significant research and evaluation of microbial cell lysis methods, we have found that mechanical lysis is the only option that can provide an unbiased or close to unbiased microbial cell lysis. To determine if a microbial DNA extraction process is biased or not, one needs a microbial sample of defined composition, and this is where the microbial community reference materials are useful. Using the ZymoBIOMICS® Microbial Community Standards, we have assessed the performance of the ZymoBIOMICS® DNA Miniprep together with the three most cited DNA extraction methods used in the field. The extracted DNA samples were then profiled using 16S sequencing. The results showed good agreement between the profile from the ZymoBIOMICS® DNA Miniprep and the theoretical composition of the standard. In contrast, dramatic bias was observed using the other three methods (Figure 5). Because these three methods are currently the three most cited protocols in the field, our data revealed the serious situation facing the field of microbiomcs as a whole. To learn more about the ZymoBIOMICS® DNA Miniprep, see page 32.

Controlling Bias and Artifacts During Library Preparation

The NGS library preparation process normally consists of enzymatic reactions that are prone to bias. For example, PCR amplification in 16S library preparation are well-known to have bias caused by PCR chimera, primer degeneracy, amplicon sequence variations, and PCR conditions. Therefore,

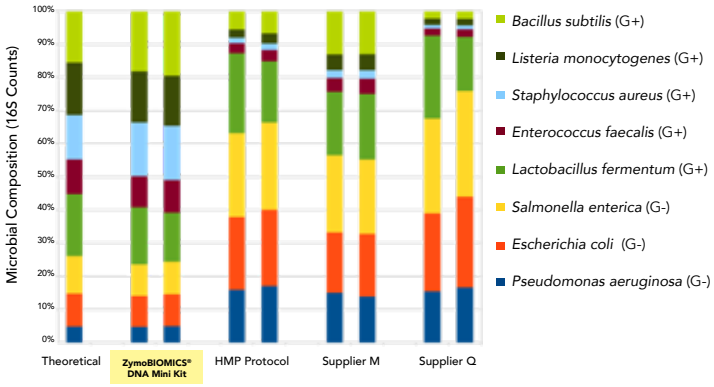

Figure 5. Assessing the performance of four different DNA extraction kits with the ZymoBIOMICS® Microbial Community Standard. The four different DNA extraction methods investigated include ZymoBIOMICS® DNA Miniprep Kit, Human Microbiome Project fecal DNA extraction protocol (HMP Protocol), a soil DNA extraction kit from "Supplier M" and a fecal DNA extraction kit from Supplier Q. DNA was extracted with ZymoBIOMICS® DNA Miniprep Kit and then subjected to 16S targeted sequencing with an internal library preparation protocol. The microbial composition was determined by mapping raw sequencing reads against reference 16S sequences of the strains contained in the standard.

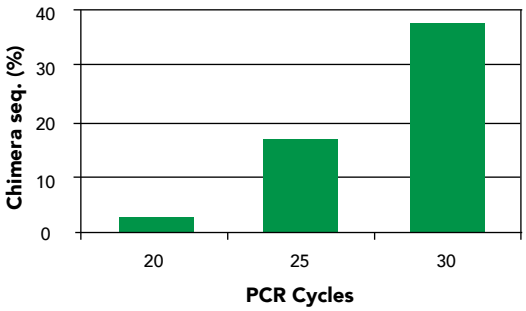

Figure 6. The effect of PCR cycles on PCR chimera formation in 16S library preparation. The 16S library preparation was run for different numbers of cycles. After that, the libraries were sequenced with MiSeq and PCR chimeric sequences were identified with Uchime, using the 16S sequences of the strains contained in the ZymoBIOMICS® Microbial Community Standard as reference.

a good DNA reference material is necessary to determine the bias of a library preparation. Using the ZymoBIOMICS® Microbial Community DNA Standard, one can accurately assess key artifacts during the library preparation process. Figure 6 shows that simply extending PCR to 30 cycles can cause PCR chimeric sequences can account for more than 35% of all sequences in the case of 16S library preparation. The value of this standard is that it allows one to accurately identify all chimeric sequences, because composition of the standard is well defined. Figure 7 shows that Nextera XT, a shotgun library preparation kit from Illumina®, resulted in an underrepresentation of the abundance of *Staphylococcus aureus* of the ZymoBIOMICS® Microbial Community DNA Standard in shotgun metagenomic sequencing results. Further investigation revealed that the Nextera XT induced bias was caused by GC content variation, with *Staphylococcus aureus* containing the lowest GC content in the standard.

Conclusions

Microbiomics is an exciting and rapidly developing field, but currently the field is plagued with poor quality data. It has been very difficult to compare microbiomics data across labs. This is because microbiomics measurements are complicated and substantial bias can be introduced by various factors in every step of the workflow. To achieve quantitative and accurate measurements, stricter requirements need to be imposed on microbiome workflows, including the use of unbiased methods and tools with low background contamination. Zymo Research has been introducing new innovative technologies to solve these technical challenges. Our goal is to provide researchers the best tools for microbiome measurements to reveal real microbial compositions rather than biased compositions.

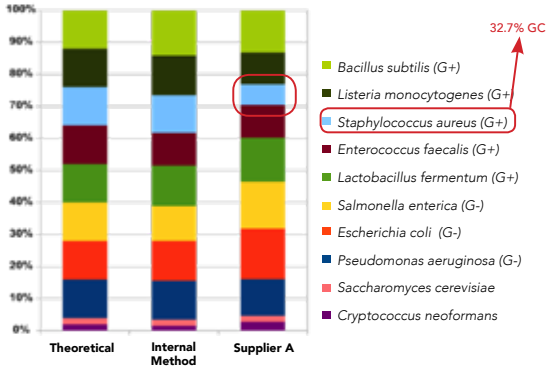

Figure 7. Assessing the performance of two shotgun library preparation methods using the ZymoBIOMICS® Microbial Community DNA Standard. The sequencing was performed on Illumina® HiSeq and the microbial composition was determined by mapping raw reads against the genomes of the strains contained in the standard.

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FEATURED ARTICLE:

High Quality Microbiome Data Through Use of Microbial Reference Controls

Scott Tighe[a]

Author Affiliations
a. Association of Biomolecular Resource Facilities Metagenomics Research Group and Extreme Microbiome Project

Until recently, the area of microbiomics and metagenomics has been relatively unrecognized as a major field in biological research with only a few dozen publications a year. However, with the advent of Next-Generation sequencing (NGS) and the ability to sequence millions of mixed DNA sequences simultaneously, microbiome and metagenomic studies have expanded into nearly every area of biological research today including patient care. Although NGS has opened the door to this data rich field, it is recognized by most experts that significant technical advancements will be required to produce accurate and valid data sets moving forward.

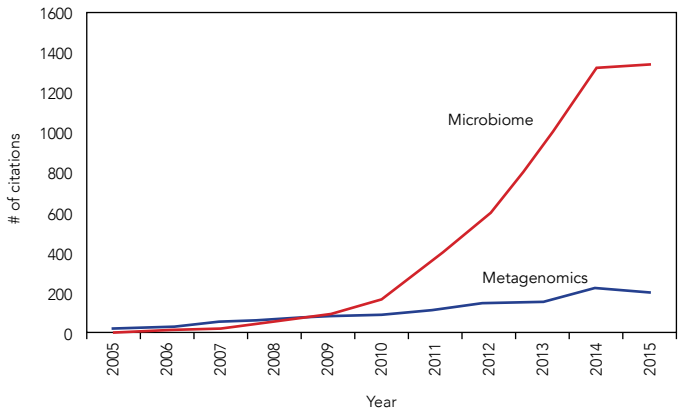

Figure 1. Search Results for Publications with Microbiome or Metagenomics in Title or Keywords Using PubMed.

Because of this rapid expansion and the demand for high-performance protocols at almost every level of sample processing, specially designed controls and reagents are needed, including sample collection, DNA and RNA extraction, NGS library preparation, and special bioinformatic software to understand the variations that can occur throughout these many steps. It is clear that both whole cell microbial reference standards as well as genomic DNA standards are required to ascertain detection limits and performance statistics with all studies including those clinical samples.

Implementing microbial reference controls into microbiome studies is a new required practice to ensure high-quality data. However, fabrication of high-quality reference controls has been difficult, with the only source being the well-known BEI control DNA controls, which are now in very limited supply. Additionally, since efficient microbial lysis is a paramount step in all microbiome studies, the need for accurately quantified DNA and cellular mixed microbial standards is also needed to determine detection limits and percent recovery of various types of organisms. Recognizing these needs, three groups addressed the challenge and created multiple microbial reference standards, including Zymo Research (ZymoBIOMICS®), the Association of Biomolecular Resources Facilities (ABRF) metagenomics research group (Class I MGRG standards) and the National Institute of Standards and Testing (NIST). While only a few of these whole cell and genomics standards are currently available, future standards including complex mixtures of both eukaryotic and prokaryotic as well as RNA are currently being developed by these various organizations.

The ZymoBIOMICS® Microbial Community Standard is a mock microbial community consisting of 8 bacterial and 2 fungal strains (3 Gram-negative, 5 Gram-positive and 2 yeasts) with 7 being human pathogens. In contrast, the ABRF MGRG controls include 10 strains belonging to Class I genomes, and include both Gram negative and positive and 1 archaea but does not include human pathogen-related strains. NIST has generated several microbial standards, with the most recent being a human microbiome-related panel of microbial DNA with a release date in late 2017. Both Zymo Research and the ABRF offer a whole cell microbial standard which is absolutely necessary for determining DNA extraction efficiency and can be used as sample/matrix spike-in for recovery determinations, which is certainly one of the largest shortcomings of most DNA kits today.

Regardless of the study, whether it be clinical FMT samples, routine metagenomic samples from soil or food, or DNA extraction efficiency studies, the use of microbial reference standards is an important ingredient to be considered for any microbiome project as it will enable biologists, software developers, and product manufactures to determine efficiencies at every point in their process.

Metagenomic Standards Across the Globe and Beyond

Ebrahim Afshinnekoo[a] and Christopher E. Mason[b]

Author Affiliations:
a. Medical Student, New York Medical College
b. Associate Professor, Weill Cornell Medicine

Microbiome and Metagenomics Research

High-throughput, Next-Generation sequencing (NGS) has revolutionized the field of microbiology and genomics, ushering a surge of microbiome and metagenomics studies. As these studies continue to grow both in number and in scope, researchers face methodological and computational challenges for experimental design and interpretation. To address this challenge, many groups including the Association of Biomolecular Resource Facilities Research Groups on Next-Generation sequencing and metagenomics[1,2], the Food and Drug Administration[3], the National Institute of Standards and Technology[4], the Genome in a Bottle standards consortium[5], and the Microbiome Quality Control Project[6] have been working and collaborating with many of the leading companies on developing standards for the field to improve methodological rigor and data utility from increasingly global and distributed studies.

MetaSUB International Consortium

In 2015, the New York City PathoMap project showed that city-scale metagenomics had arrived[7], and shortly after the International MetaSUB Consortium[8] was founded to explore the molecular dynamics of cities and urban biomes around the world. Their goal is to study the metagenome of city mass transit systems and urban ecosystems, scanning for new biology, antimicrobial resistance markers, and novel biosynthetic gene clusters that can be used for drug development. For this massive endeavor, the consortium needed to develop standardized protocols for sample collection, processing, and analysis across over 70 cities and laboratories.

On the longest day of the year, June 21st, 2016, in collaboration with Ocean Sampling Day,[9] MetaSUB launched a global City Sampling Day (CSD), where "swab squads" across the globe geared up with their sampling kits, mobile-phone collection app, and gloves. They worked to collect over 7,500 samples in one day. To standardize this massive endeavor, MetaSUB worked closely with Zymo Research to develop certain standards and controls that could be utilized throughout the world's cities and the hub labs. The issues of kit and human contamination during DNA extraction/sequencing and

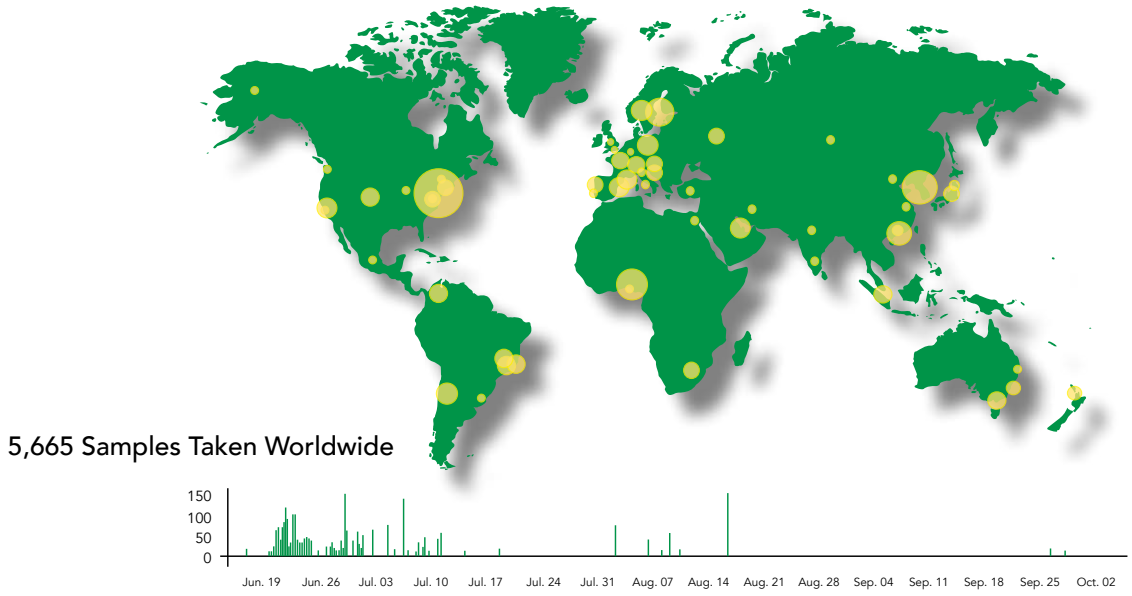

Figure 1. Samples collected during the first annual global City Sampling Day (CSD). Circles are proportional to the number of samples collected at each city.

challenges in accurate and precise taxa classification demanded standards to ensure these protocols are run efficiently and effectively.

The ZymoBIOMICS® Microbial Community Standard (D6305 & D6306)[10] was utilized as the positive control for the international MetaSUB study, it contains both a cellular sample and purified DNA. Since it is known precisely which organisms are in the standard, and at what relative abundances, the standard will establish if there were any kit contaminants during the extraction of the CSD samples. Also, when using tools for taxonomic classification, appropriate filters can then be determined to remove background noise. This is essential due to the wide variety of computational tools now available for metagenomics, which show a large range of sensitivity, specificity, precision, and accuracy[11]. The ZymoBIOMICS® Microbial Community Standards (D6300) have enabled a comprehensive test to monitor the reliability of current metagenomics tools for measures of species' presence, abundance, false positives, and false negatives[11]. MetaSUB is continuing to work with Zymo Research to develop this standard, our protocols for CSD 2017, and plans to use it annually for global sampling days until 2020.

Extreme Spaces and the Final Frontier

While we continue to create metagenomics profiles of places on Earth, DNA sequencing devices have now gotten small enough to begin sequencing in zero gravity and in space[12]. This is part of an ongoing NASA project called the Biomolecule Sequencer (BSeq), that synthesizes ideas from engineers, scientists, astronauts, and geneticists from NASA, Weill Cornell Medicine, and UCSF to enable real-time diagnostics of infections and samples while in space[13]. Also, work from the Earth Microbiome Project[14] and the Extreme Microbiome Project[15] is examining environments on Earth that mimic extreme environments to understand the mechanisms extremophiles utilize to live in such milieus. Members of the Extreme Microbiome Project have recently used portable nanopore sequencing in Antarctica, demonstrating that portable sequencing and metagenomics has truly encompassed all seven continents and the International Space Station above it.

For all of these sites, the ZymoBIOMICS® Microbial Community Standards (D6300) were, or will be, used in the experimental work as a critical positive control for sample collection, extraction, preparation, and sequencing. Indeed, these controls ensure that well-characterized, titrated mixtures of micro-organisms can be accurately sequenced and their genetic proportions fully recapitulated, even when spanning multiple Kingdoms of Life. Without them, data processing and interpretation of samples collected from these rare sites would be bereft of true positives, which are essential for methodological quality control. Similarly, the discovery of new genetic strains or epigenetic states (such as methyl-6-adenosine or other base modifications) from new organisms found at these sites requires the validation of the known molecular states of the Zymo controls' nucleic acids.

Once validated, non-canonical bases and novel organisms can be discovered and quantified, perhaps as far away as Mars. While Star Trek was ahead of its time when it posited that space is the final frontier, it seems that now researchers can aim to boldly sequence any metagenome, anywhere, while discovering new life and new (microbial) civilizations.

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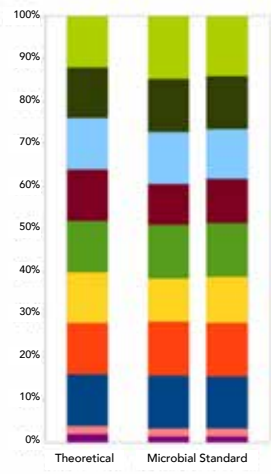
ZymoBIOMICS® Microbial Community Standard

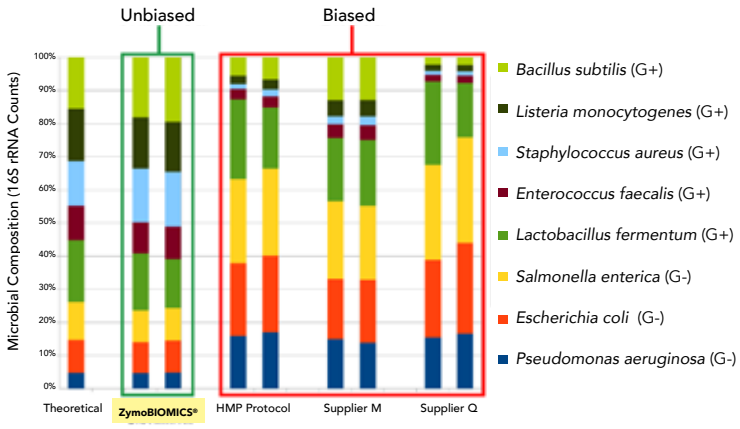
- Mock microbial community of well-defined composition.
- Ideal for the validation, optimization, and quality control of microbiomics and metagenomic workflows.
- Perfect for assessing bias of DNA extraction methods since it contains both tough- and easy-to-lyse microbes.



Species	Avg. GC (%)	Gram Stain	gDNA Abun. (%)
Pseudomonas aeruinosa	66.2	-	12
Escherichia coli	56.8	-	12
Salmonella enterica	52.2	-	12
Lactobacillus fermentum	52.8	+	12
Enterococcus faecalis	37.5	+	12
Syaphylococcus aureus	32.7	+	12
Listeria monocytogenes	38.0	+	12
Bacillus subtilis	43.8	+	12
Saccharomyces cerevisiae	38.4	Yeast	2
Cryptococcus neoformans	48.2	Yeast	2

Accurate Characterization

Containing three easy-to-lyse Gram-negative bacteria, five tough-to-lyse Gram-positive bacteria, and two tough-to-lyse yeasts, the ZymoBIOMICS® Microbial Community Standard is perfect for assessing bias in various DNA extraction methods. The microbial standards are accurately characterized, with a wide GC range (15%-85%) and contain negligible impurities (<0.01%), enabling easy exposure of artifacts, errors, and bias in microbiomics or metagenomic workflows.



Find Your Bias & Eliminate It

ZymoBIOMICS® Microbial Community Standard was used to compare different DNA extraction protocols. DNA samples were profiled by 16S rRNA gene targeted sequencing.

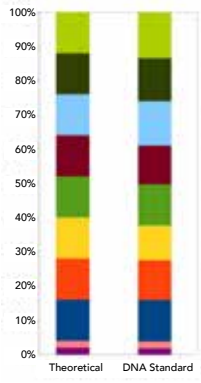
Learn more at
www.zymoresearch.com/zymobiomics

Product	Cat. No.	Size
ZymoBIOMICS® Microbial Community Standard	D6300	10 preps.

ZymoBIOMICS® Microbial Community DNA Standard

- A DNA standard of well-defined composition.
- Ideal for the validation, optimization, and quality control of microbiomics and metagenomics workflows.
- The DNA has a wide GC range of 15% – 85%.

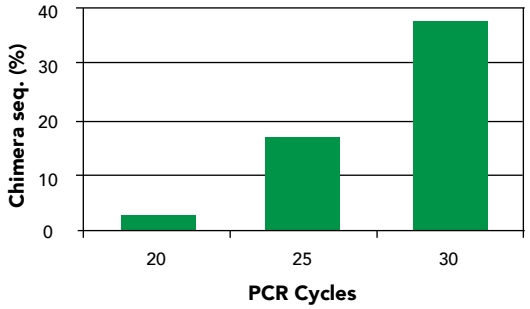
Accurate Characterization



Species	Avg. GC (%)	Gram Stain	gDNA Abun. (%)
Pseudomonas aeruinosa	66.2	-	12
Escherichia coli	56.8	-	12
Salmonella enterica	52.2	-	12
Lactobacillus fermentum	52.8	+	12
Enterococcus faecalis	37.5	+	12
Syaphylococcus aureus	32.7	+	12
Listeria monocytogenes	38.0	+	12
Bacillus subtilis	43.8	+	12
Saccharomyces cerevisiae	38.4	Yeast	2
Cryptococcus neoformans	48.2	Yeast	2

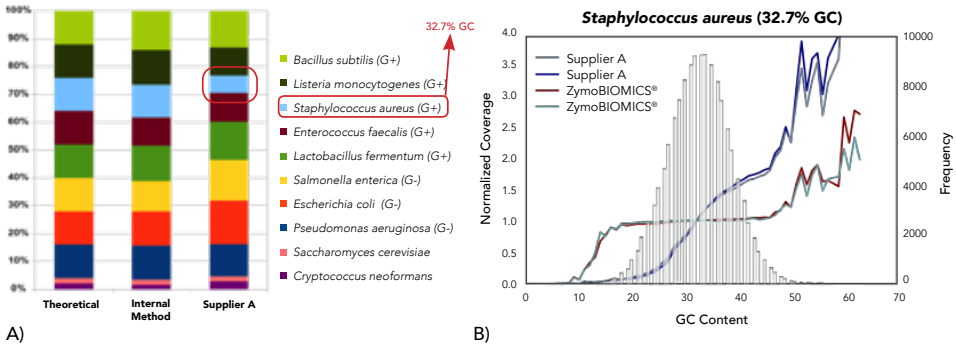
DNA from three Gram-negative bacteria, five Gram-positive bacteria, and two tough-to-lyse yeasts. The ZymoBIOMICS® Microbial Community DNA Standard are perfect for assessing bias in popular extraction methods. The microbial standards are accurately characterized, with a wide GC range (15%-85%) and contain negligible impurities (<0.01%), enabling easy exposure of artifacts, errors, and bias in microbiomics or metagenomic workflows.

Address & Reduce PCR Chimera



PCR chimera increase the number of PCR cycles during the library preparation step of 16S rRNA gene targeted sequencing. 20 ng ZymoBIOMICS® Microbial Community DNA Standard was used as a template. The PCR was performed with ZymoBIOMICS® PCR PreMix master mix and with primers that target the V3-V4 region of 16S rRNA gene. Chimera percentage was determined with Uchime and using the 16S rRNA genes of the 8 bacterial strains in the standard as reference.

Assess GC Bias & Eliminate It



A) Assessing bias of two different library preparation processes in shotgun metagenomic sequencing using ZymoBIOMICS® Microbial Community DNA Standard. Compared to our ZymoBIOMICS® Services, the Supplier A kit has some bias due to GC content variation. Sequencing was performed on MiSeq (2 x 150 bp). **B) Raw reads were mapped to the 10 microbial genomes to evaluate the potential effect of GC content on sequencing coverage.** Normalized coverage was calculated by normalization with the average sequencing coverage of each genome.

Product	Cat. No.	Size
ZymoBIOMICS® Microbial Community DNA Standard	D6305	200 ng
	D6306	2,000 ng

Learn more at
www.zymoresearch.com/zymobiomics

FEATURED ARTICLE:

Guideline for Use of the ZymoBIOMICS® Microbial Community Standard

Shuiquan Tang

How to use the ZymoBIOMICS® Microbial Community Standards

The ZymoBIOMICS® Microbial Community Standard and DNA Standard can be used as a defined input to assess microbiomics workflows. The standards aim to help assess how accurately the microbial composition is measured. They can be used in two major applications: (1) the establishment and optimization of an accurate and reliable microbiomics workflow, and (2) the routine quality control of an established microbiomics workflow.

Microbial Community DNA Standard

The ZymoBIOMICS® Microbial Community DNA Standard (D6305) can be used to determine differences between library preparation protocols by fixing other variables, such as sequencing platforms and bioinformatics analysis methods. The DNA standard is also ideal for optimizing conditions in the library preparation process, e.g. PCR cycle numbers, PCR annealing temperature, 16S primers etc. To use the ZymoBIOMICS® Microbial Community DNA Standard (D6305), simply thaw the standards and use the recommended amount of DNA input for your library preparation process. We recommend first time Microbial Community Standard users to start off with this standard to optimize and validate their sequencing and analysis methods before addressing bias in extraction protocols.

Microbial Community Standard

While it is highly recommended to use the standards in conjunction with each other, they can also be used independently. The ZymoBIOMICS® Microbial Community Standard (D6300), which is a mock microbial community containing known quantities of ten different microbes, is used to measure the accuracy of a DNA extraction method. Simply process 75 µl of the microbial community standard, treating it as if it were just another one of your actual microbial samples to reliably determine the accuracy of your DNA extraction protocol. After extracting the DNA from the microbial community standard, send the DNA through your pre-optimized library preparation, sequencing and data analysis process. We have provided the theoretical composition of the microbial community standard to compare against the sequenced DNA. If there are any major discrepancies between the theoretical standard and your sequenced standard, you will be able to identify the flaws within the extraction protocol.

How to Establish an Accurate Microbiomic Workflow in Your Lab

How can the ZymoBIOMICS® Microbial Community and DNA Standards help establish an accurate microbiomics workflow? Let's assume two cases: User A wants to establish a microbiomics workflow in a new lab, and

user B wants to optimize an existing microbiomics workflow. We recommend both users apply the ZymoBIOMICS® Microbial Community DNA Standard (D6305) first to determine best practices in the workflow, post-DNA extraction. To determine which library preparation protocol to use, you can compare different library preparation protocols by fixing other variables, such as sequencing platforms and bioinformatics analysis methods. For example, in the case of 16S rRNA sequencing, you can use the standard to compare different library preparation protocols, such as the HMP protocol and EMP protocol. Also note that Zymo will soon release a library preparation kit for targeted sequencing. You can also use the ZymoBIOMICS® Microbial Community DNA Standard (D6305) to optimize conditions within the library preparation process, e.g. PCR cycle numbers, PCR annealing temperature, 16S primers etc.

After you determine the best practices for library preparation, you can begin to optimize the DNA extraction step using the ZymoBIOMICS® Microbial Community Standard (D6300) cellular format. The microbial community standard enables you to compare different DNA extraction protocols or commercial kits for accuracy. DNA samples isolated with different DNA extraction methods can go through the same, pre-optimized library preparation process, sequencing and data analysis. The ZymoBIOMICS® DNA Standard allows you to compare the results between the ZymoBIOMICS® Microbial Community Standards and the DNA Standard. If the results of the DNA standard and the microbial community standard match, this indicated minimal bias in the DNA extraction step; and if they both agree with theoretical values of the standard, there is minimal bias throughout the entire workflow.

After the best practices for the entire workflow are determined, the ZymoBIOMICS® Microbial Community Standard (D6300) can be used as a quality control. For example, it is good practice to include a positive control (such as the ZymoBIOMICS® Microbial Community Standard, D6300) and a negative control (blank control) in each batch of DNA extractions. The positive control will show you how consistently and accurately your workflow performs. The negative control can help you assess the total bioburden (or contaminations) of your workflow. Including a negative control is critical to the analysis of low-biomass samples (e.g. skin swabs).

How to Analyze the Sequencing Data from the ZymoBIOMICS® Standard

For both the microbial community and DNA standards, the percentage genomic DNA abundance of the microbial composition is certified. With genome size, ploidy, and 16S/18S copy numbers of each microbe given in the manual of the product, you can transform percentage genomic DNA abundance into percentage abundance by 16S copy number or by genome copy number with basic assumptions.

Analyzing 16S Sequencing Data of the ZymoBIOMICS® Standard

When sequencing the ZymoBIOMICS® standards, analyze them using regular 16S rRNA analysis pipelines, such as Qiime[1] and Mothur[2]. You can compare the measured composition with the theoretical composition of the standard. Questions that should be kept in mind during this comparison include: (1) whether your measurement covers all strains with the proper taxonomy assignment and with correct abundance, (2) whether your measurement indicates the presence of foreign taxa with significant abundance. Taxonomy assignment might be incorrect or improper because of problems in the reference database. Abundance estimation might be off because of bias in DNA extraction, bias in library preparation, poor quality of MiSeq runs, etc. The presence of foreign taxa might indicate process contamination, poor sequencing quality, PCR chimera in library preparation, defects in bioinformatics analysis, defects in the reference database, etc. Both the ZymoBIOMICS® Microbial Community Standard and the DNA Standard are certified to have low impurity levels (<0.01% by DNA abundance). Any foreign taxa with abundance higher than 0.01% are derived from artifacts in the workflow.

Both Qiime and Mothur analyses are built upon sequence clustering for OTU analysis. This process is known to be unstable[3] and can bias final results. Since the 16S rRNA genes of the microbes contained in the standard are known, the more accurate and straightforward way to calculate the abundance of different organisms is by mapping the raw sequencing reads to these 16S sequences. Given the 16S rRNA genes of the strains contained in the standards as references, you can also accurately determine the percentage of PCR chimeric sequences using tools like Uchime[4].

Analyzing Shotgun Metagenomic Data from the ZymoBIOMICS® Standard

In terms of the accuracy of the measurement of microbial composition, we found shotgun metagenomic sequencing is generally more accurate than targeted sequencing, including 16S rRNA sequencing. This increase in accuracy can be attributed to shotgun sequencing library preparation protocols requiring fewer PCR cycles or even PCR-free protocols, while 16S library preparations are solely PCR based. With that being said, shotgun metagenomic sequencing can also experience bias. The ZymoBIOMICS® Microbial Community DNA Standard (D6305) can easily help you elucidate this bias. For example, using the ZymoBIOMICS® Microbial Community DNA Standard (D6305), we have observed that the shotgun library preparation kit from Nextera® XT (Illumina®, CA, US) resulted in lower sequencing coverage for both low GC content regions and high GC content regions.

In order to infer microbial composition from shotgun metagenomic sequencing, there are two types of analyses based on whether or not sequence assembly is applied. However, as metagenomic assembly with short reads from NGS data remains computationally challenging, assembly-free methods have gained popularity, including MetaPhlan[5], PhyloSift[6], and mOTU[7]. Most of these programs infer microbial abundance based on sequencing depth or coverage of marker genes; the calculated composition is similar to microbial composition by genome copy number. The ZymoBIOMICS® Microbial Community Standard certifies composition by total genomic DNA abundance. When you are using these assembly-free programs to analyze the sequencing data of the ZymoBIOMICS® Microbial Community Standard (D6300), it is important to convert microbial composition based on total genomic DNA abundance considering genome copy number with the genome size and ploidy of the strains given in the user manual.

However, if the purpose of your experiment is to test whether a DNA extraction method or shotgun library preparation method is biased, we recommend an alternative analytical method. Since the genomes of the strains contained in the standard are provided, the most accurate way to determine the microbial abundance is to map the raw reads directly to these known genomes and determine the abundance based on the number of reads mapped to each genome. As mentioned, most assembly-free programs are based on comparing the raw sequences, or K-mers, to marker genomes rather than whole genomes. This process undoubtedly can lead to bias.

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INSIGHTS:

A Fireside Chat with Dr. Jonathan Eisen on the Fields of Microbiomics and Metagenomics

Dr. Jonathan Eisen

Zymo Research: What are you most excited about in the field of microbiomics and metagenomics?

Dr. Jonathan Eisen: I'm excited about a few different areas in this field. One area I think is evolving and becoming more interesting is genetic studies of the interactions between hosts and their microbiomes. This would include studies like QTL mapping or genome-wide association where the microbiome is the trait. Then like you would with any other trait, such as height, weight, or heart disease, you look for factors in the host genome that affect the relative abundance or even predicted function of members in the microbial community on that host. I find this really inspiring because for the last 40 or 50 years people have been developing innovative tools to study genetics of hosts as they regulate particular traits. We now have the ability to characterize the microbiome in different parts of the organism or at different time points or different conditions.

Another general area that I find exciting is the move from characterizing microbial communities towards manipulating the microbial community in some way. We can now try and understand the factors that regulate the community, instead of just documenting what microbes are there and what they are correlated to. I could go on-and on about research I'm excited about; there are roughly 10 areas in the field that I find truly interesting and very important. I think the fact that technology allows people to treat microbial communities as a piece of data that they can gather info about is what is going to allow many areas of research to move from observing to understanding what factors control microbial communities.

Zymo Research: What are the greatest technical challenges facing the field of microbiomics and metagenomics today?

"We can now try and understand the factors that regulate the community, instead of just documenting what microbes are there and what they are correlated to."

Eisen: [Laughs] I think there are many. One thing that I care about is the over-interpretation, or as I call it the overselling, of the microbiome. I think this is partly due to people being careless, but also partly due to the technical challenges we face in studying microbial communities. A statistical technical challenge is the problem with false positives and associations. When people analyze correlations or even an experimental manipulation, and record data about the microbial community, they get information about thousands of species or predicted function. This provides you with tens of thousands of variables in that sense, and you're trying to ask the question "is any one of those variables correlated with something I was observing in the system?" Within observations of the metadata - some other piece of data about the system such as health status of individual or punitive function of the community - you're always going to find things that are perfectly correlated because you have thousands and thousands and thousands of recordings of the microbial community. I think there is an immense challenge in figuring out how to design experiments and analyses that aren't misdirected by the false positives that inevitably occur.

Once you get beyond that, there are obviously plenty of technical challenges in doing work on microbial communities. For example, one massive technology challenge is predicting functions of the community. The way people did this, and many still are doing it, is by identifying which taxa are present in the community. Then based upon either literature or some information about those taxa, they are trying to predict the functions that are present in the community. This only works well if there is robust literature of organisms closely related to sequences that are found in the community, or if you have tens to hundreds to thousands of complete genomes for relatives of the organisms within the community. The problem is we basically only have that information for the human microbiome. It's sort of the equivalent of going out to a rainforest and creating a catalog of a couple organisms that live there, and then trying to predict the function of an entire ecosystem with your field guide that only has two organisms in it.

It's incredibly hard to do anything in many of these communities where we don't have a lot of reference information on relatives of the organisms within the community. From a sequencing point of view, one way to get around this barrier is to try and sequence metagenomes instead of phylogenetic marker genes. And while that helps, we still don't have any idea of what 20-40% of the genes do in even well-studied, cultured model organisms. So imagine when you're sequencing tiny little fragments from an environmental community for which we don't have genomic data for close relatives from 75% of the organisms in the environment. The functional predictions you could make are pretty poor and probably not very precise. That creates another massive challenge of how do you make useful functional predictions from that data?

What I think is really interesting in the field and about the technical challenges is moving beyond functional predictions to actual functional studies. These studies can include cultured representatives of communities, which is, in essence, what people have been doing for 100 years, or *in situ* function studies with stable isotope probing or microscopy, or metabolomics, or any other experimental methods where you are trying to measure something direct about what's going on in the community. Hopefully, that will improve our ability to make function predictions and allow us to ask what the community is actually doing as opposed to what it is predicted to be doing.

"What I think is really interesting in the field and about the technical challenges is moving beyond functional predictions to actual functional studies."

Zymo Research: Can you explain the pros and cons of 16S and Shotgun Sequencing? Also can you tell us how to determine what sequencing method would be right for your research?

Eisen: I'll answer the second question first. Which method of any kind including sequencing is right for your research depends on the questions that you're asking and the system you have. If you are doing experiments on the human microbiome, especially the gut, you can use short read Illumina® sequencing of ribosomal RNA sequences of metagenomes in order to count members of the community that you already have data about. In that case, you are basically using sequencing in a way that people are using RNA-seq in transcriptome studies. You may have a reference database that is very robust and using sequences to count either taxa, genes, or population variance within a taxa, because you have such great reference data. But if you are doing experiments in any other organisms, even mice, the reference data is nowhere near as good. You might have literally the exact same question that you were trying to ask in the human system, but you would have to take a different approach because the reference data isn't as good. If you're conducting research like what my lab is doing with sea grass or wild cats or in other systems where reference data is even poorer, you could yet again have the exact same question as before and it's going to change the approach you're doing. I think you begin by determining the question you want answered, and the comparative information you can leverage to answer that question. This should guide what approach you should use.

Then you arrive at the technical question, "I think I'm going to use sequencing for some of this. How can I make this sequencing work better?"

To answer this I think it's useful to draw out the entire workflow. Let's say you imagine an experiment on a new species of bird that has just been discovered in a tropical rainforest and you don't have any reference information about it. What are you going to do if you want to study the microbiome of this bird? You should first outline your experiment. Let say you're interested in the microbiome of this bird eating some leaves from some strange tree. The next step would be to outline how much money you have to put into this experiment. You should think of the big picture, for example you might end up concluding taxonomic PCR surveys, like 16S sequencing surveys, are where you want to start with, but you might realize that there are no reference genomes from this bird. It wouldn't be totally crazy to have a project on culturing organisms from this bird and sequencing a few genomes. Right now it cost us about $100 to sequence a genome, and that is certainly going to make your functional interpretations of the 16S data better. The same goes for shotgun sequencing. If you decide that you want to do shotgun sequencing from the system, having some reference genomes to tile the reads from the shotgun sequencing data is also going to help. I wouldn't limit the decision to what you're going to do with the actual microbiome sample that you were interested in. Instead, I would design it from the total experiment point of view and say, "What other information do we need?"

Maybe start out with some taxonomic survey and get a lay of the landscape and then say, "My system is overrun by members of the mycoplasma genus. Do I think I have enough for a reference genome of that group to make something useful from the data I have?"

I think it is an iterative process and it depends on the organism that you're working on and how much reference information you have. For my projects and my labs, if we can, we are moving away from 16S as the first characterization of a system. Sequencing is cheap enough to do shotgun sequencing in many of these cases where you might have done just 16S in the past, and that gets you taxonomic, phylogenetic, and functional prediction information.

However, this depends on the system. If you are working on a system where you are interested in using DNA sequencing to characterize a microbiome, there might be a problem in that when you collect a sample most of the material you collect is host DNA. Shotgun sequencing is going to be very expensive to get information about the microbial components of that mix of DNA. So in those cases you are probably much better off doing 16S, ITS, or some other survey. But if you can get a sample where most of the material is the microbial material that you're interested in, I think there are enough benefits to doing shotgun sequencing, where the cost per sample is higher and the benefit per sample is also higher. It is really context dependent, scientific question dependent, and total project dependent to make these decisions. If

your samples are rich in microbes, in many cases, it now makes sense to do shotgun sequencing instead of doing any PCR surveys.

Zymo Research: Can you comment on the use of third generation sequencing, such as PACBIO® Sequencing, in microbiomics applications or research?

Eisen: If we can talk about second generation sequencing for a minute. I think the benefit in second generation sequencing is the massive number of reads it provides at low cost. That can be used for many purposes, including doing surveys across large amounts of samples, doing deep surveys, doing genomic type of categorization and counting things. The disadvantage is that you generally lose the linkage information between different fragments. You can recover that by assembly or binning but that doesn't always work for every sample, and you can miss out on some of the information. I think third generation (long read sequencing) methods seem the most promising, because they allow you to get around not having to do complicated informatics to recover information about linkage.

We've done a bunch of stuff with PACBIO®, and I know other people have done long read sequencing for microbial communities and it can be incredibly beneficial. Again, you have to evaluate it in a context of your scientific question. If your question was about trying to distinguish relative abundance of particular organisms that are rare in your sample, long read sequencing will be too expensive for that. Long read sequencing can be really powerful if you want to assemble genomes or detect lateral gene transfer or linkage information.

Then you get to questions, "Which method do I want to use? What is the error rate of this method"?

PACBIO® has another advantage in that it allows you to detect methylation and other types of bases modification in the samples, which has turned out to be very useful. Oxford Nanopore® sequencing has the advantage to be able to work in the field. I'm still amazed that people can do this but it honestly looks like a real option for microbiome samples. You can get data in a relatively short amount of time without having to bring samples back to the lab. So if you are doing field work in some remote location, like the space station or Antarctica, tools like that will be a huge advantage. However, it is important to remember that these are all tools, not answers in and of themselves. Any tool can be used well or used poorly. You have to think about it in context.

Another thing that is really interesting, but I don't think this is a third generation sequencing method, is the methods to use Illumina® sequencing but in a way that you get linkage information; this would be the 10X genomics or the dovetail or other Hi-C or variance of Hi-C sequencing. So there you are using short read Illumina® sequencing, but you are making your library in a way that the short read sequencing and the bar coding that you get from some library construction method tells you linkage information. I think all of these things are very promising and serve many uses in microbiome research.

"I think in situ *functional assays and the manipulation of the microbial community with more precise manipulation than what is currently being done with antibiotics is going to be one of the most exciting areas of research in the next five years."*

Zymo Research: Where do you see the field of microbiomics and metagenomics going in the next five to ten years?

Eisen: Five years is almost as far as I could even imagine thinking. There is no way that I could predict the next 10 years [laughs]. I think, as I was hinting at earlier, the field is making progress toward filling out the reference information for model organisms, so that anybody can do microbiome studies without having to collect all of that reference information. Even for humans, there is not a lot of data. For example, there is little information on the gut microbiome of any people who are not from North America or Western Europe. So the reference data is really narrow. There is a lot more diversity that needs to be characterized for the reference information, and there is not a lot of reference information for microbiomes other than the gut. The oral microbiome has been less deeply characterized, the skin even less so, and the

vaginal microbiome even less so. And there are microbial communities that differ within different parts of the gut that we probably haven't characterized in much detail. For the viral community we probably don't have enough reference data yet, even for humans. The fungal community we don't have enough reference data, and the protist community we don't have enough reference data, that's even for humans. So if you're interested in Arabidopsis or mouse or corn, or other so called model organisms, we're still missing massive amounts of reference data. Then if you go beyond the model organisms, there is very little. In the next five years we will see a filling out of the reference data for many of the model organisms, and eventually for many of the non-model organisms.

I think that even without the reference data, we're seeing new technologies being brought in that do not need all of the reference data for some experiments. Again, to what I was referring to earlier, what is most exciting is the *in situ* functional studies, where you might take advantage of some sequencing data but the actual clinching experiment is not a sequencing, it's a NanoSIMS microscopy experiment, or a stable isotope probing to track a movement of nitrogen in a system, or it's some fluorescent assay where you're looking at the interconnectedness of molecules between different cells, or it's a manipulative experiment where you're knocking out specific members within the microbial community by a bacterial virus. I think *in situ* functional assays and the manipulation of the microbial community with greater precision than what is currently capable with antibiotics is going to be one of the most exciting areas of research in the next five years.

Another thing I believe will be exciting in years to come, and some of this is happening now, which I don't want to discredit, is a total-systems level approach. So far we have not done a very good job of trying to characterize all of the inputs and outputs of a microbial community in a particular system. We have a decent idea of how human babies get colonized from their mothers during vaginal birth and how breastfeeding impacts the microbial community. We have a much poorer understanding of how the environment shapes the microbial community, of how we get microbes into our gut from our food, our buildings, our dogs, and our friends, and all of that total systems-level approach to the microbial community will also be very important. After a year when a baby is starting to be colonized by everything in its environment, why do some things take hold and some don't? What shapes why there are changes in the microbial community over time or over space or in response to diet? I think discovering those dynamics will be interesting, and understanding the inputs and outputs will be incredibly important.

Zymo Research: Are there any other comments you would like to make or anything else you would like to discuss?

Eisen: I think it's always important to temper the hype a little bit. I work in this area so I obviously think microbial communities are very important and interesting, but at the same time they are not the only thing to study in these systems. They're not the only thing that impacts the host they live on or, if they are free living, the ecosystems of the planet. They are complicated and that makes them interesting to me and they're likely important in many systems, but they are not the only thing. There is a bit of a backlash, that is somewhat deserved, where people are saying, "Oh, no, not another microbiome project". We need to be careful about overselling the findings of microbial communities. Where again, going back to the systems-level approach, a multicellular organism like a human or *Arabidopsis* plant, the microbial parts of that organism probably impacts much of that biology.

However, some people have taken this notion to the extreme to literally say things like "the human genome does not impact human biology". The more we oversell it, the bigger the risk is that people will start to discount even the important and compelling discoveries that come out in the field.

One other thing I think is really exciting and interesting is the engagement of the public and citizen science in studies of microbial communities, whether that's on people or the oceans or the water associated in Flint, Michigan, or the roses in your backyard. There are a lot of projects that people have already started where the public is being engaged in microbiome research. This is truly important because of the importance of microbial communities and also because they are difficult to wrap your head around, given that we can't see them and they are very complicated. So I think getting the public involved in thinking about this hidden world is very important.

Functional Metagenomic Approaches for Studying and Combating the Antibiotic Resistome

Andrew J. Gasparrini,[a] Gautam Dantas[a,b,c,d]

Author Affiliations
a. Center for Genome Sciences and Systems Biology, Washington University School of Medicine, 4515 McKinley Avenue, Campus Box 8510, St. Louis, MO 63110, USA.
b. Department of Pathology & Immunology, Washington University School of Medicine, 660 South Euclid Ave, St. Louis, MO 63110, USA
c. Department of Biomedical Engineering, Washington University in St. Louis, 1 Brookings Drive, St. Louis, MO 63130, USA
d. Department of Molecular Microbiology, Washington University School of Medicine, 660 S. Euclid Ave, St. Louis, MO 63110, USA.

Abstract

As the incidence of antibiotic-resistant infections has increased, the study of the antibiotic resistome in diverse microbiomes has emerged as an important basic science and translational research priority. Recent computational and technical advances have facilitated a dramatic increase in the resolution and throughput of resistome studies, illustrating the ubiquity of functional antibiotic resistance reservoirs across diverse habitats and ecosystems. Surveys of resistomes have allowed us to characterize networks of resistance transmission, assess the risk of cross-habitat dissemination of resistance genes, identify novel resistance determinants, and design novel therapeutic strategies to combat resistant pathogens[1-4]. In this article, we review technical approaches to the study of resistomes and describe advances that have emerged from application of these techniques.

"A recent report estimated that unless the current trajectory is altered, by the year 2050 antibiotic resistant infections will claim 10 million lives per year..."

Introduction

The evolution and spread of antibiotic resistance paired with the dearth of approvals of new antibiotics jeopardizes the effective treatment and prevention of bacterial infections. A recent report estimated that unless the current trajectory is altered, by the year 2050 antibiotic resistant infections will claim 10 million lives per year (1 death every 3 seconds) and cost the global economy 100 trillion US dollars (roughly equivalent to the last 6 years of the total US GDP)[5]. Already, antibiotic resistant infections are directly responsible for at least 23,000 annual deaths in the US alone[6]. Furthermore, antibiotic resistance in both the environment[7] and in pathogens[8] has been steadily increasing over the past several decades. As a result of the human and economic cost of antibiotic-resistance, it has become critically important to understand the antibiotic resistance landscape across habitats to improve and inform stewardship of existing antibiotics, development of new antibiotics, and treatment of antibiotic-resistant infections.

The Antibiotic Resistome

The antibiotic resistome is defined as the universe of antibiotic resistance genes in a given microbial habitat[9]. While classical studies of antibiotic resistance focused on single resistance genes harbored in pathogenic organisms, recent studies have taken a systems-level approach to characterize the resistomes of microbial communities. This approach has revealed diverse and extensive resistomes in nearly all habitats queried. Interestingly, even habitats devoid of exposure to commercially produced antibiotics, such as a cave isolated from humans for the past four million years[10], 30,000 year old permafrost sediments[11], and the gut microbiota of previously uncontacted Amerindians[12], harbor diverse resistomes. Such systems-level analyses of microbial communities broadly fall into the category of metagenomics, or the study of microbial communities using DNA sequencing. Many recent resistome analyses have been powered by advances in sequencing technologies and concomitant dramatic drops in sequencing costs that have occurred over the past decades.

Functional Metagenomics Reveals Known and Novel Resistance Genes

Functional metagenomics is a powerful method for accessing both known and novel resistance genes. The method involves shearing metagenomic DNA to a

Figure 1: The Dantas lab pairs classical culture-based techniques (top left) with next-generation sequencing to study the antibiotic resistomes of diverse microbial ecosystems, including the Neonatal Intensive Care Unit (top right) and rural and peri-urban dwellings in El Salvador and Peru (bottom). Photo credit: Pablo Tsukayama.

desired size distribution, shotgun cloning these fragments into a suitable screening vector, and transforming the resultant library into a heterologous host. The library is then screened for a phenotype of interest, such as antibiotic resistance. Sequencing transformants that survive antibiotic selection can reveal both known and novel resistance genes. Recent improvements in Next-Generation sequencing, computational assembly, and annotation pipelines have enabled our group (www.dantaslab.org) to improve the throughput of functional metagenomic selections, permitting robust characterization of resistomes from diverse microbial habitats[13].

"Identification of such emerging resistance threats prior to their spread to pathogens is critical because it enables proactive surveillance and mitigation of the novel resistance gene."

An important advantage of functional metagenomics is that it removes any requirement for culturing the original host of the resistance gene. This has empowered characterization of resistomes of environments in which the majority of bacteria are difficult to culture in the laboratory setting, such as the soil. Our interrogation of soil resistomes by functional metagenomics provided the first evidence for multiple antibiotic resistance genes in benign soil bacteria that are identical to those in several human pathogens[13]. This suggests recent exchange of genetic material between the soil and clinical resistomes, highlighting the importance of expanding our study of resistomes beyond the clinical setting.

A further strength of functional metagenomics is that it enables identification of resistance genes without any prior knowledge of that gene sequence. Indeed, we have shown that resistance determinants uncovered by functional metagenomics frequently have low identity to genes in existing antibiotic resistance databases, illustrating the power of the method for uncovering genes to which a resistance function has not previously been ascribed[14]. This allows the discovery of novel resistance genes. For example, we recently discovered a novel family of tetracycline inactivating enzymes in the soil using functional metagenomic selections[15], which could compromise a number of new tetracycline derivatives in current late-stage clinical development. Identification of such emerging resistance threats prior to their spread to pathogens is critical because it enables proactive surveillance and mitigation of the novel resistance gene.

A final advantage of functional metagenomics is the ability to provide quantitative information on the risk for transmission of resistance genes between habitats. In a recent study, our group used functional metagenomics to characterize the resistomes of hundreds of fecal and environmental samples from rural and peri-urban dwellings in El Salvador and Peru[16]. By complementing these analyses with 16S phylogenetic profiling and whole

metagenome shotgun sequencing, we were able to infer that resistomes are generally structured by microbial phylogeny and habitat, and were able to measure the abundance of all characterized resistance genes across all samples. By including multiple microbial habitats in the resistome analysis, we identified wastewater treatment plants and chicken coops as areas in which resistance gene exchange might be enriched. Furthermore, examining the context in which a resistance gene occurs can provide evidence for past horizontal gene transfer. For example, our El Salvador and Peru resistome study identified a single β-lactamase (TEM-1) encoded in 25 different genetic contexts. In these contexts, TEM-1 was often syntenic with mobile genetic elements such as integrases, transposases, and resolvases, suggesting that it is highly mobile.

Conclusions and Future Directions

Functional metagenomics is a powerful method for characterizing the antibiotic resistomes of diverse microbial habitats in a sequence- and culture-unbiased manner. Importantly, functional metagenomics enables researchers to survey the resistome of communities containing uncultivable microbes, to identify novel resistance determinants, and to describe the threat for dissemination of antibiotic resistance genes across habitats. Complementing this method with 16S phylogenetic profiling and whole metagenome shotgun sequencing can enable inference of the host taxa of specific resistance determinants, determination of the abundance of these taxa and their resistomes, and modeling of evolution and horizontal transfer of resistance determinants over time in longitudinally-sampled cohorts. Future resistome studies should explore phylogenetically diverse functional metagenomic hosts to appropriately capture the host specificity of the resistome and to expand our knowledge of resistome beyond genes functional in commonly used Gram-negative lab strains. Additionally, it is important that studies consider the genomic context of functionally selected resistance genes, with particular attention paid towards mobile genetic elements. This will allow us to narrow our focus to the intersection of the resistome and the mobilome (i.e. the universe of mobile genetic elements in a genome), prioritizing those resistance genes that pose the greatest threat for future dissemination.


The Dantas Lab

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Sample Collection and Storage Considerations for Microbiomics

As the beginning of the entire workflow, sample collection and preservation is one of the most critical steps for achieving high quality, reproducible results. Yet, sample collection can vary greatly between labs. When a sample is stored or transported at ambient temperature, without a protective mechanism in place (e.g. preservation reagents or effective cold chain), microbes have markedly varied growth and survival rates. This can lead to drastically altered community profiles. Nucleic acid profiles can rapidly change due to degradation or transcription in response to environmental changes. To achieve an accurate representation of the original sample, collection and storage methods need to prevent the alteration of the nucleic acid profile to avoid inaccuracies and biases. While freezing samples at -80°C on site is the most ideal solution, access to freezers is inconvenient or unfeasible in many situations, and transporting samples that require refrigeration or freezing is costly. Some preservation reagents also require reagent removal that can introduce bias by inadvertently causing uneven partitioning of the sample. When and how a sample is collected can also affect observed microbial profiles and should be carefully considered when designing a study.

Zymo's Research DNA/RNA Shield™ was designed for microbiomic applications and satisfies all of the requirements for accurate community profiling, including preservation of nucleic acids at ambient temperature, inactivating organisms, and enabling high-throughput streamlined purification.

How To Preserve Microbial Composition at Ambient Temperature

The quality of the collection and storage methods can greatly influence the growth and decay of certain microbes, leading to composition shifts after the time of collection. Sample collection and storage can vary greatly between labs - from the handling of samples collected in the field, to the accessibility of storing samples in -80°C freezers. When stored at ambient temperatures, bacterial species have markedly varied growth and survival rates. Nucleic acids can also degrade during this step. Lysis of fragile cells during freeze thaw may also lead to degradation of nucleic acids that leak out during the thawing step, which leads to misrepresentation of the community profile at the time of collection.

To demonstrate, stool samples suspended in DNA/RNA Shield™ (R1100-50) and stored at room temperature were compared to stool without preservative for one month (Figure 1). They were sampled at the indicated time points and processed with the ZymoBIOMICS® DNA Miniprep Kit (D4300). The extracted DNA was then subjected to microbial composition profiling via 16S rRNA gene targeted sequencing. Samples stored with DNA/RNA Shield™ had a constant microbial composition while the samples stored without shifted dramatically.

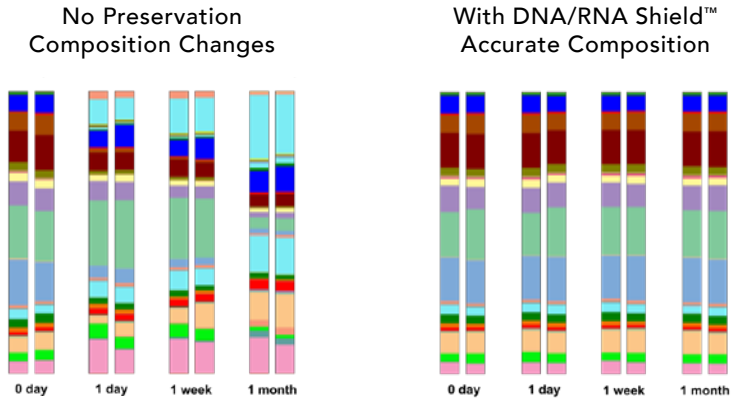


Figure 1: Microbial composition of stool is unchanged after one month at ambient temperature with DNA/RNA Shield™.

Safely Handle, Process, and Transport Sample to Prevent Spread of Pathogens

Transporting and mailing samples can often be challenging or not possible, especially when crossing borders. DNA/RNA Shield's™ ability to inactivate organisms (bacteria, fungi, virus, etc.) including pathogens contained in a sample eliminates safety concerns during transportation (*e.g.* border crossing) and sample processing (*e.g.* accidental leakage or spills in DNA extraction). DNA/RNA Shield™ has been shown to inactivate pathogens (Figure 2) such as Influenza, Ebola, HIV-1, *M. tuberculosis*, *E. coli*, and *C. neoformans*

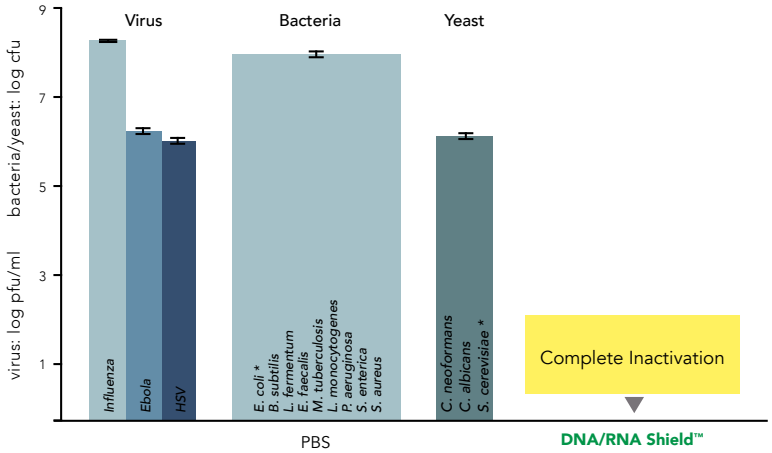


Figure 2: Viruses, bacteria and yeast are effectively inactivated by DNA/RNA Shield™. Samples containing the infectious agent (virus, bacteria, yeast) were treated for 5 minutes with DNA/RNA Shield™ or mock (PBS). Titer (PFU) was subsequently determined by plaque assay. Validated by: Influenza A - D. Poole and Prof. A. Mehle, Department of Medical Microbiology and Immunology, University of Wisconsin, Madison; Ebola (Kikwit) - L. Avena and Dr. A. Griffiths, Department of Virology and Immunology, Texas Biomedical Research Institute; HSV-1/2 - H. Oh, F. Diaz and Prof. D. Knipe, Virology Program, Harvard Medical School; *E. coli, L. fermentum, B. subtilis, S. cerevisiae* – Zymo Research Corporation).

*Disclaimer: Only initial growth levels or values of *E. coli displayed.* All microbes were tested independently and were combined into one graph for brevity. Bacterial cultures were grown between 10^8 - 10^9 cells and yeast cultures were grown between 10^7 - 10^8 cells.

Store and Transport DNA and RNA at Ambient Temperatures for One Month

Ambient temperature storage/transportation is a major concern for sample integrity when no cold chain is available.

To demonstrate the stabilization power of DNA/RNA Shield™, DNA and/or RNA was isolated from various samples including stool, saliva, blood, and cells over a one month time-frame. Nucleic acids were analyzed using PCR, RT-PCR, or gel electrophoresis showing no significant degradation during this time frame (Figure 3).

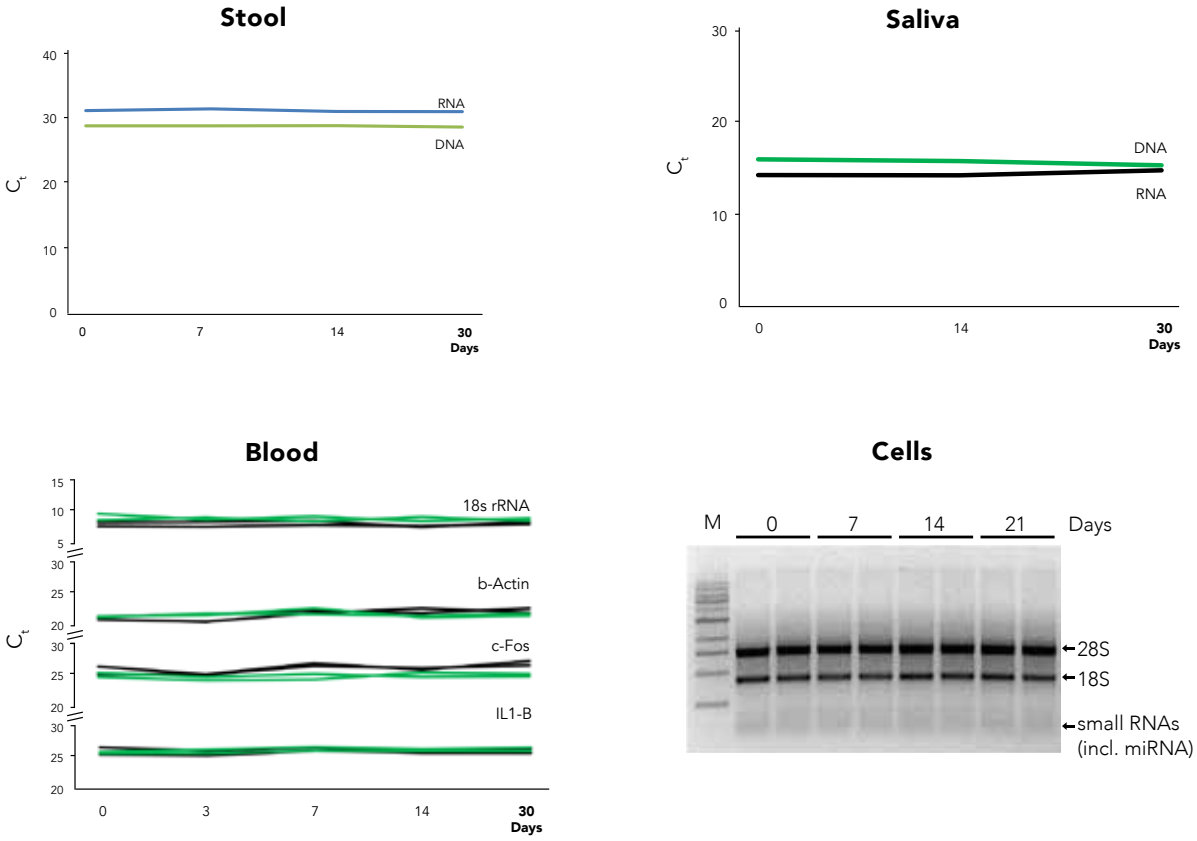


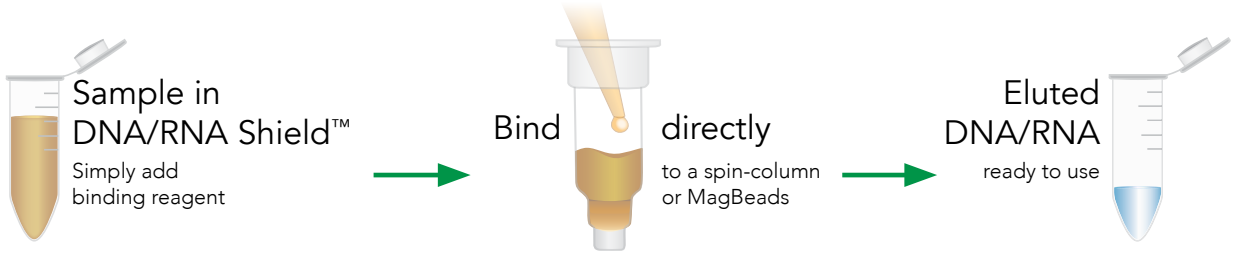
Figure 3: DNA and RNA are stable for one month at ambient temperatures using DNA/RNA Shield™. Nucleic acids were isolated from stool, saliva, blood, and cells and analyzed using PCR, RT-PCR, or gel electrophoresis. No significant decrease of C_t value or degradation of RNA bands is noted. M is a 1kb marker.

How to Streamline Your Purification

Preprocessing, such as reagent removal, complicates high throughput automation and introduces potential biases associated with phase separations. Phase separation (e.g. precipitation) has been shown to bias downstream analyses as not all nucleic acids fully separate during phase separation. Small nucleic acids (e.g. miRNA) are particularly vulnerable to such biases and/or complete signal loss, because of their aberrant behavior when compared to larger nucleic acids.

Samples in DNA/RNA Shield™ can be immediately used in all ZymoBIOMICS® isolation kits and are universally compatible with all available commercial isolation products. This greatly reduces the amount of handling steps and processing time, allowing for a simplified, streamlined workflow.

No Reagent Removal. Compatible with ZymoBIOMICS® Purification Products.



Accommodates Any Sample

DNA/RNA Shield™ collection devices are compatible with any sample, including:

- stool
- soil
- vaginal swabs
- nasal swabs
- endocervical swabs
- buccal swabs
- naso-pharyngeal swabs
- saliva
- whole blood
- tissue biopsies
- insects
- plant tissue
- and more!

DNA/RNA Shield™ Minimizes Microbial Composition Changes Caused by Freeze-Thaw Cycling

As the first step of any microbiomics workflow, sample collection and preservation is critically important. Any bias introduced in this step will be carried through the whole workflow and it is often difficult to repeat the sample collection step. Additionally, it is well understood that microbes can react very rapidly due to the change of environmental conditions, such as the change of temperature or oxygen concentration during sample collection and transportation. Therefore, to achieve accurate microbiome measurements, it is necessary to implement certain microbial preservation measurements immediately after sample collection to prevent potential bias caused by undesired microbial growth or decay, or undesired degradation of nucleic acids.

There have been many discussions in the literature regarding the best way to preserve microbial samples for microbiome measurements[1-10]. Most microbiologists believe the best way to preserve microbial samples is to freeze them immediately. Unfortunately, resources to freeze samples may be impossible to access or too costly in many scenarios, such as in sample transportation and collection of samples in the wild. For this specific purpose Zymo has released DNA/RNA Shield™, a liquid preservation reagent that can preserve microbial DNA and RNA at ambient temperature for several months. Also, even if access to freezing resources is not an issue and you prefer to freeze your microbial samples during sample collection, you may still consider saving your samples in this reagent because DNA/RNA Shield™ also helps prevent microbial composition change caused by freeze-thaw cycling.

It is clear that problems can arise from freeze-thaw cycling such as damage to nucleic acids and reduction of the viability of microbes. Freeze-thaw cycling can also cause dramatic changes in microbial composition. For example, it has been reported that freeze-thaw cycling of fecal samples can dramatically reduce the DNA recovery of *Bacteroidetes*, a dominant phylum in gut microbiota[3,4]. In one experiment we performed, five freeze-thaw cycles completely erase the *Bacteroidetes* community in a fecal sample (Figure 1). In contrast, when the same fecal sample was saved in DNA/RNA shield, the *Bacteroidetes* community can be preserved even after ten freeze-thaw cycles (Figure 1). For more details about DNA/RNA Shield™, go to pages 29-30.

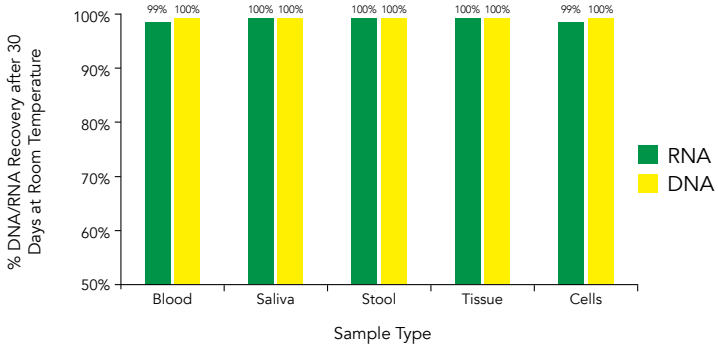
Figure 1. DNA/RNA Shield™ minimizes microbial composition changes caused by freeze-thaw cycling. Aliquots of a fecal sample, some saved in DNA/RNA Shield and some without, were subject to freeze-thaw cycling. DNA extraction was performed with the ZymoBIOMICS DNA Miniprep. Microbial composition was determined by 16S rRNA gene targeted sequencing.

References:
1. Gray MA, Pratte ZA, Kellogg CA: Comparison of DNA preservation methods for environmental bacterial community samples. FEMS microbiology ecology 2013, 83(2):468-477.
2. Mitchell KR, Takacs-Vesbach CD: A comparison of methods for total community DNA preservation and extraction from various thermal environments. Journal of industrial microbiology & biotechnology 2008, 35(10):1139-1147.
3. Hsieh YH, Peterson CM, Raggio A, Keenan MJ, Martin RJ, Ravussin E, Marco ML: Impact of Different Fecal Processing Methods on Assessments of Bacterial Diversity in the Human Intestine. Frontiers in microbiology 2016, 7:1643.
4. McKain N, Genc B, Snelling TJ, Wallace RJ: Differential recovery of bacterial and archaeal 16S rRNA genes from ruminal digesta in response to glycerol as cryoprotectant. Journal of microbiological methods 2013, 95(3):381-383.
5. Lauber CL, Zhou N, Gordon JI, Knight R, Fierer N: Effect of storage conditions on the assessment of bacterial community structure in soil and human-associated samples. FEMS microbiology letters 2010, 307(1):80-86.
6. Song SJ, Amir A, Metcalf JL, Amato KR, Xu ZZ, Humphrey G, Knight R: Preservation Methods Differ in Fecal Microbiome Stability, Affecting Suitability for Field Studies. mSystems 2016, 1(3).
7. Saito MA, Bulygin VV, Moran DM, Taylor C, Scholin C: Examination of microbial proteome preservation techniques applicable to autonomous environmental sample collection. Frontiers in microbiology 2011, 2:215.
8. Gorzelak MA, Gill SK, Tasnim N, Ahmadi-Vand Z, Jay M, Gibson DL: Methods for Improving Human Gut Microbiome Data by Reducing Variability through Sample Processing and Storage of Stool. PloS one 2015, 10(8):e0134802.
9. Hale VL, Tan CL, Knight R, Amato KR: Effect of preservation method on spider monkey (Ateles geoffroyi) fecal microbiota over 8 weeks. Journal of microbiological methods 2015, 113:16-26.
10. Tedjo DI, Jonkers DM, Savelkoul PH, Masclee AA, van Best N, Pierik MJ, Penders J: The effect of sampling and storage on the fecal microbiota composition in healthy and diseased subjects. PloS one 2015, 10(5):e0126685.

DNA/RNA Shield™ Sample Collection Devices

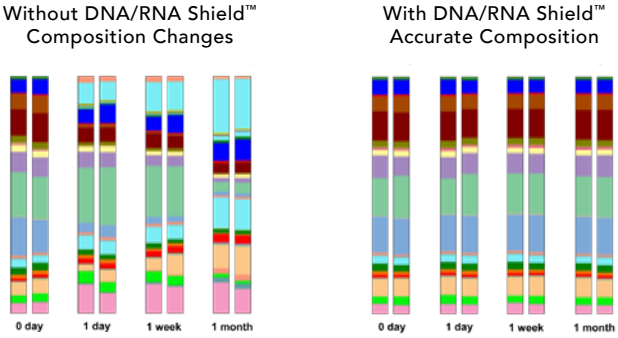
Safe Transport and Storage at Ambient Temperature

DNA/RNA are Stable at Ambient Temperature:
No refrigeration required. At ambient temperature DNA stability exceeds 1 year and RNA is stable up to 30 days.



DNA and RNA are effectively stabilized for one month at room temperatures. The above figure shows DNA and RNA quantified using PCR comparing recovery at 0 and 30 days after collection.

Microbiome Community Profile is Preserved:
Samples in DNA/RNA Shield™ accurately reflect the composition of the sample at the time of collection.

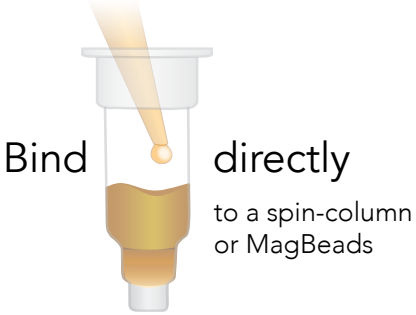


Microbial composition of stool is unchanged after one month at ambient temperature with DNA/RNA Shield™.

Microbial Pathogen Inactivation:
Safely store, transport, and process samples collected in DNA/RNA Shield™ including:

- Influenza
- Ebola
- HSV
- *E. coli*
- *M. tuberculosis*
- *C. neoformans*
- And More!

Streamlined Protocol:
No reagent removal. No precipitation. Universally compatible with commercial DNA/RNA Isolation kits.



Multiple Formats Available for Microbial Specimen Collection

DNA/RNA Shield™ - Swab & Collection Tube

- A sterilized 12 x 80 mm screwcap vial prefilled with 1 or 2 ml of DNA/RNA Shield™
- Contains a sterile HydraFlock® swab with short (80 mm) breakpoint
- Ideal for the general collection of swab samples (i.e., nose, mouth, throat)

Product	Cat. No.	Size
DNA/RNA Shield™ - Swab & Collection Tube	R1106	10 pack (1 ml fill)
	R1107	50 pack (1 ml fill)
	R1108	10 pack (2 ml fill)
	R1109	50 pack (2 ml fill)

DNA/RNA Shield™ - Fecal Collection Tube (with scoop)

- A 15 ml tube prefilled with 9 ml of DNA/RNA Shield™
- The tube is equipped with a scoop attached to its screwcap for convenient sample collection
- The tube can collect up to 1 g or 1 ml of fecal specimen

Product	Cat. No.	Size
DNA/RNA Shield™ - Fecal Collection Tube	R1101	10 pack

DNA/RNA Shield™ - Lysis Tube (Microbe)

- A 2 ml tube prefilled with 1 ml of DNA/RNA Shield™
- Contains ultra-high density BashingBeads™ for homogenization

Product	Cat. No.	Size
DNA/RNA Shield™ - Lysis Tube (Microbe)	R1103	50 pack
DNA/RNA Shield™ - Lysis Tube (Microbe) with Swab	R1104	50 tubes/50 swabs

Compatible Isolation Kits

Sample Type	DNA	RNA	Total Nucleic Acid
Microbiomic Samples (including feces, soil, water, etc.)	ZymoBIOMICS® DNA Kit	ZymoBIOMICS® RNA Kit	ZymoBIOMICS® DNA/RNA Kit

Hydraflock® is a registered trademark of Puritan Medical

Validated, Accurate DNA/RNA Isolation

Bias in nucleic acid extraction procedures is a major contributor to inaccurate microbial profiling due to inferior cell lysis methods failing to extract DNA uniformly from diverse microbes. Researchers have evaluated many different cell lysis mechanisms including mechanical, chemical, thermal, and enzymatic. Processes that involve chemical or thermal lysis often cause over-representation of easy-to-lyse organisms (e.g. Gram-negative bacteria) due to poor liberation of DNA from hardy, tough-to-lyse organisms (e.g. Gram-positive bacteria and yeast). Enzymatic lysis suffers from its inherent non-stochastic nature. Enzymes make this method particularly vulnerable to bias, especially from highly diverse sample inputs such as soil. Mechanical lysis methodologies (e.g. sonication, blending, liquid nitrogen/mortar and pestle, French pressing, and bead-beating) are considered the best approach due to their stochastic nature, with bead-beating accepted most widely in the community as the gold standard. However, not all methods perform equally, and each can suffer from specific problems such as low yields, excessive nucleic acid shearing, and non-uniform lysis. Even bead-beating methodologies that have not been fully optimized, characterized, and validated for microbiomic applications can be biased. Simply combining an array of cell lysis mechanisms to achieve unbiased lysis does not necessarily reduce bias, despite potentially improving yields. When performing microbial composition profiling, combining more cell lysis mechanisms might only introduce additional types of bias into the process as opposed to reducing the bias overall. Therefore, for community profiling (e.g. microbiomics, metagenomics, etc.) the use of of nucleic and microbial community standards are critical for validation of a method.

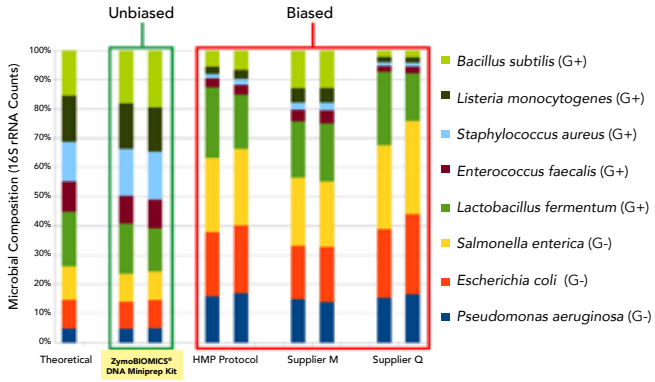
For nucleic acid extraction, Zymo offers unique technologies designed specifically for microbiomics and validated using a mock microbial community standard. ZymoBIOMICS® DNA and RNA Kits were developed to achieve uniform cell lysis from a wide range of organisms (e.g. Gram-negative/positive bacteria, fungus, protozoans, and algae) to ensure accurate microbial profiling. ZymoBIOMICS® DNA (D4300) and RNA (R2001) Kits achieve this by utilizing Zymo's unique bead-beating matrix (featuring ultra-high density mixed beads) and novel chemistry that protects DNA against severe fragmentation during bead-beating. The nucleic acid extraction kits are also equipped with our unique OneStep™ PCR Inhibitor Removal (D6030) spin-column, allowing ultra-pure DNA extraction from a variety of sample types, including feces, saliva, swabs, soil, water, sediments, biofilms, etc. The extracted DNA is ready for any downstream applications, including 16S rRNA gene sequencing and shotgun metagenomic sequencing. Another important feature of this DNA extraction kit is that it is built to have low bioburden, which makes it extremely useful when dealing with samples of low microbial biomass.

ZymoBIOMICS® DNA Kits

For processing feces, soil, water, biofilms, body fluids, etc.

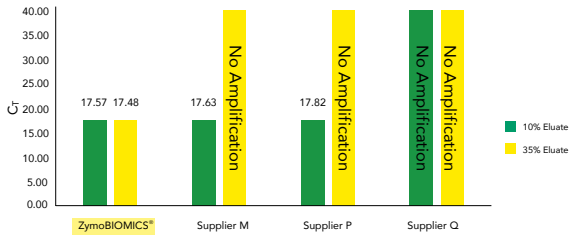
- **Validated Unbiased For Microbiome Measurement:** Unbiased cellular lysis was validated using the ZymoBIOMICS® Microbial Community Standard.
- **Inhibitor-Free DNA From Any Sample:** Isolate ultra-pure DNA from any sample that is ready for any downstream application.
- **Certified Low Bioburden:** Boost your detection limit for low-abundance microbes.
- **Simple Workflow:** Simply bead-beat sample, purify via spin-column, and filter to remove PCR Inhibitors. No precipitations or lengthy incubations!

Accurate Community Profiling



The ZymoBIOMICS® DNA Miniprep Kit provides accurate representation of the organisms extracted from the ZymoBIOMICS® Microbial Community Standard.

Ultra-Pure DNA from Inhibitor-Rich Samples



The ZymoBIOMICS® DNA Miniprep Kit provides inhibitor-free DNA even when challenged with extremely inhibitor-rich samples. Real-time PCR was used to evaluate eluates recovered using the ZymoBIOMICS® DNA Miniprep Kit, or Suppliers M, P, and Q. Reaction volumes consisted of either 10% or 35% of the eluate from each kit to detect the presence of PCR inhibitors. Each reaction contained 25 ng of *Brettanomyces* DNA. Delayed and/or no amplification indicates PCR inhibition from inefficient inhibitor removal.

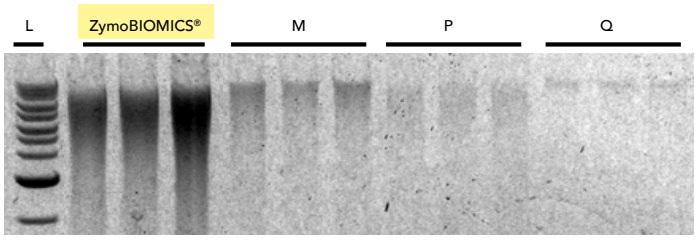
Streamlined Workflow

Accurate lysis using ZR BashingBead™ Lysis Tubes

Superior yields and integrity with Zymo-Spin™ technology

PCR inhibitor removal eliminates polyphenolics, humic/fulvic acid, and melanin

Superior Yields and Purity



The ZymoBIOMICS® DNA Miniprep Kit provides superior yields when compared to Suppliers M, P, and Q.

Learn more and view additional formats at www.zymoresearch.com/zymobiomics

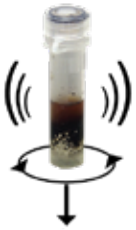
Product	Cat. No.	Size
ZymoBIOMICS® DNA Miniprep Kit	D4300	50 preps.
	D4300T	10 preps.
ZymoBIOMICS® DNA Microprep Kit	D4301	50 preps.
ZymoBIOMICS® 96 DNA Kit (includes ZR BashingBead™ Lysis Rack)	D4303	2 x 96 preps.
ZymoBIOMICS® 96 DNA Kit (includes ZR BashingBead™ Lysis Tubes)	D4309	2 x 96 preps.

ZymoBIOMICS® RNA Miniprep Kit

For processing feces, soil, water, biofilms, body fluids, etc.

- **Validated Unbiased Lysis for Microbiome Profiling:** Unbiased cellular lysis was validated using the ZymoBIOMICS® Microbial Community Standard.
- **Inhibitor-free RNA from Any Sample:** Isolate ultra-pure RNA from any sample that is ready for any downstream application.
- **Simple Workflow:** Simply bead bash sample, purify via spin-column, and filter to remove PCR Inhibitors. No precipitations or lengthy incubations!
- **RNA is free of DNA Contamination:** DNase I included.

Streamlined Workflow



Accurate lysis using DNA/RNA Shield™ Lysis Tube (Microbe)

Purification

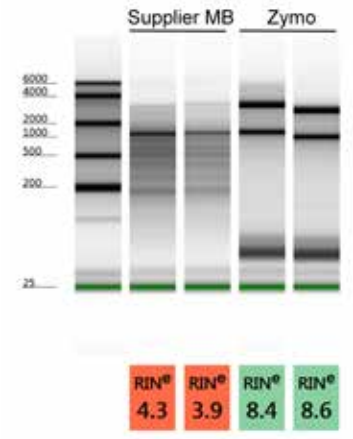
Spin
Wash
Elute

DNA-free RNA

Complete PCR inhibitor removal using Zymo-Spin™ IV-HRC Spin Filters

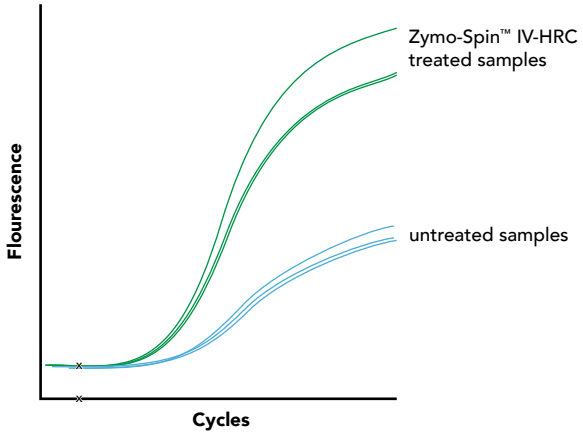
Ultra-pure Total RNA

Ultra-pure RNA from Inhibitor-rich Samples



Human stool RNA isolated with the ZymoBIOMICS® RNA Miniprep Kit is higher quality (right) compared to Supplier MB procedures (left). Quality assessed by Agilent 2200 TapeStation®.

Ultra-pure RNA from Inhibitor-rich Samples



Total RNA isolated from human stool with or without inclusion of the Zymo-Spin™ IV-HRC Spin Filter during the ZymoBIOMICS® RNA Miniprep Kit protocol. Earlier amplification cycles indicate complete removal of PCR inhibitors.

Product	Cat. No.	Size
ZymoBIOMICS® RNA Miniprep Kit	R2001	50 preps.

Learn more and view additional formats at www.zymoresearch.com/zymobiomics

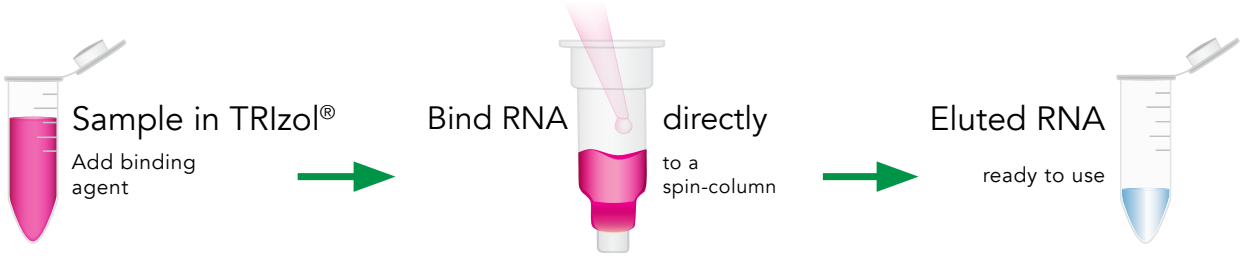
Direct-zol™ RNA Kits

Isolation of RNA from sample in TRIzol

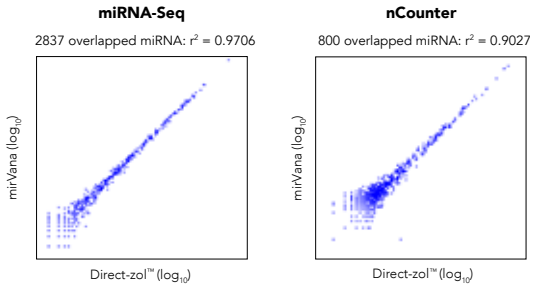
- **TRIzol® to RNA in 7 minutes:** Purify RNA directly from TRIzol® with a spin-column. No phase separation. No precipitation.
- **NGS Ready (DNA-Free) RNA:** Ultra-pure RNA is free of phenol and DNA contamination.
- **Validated Unbiased Lysis for Microbiome Profiling:** Unbiased lysis of microbes is achieved using novel BashingBead™ Technology.

Innovation. Pure & Simple.™

TRIzol® to RNA in only 7 minutes!

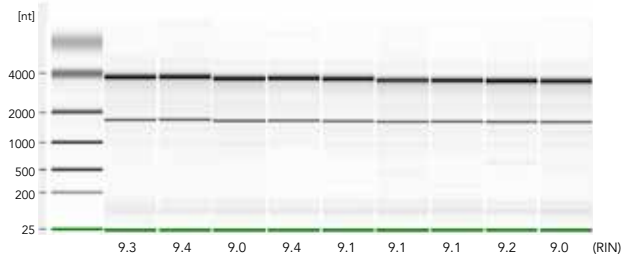


Efficient Small RNA Recovery



The data shows RNA purified from TRIzol® samples using the Direct-zol™ RNA Miniprep compared to an unbiased method (mirVana™, Ambion). Micro-RNA analysis was performed using miRNA-Seq (MiSeq®, Illumina) and a direct hybridization assay (nCounter®, Nanostring).

High-Quality RNA



High RNA integrity number (RIN > 9; Bioanalyzer®, Aligent Technologies Inc.) indicates high-quality RNA was purified from human epithelial cells using the Direct-zol™ RNA Kit.

Accommodates any Sample

in TRIzol®, TRI Reagent®, etc.

Learn more and view additional Direct-zol™ Kit formats at http://www.zymoresearch.com/rna/rna-purification

Product	Cat. No.	Size
Direct-zol™ RNA Microprep Kit	R2060, R2061*	50 preps.
	R2062, R2063*	200 preps.
Direct-zol™ RNA Miniprep Kit	R2050, R2051*	50 preps.
	R2052, R2053*	200 preps.
Direct-zol™ RNA Miniprep Plus Kit	R2070T	10 preps.
	R2070, R2071*	50 preps.
	R2072, R2073*	200 preps.
Direct-zol™ 96 RNA Kit	R2054, R2055*	2 x 96 preps.
	R2056, R2057*	4 x 96 preps.

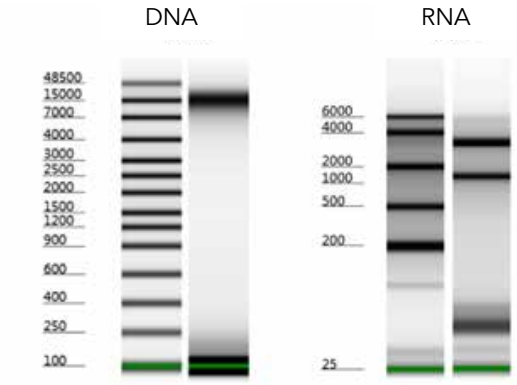
*Supplied with TRI Reagent®. MagBead formats also available. Beads sold separately.

ZymoBIOMICS® DNA/RNA Miniprep Kit

For processing feces, soil, water, biofilms, body fluids, etc.

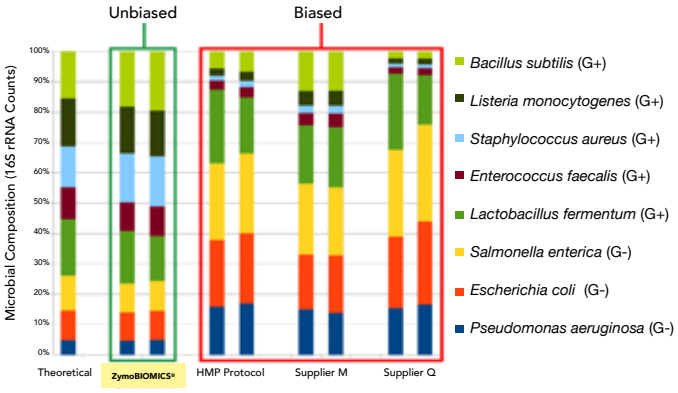
- **Validated Unbiased for Microbiome Profiling:** Unbiased cellular lysis was validated using the ZymoBIOMICS® Community Standard.
- **Inhibitor-free DNA/RNA from Any Sample:** Isolate ultra-pure DNA and RNA from any sample that is ready for any downstream application.
- **Simple Workflow:** Simply, bead bash sample, purify via spin-column, and filter to remove PCR Inhibitors. No precipitations or lengthy incubations!

Superior Yields



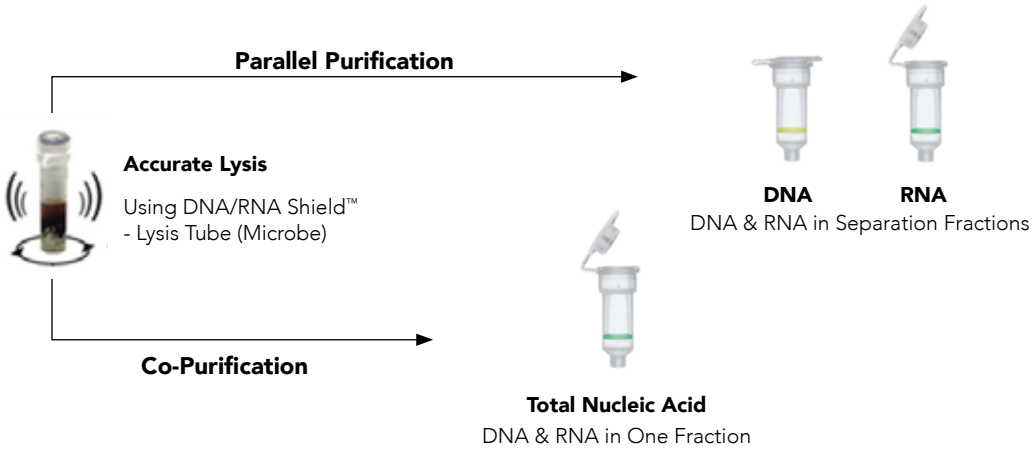
Human stool genomic DNA and total RNA isolated with the ZymoBIOMICS® DNA/RNA Miniprep Kit is highly intact. Quality assessed by Agilent 2200 TapeStation®.

Accurate Community Profiling



The ZymoBIOMICS® DNA/RNA Miniprep Kit provides accurate representation of the organisms extracted from the ZymoBIOMICS® Microbial Community Standard.

Streamlined Workflow



Product	Cat. No.	Size
ZymoBIOMICS® DNA/RNA Miniprep Kit	R2002	50 preps.

Learn more and view additional formats at www.zymoresearch.com/zymobiomics

Tips and Tricks for Processing Difficult Samples with the ZymoBIOMICS® workflow

The ZymoBIOMICS® DNA product line is capable of handling samples from a variety of sources. Below are tips & tricks for dealing with difficult samples.

DNA Viruses

For viruses enveloped in a nuclear envelope, we recommend adding a Proteinase K digestion after bead-beating to ensure efficient lysis of the nuclear envelope. Proteinase K digestions can be added as part of the ZymoBIOMICS® DNA Kit protocol to ensure effective isolation of DNA from enveloped viruses.

Cheese and Protein Rich Biofluids (e.g. Milk, Sputum, Saliva, Spinal Fluid, and Serum)

Samples such as cheese or sputum can be rich in proteins. An additional Proteinase K digestion after bead-beating is recommended to substantially improve purification efficiency with increased yield and purity.

Tissue and Insect Samples

Microbes can be present in tissue and/or insect samples (e.g. gut microbiome), and typically require additional pre-processing to release the microbes from the tissue. Insects will require mechanical homogenization while mammalian tissues can be digested by proteinases. To ensure complete lysis of these samples, pre-process with an enzymatic digestion (e.g. Proteinase K) or mechanical homogenization (e.g. mortar and pestle or bead-beating with ZR BashingBead™ Lysis Tubes (2.0 mm, Cat. No. S6003-50)). After the tissue is homogenized the sample can be processed using ZymoBIOMICS® DNA Kits or other commercially available products.

Plant Tissue (Leaves and Other Plant Material)

Depending on whether processing just the plant surface or the entire sample, there are different pre-processing steps. A major issue in working with microbes from plants is that plant derived DNA can overwhelm the sequencing reads. To prevent the host plant DNA from overwhelming the microbial DNA, users would need to either forgo processing the plant tissue or use a targeted approach in the downstream analysis (e.g. 16S rRNA gene seq.).

- Surface microbes: Users can exclude processing the host plant tissue and instead remove the surface microbes by washing or sonicating the tissue into an isotonic solution. Alterna-

tively, the ZymoBIOMICS® Lysis Solution or DNA/RNA Shield™ can be used to release the microbes from the surface. Subsequently the solution can be processed with the ZymoBIOMICS® DNA Miniprep Kit (D4300) directly or other commercially available products.

- Total Sample: The host plant's mitochondrial and chloroplast DNA will overwhelm the bacterial 16S rRNA gene, so a more targeted approach in quantifying the bacterial DNA is needed. Users should process the plant tissue using mechanical methods such as grinding with mortar and pestle or bead-beating with the ZR BashingBead™ Lysis Tubes (2.0 mm, Cat. No. S6003-50). The lysate can then be processed with the ZymoBIOMICS® DNA Kits or other commercially available products for total DNA isolation, including plant and bacterial DNA.
- Plant roots can be processed directly with the ZymoBIOMICS® DNA Kits after cutting the roots into small pieces. We recommend using a low-speed bead-beating device to avoid host plant tissue contamination.

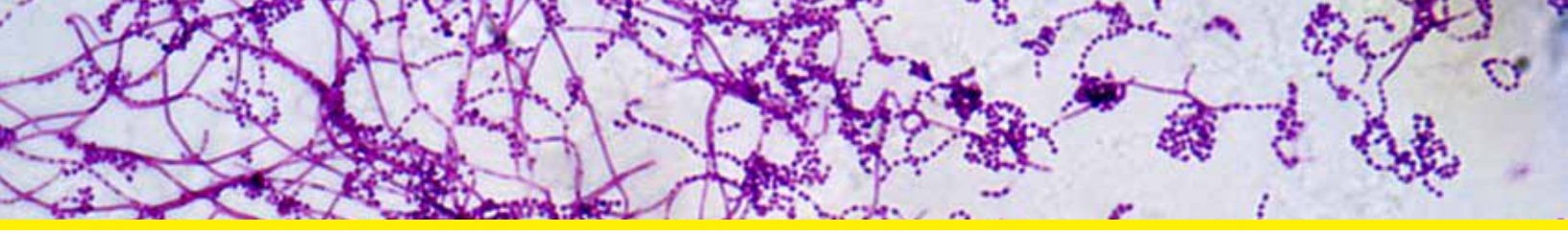
Water, Air, and Large Soil Samples

For samples with low biomass such as water, air, and some soil samples, we recommend concentrating the microbes onto a non-silica based filter. Cut the filter into small pieces, add to ZR BashingBead™ tubes (S6012-50), and then process the filter pieces directly with the ZymoBIOMICS® DNA Kits or other commercially available products.

Urine

Microbial cells from urine samples can be processed in multiple ways. Users can centrifuge at high speeds to pellet down the microbial cells, while lysing the host cells in the urine supernatant. Simply remove the supernatant so that the microbial cells remain. Microbes can be processed immediately using the ZymoBIOMICS® DNA Miniprep Kit (D4300), or other commercially available products or frozen for later processing.

Alternatively, if the microbes cannot be pelleted immediately, Zymo Research's Urine Conditioning Buffer (D3061-1-140) can stabilize urine at room temperature for up to 1 month. When samples are ready to be processed, centrifuge urine at a high speed to pellet down microbial cells and discard urine supernatant. Process the microbial cell pellet with the ZymoBIOMICS® DNA Kits.



Application Note:

An Optimized Workflow for DNA Isolation from Spores

Bacterial spores can be exceptionally tough, remaining viable even after exposure to extreme conditions due to their resistance to enzymatic lysis, desiccation, radiation, high temperatures, and chemical treatments such as disinfectants and denaturants.

When thriving, vegetative cells are endangered by harsh conditions and nutritional restrictions, bacteria can form endospores, to survive the extreme stress. The outer most coating of the endospore is a proteinous layer that provides substantial enzymatic and chemical resistance. Beneath this layer is the peptidoglycan cell wall called the cortex, followed by a germ cell wall, and an inner membrane which further provides a physical and chemical barrier. Within the inner membrane is the core which contains the DNA/Ribosomes and additional protective elements including dipicolinic acid and proteins that further protect the DNA from radiation and chemical damages.

Due to the extreme hardiness of spores, they are highly resistant to lysis, which can lead to inefficient lysis and consequently misrepresentation of the microbial community and very low (or no) DNA recovery. Heat treatments tend to be ineffective at liberating DNA from endospores. Enzymatic methods are dependent on an organism's lytic susceptibility and spores tend to be highly resistant even if the vegetative bacterium was susceptible, thus generating bias in DNA recovery and community profiling. Mechanical lysis, which has been identified widely as the most effective method to isolate DNA for community profiling, was used by Zymo Research to examine the lysis efficiency of bead-beating bacterial endospores.

Spore Induction and Indirect Quantification

Bacterial spore formation was induced by inoculating *Bacillus subtilis* cells growth/sporulating medium with *Bacillus subtilis* and incubating for several days. Successful bacterial spore formation was determined by using Schaeffer and Fulton Spore Stain Kit (Sigma Aldrich) and viewed by microscopy. Bacterial spores were then concentrated and frozen in a PBS/glycerol solution. Cell counting was not possible due to the extremely small size of the bacterial spores, so indirect counting was performed via plating serial dilutions of bacterial spore suspension.

High-Quality DNA

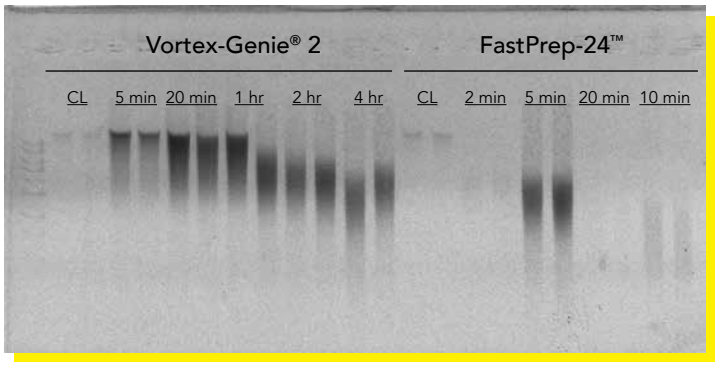


Figure 1. *B. subtilis* spores were homogenized in DNA/RNA Shield™ Lysis Tubes (Microbe) containing 0.1 & 0.5 mm beads paired with the ZymoBIOMICS® DNA Miniprep Kit. Both the Vortex-Genie® 2 (low-speed) and FastPrep-24™ (high-speed) were capable of successfully recovering DNA when utilized with the ZymoBIOMICS® DNA Miniprep Kit (D4300). CL = chemical lysis was applied.

Heat Inactivation Efficiency

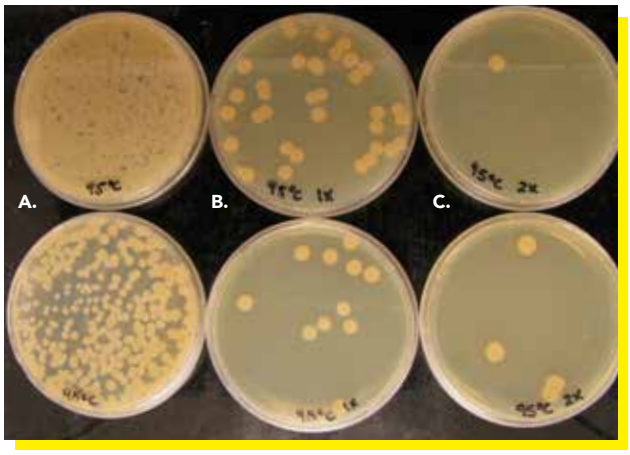


Figure 2. *B. subtilis* spores were incubated at 95°C for one hour to determine effciency of heat inactivation. Spores were plated on BHI media and incubated overnight; heat treatment of spores resulted in a 10^6 decrease in cell growth. (A) Direct plating of cell suspension (B) tenfold dilution of cell suspension (C) 100-fold dilution of cell suspension.

Methods Surveyed

Chemical Lysis: Chemical lysis was attempted using several common commercially available lysis buffers (e.g. Zymo Research Genomic Lysis Buffer, Qiagen Buffer AL, and Qiagen Buffer AVL) which led to no significant DNA recovery, as anticipated (data not shown). This does not reflect a comprehensive review of chemicals with the potential to lyse endospores, it is just an evaluation of some of the most used cellular lysis buffers.

Mechanical Lysis: The ZymoBIOMICS® DNA Miniprep Kit which contains a mixture of high density BashingBeads™ (0.1 and 0.5 mm) was evaluated in the context of two different types of homogenization systems, classified as high speed and low speed. It was found that the ZymoBIOMICS® DNA Miniprep kit effectively lysed *Bacillus subtilis* endospores using a low speed homogenizer after 20 minutes (3,200 rpm; Disruptor Genie 2) and using a high speed homogenizer after 5 minutes (1 minute interval at 6.5 m/s with 5 minutes rest; FastPrep-24™) (Figure 1). Increased bead-beating duration beyond 20 minutes on the low-speed device resulted in negligible changes in yield and minimal DNA loss/shearing. However, increased bead-beating duration beyond 5 minutes on the high-speed device resulted in substantial DNA degradation and loss of DNA. It is of note, that the high-speed device generated significant heat within the ZR BashingBead™ Lysis Tubes, which may have been a cause of the substantial DNA degradation.

Thermal Lysis: *Bacillus subtilis* spores were treated at room temperature (RT), 55 ˚C, 75 ˚C, and 95 ˚C for 1 hour. In all instances no substantial quantities of DNA were recovered in the purification, however spores treated at 95 ˚C for 1 hour experienced 10^6 decrease in cell growth when plated, in comparison to other spore treatments (Figure 2).

Mechanical Lysis Comparison

The ZymoBIOMICS® DNA Miniprep was compared to the DNeasy PowerSoil (Qiagen) kit to evaluate the lysis efficiency and recovery of DNA from solutions containing 6.0 x 10^8 CFU of *B. subtilis* endospores. The ZymoBIOMICS® DNA Miniprep was consistently able to lyse the *B. subtilis* endospores and recover the DNA, while the DNeasy PowerSoil kit was incapable of recovering measurable quantities of DNA (Figure 3).

Conclusion

The ZymoBIOMICS® DNA Miniprep's high density bead formulation (0.1 & 0.5 mm) was shown to be effective in lysing *B. subtilis* endospores with high and low speed disruptors indicating the versatility of the kit. Furthermore, lysis efficiency was determined to be greater than 99% as determined by plating spore lysates. The number of viable colony forming units plated after lysis was minimal and equated to picograms of unrecoverable DNA, suggesting that lysis using high density BashingBeads™ (0.1 and 0.5 mm) was nearly 100% efficient.

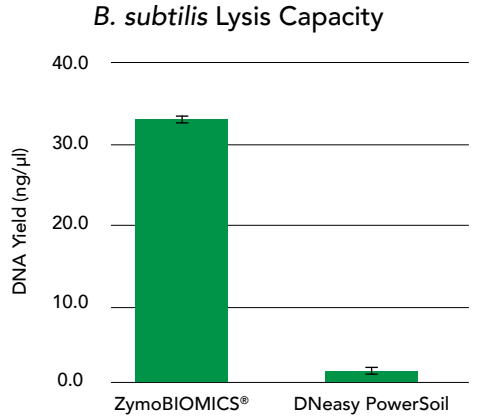


Figure 3. DNA extractions were performed using ZymoBIOMICS® DNA Miniprep and DNeasy PowerSoil with 6 x10^8 *B. subtilis*. DNeasy PowerSoil was unable to recover quantifiable amounts of DNA, while the ZymoBIOMICS® DNA Miniprep Kit was capable of recovering 33 ng/µl in a 15 µl elution. Extractions were performed in triplicates and quantified by Nanodrop.

Figure 4. *B. subtilis* spores were homogenized using ZymoBIOMICS® DNA Miniprep Kit on a high-speed instrument. 99% lysis efficiency was determined by plating spore lysate on BHI media and grown overnight. (A) Lysis Solution negative control, no bacterial spore input. (B) *B. subtilis* spores lysed with high speed instrument for 5 minutes at 6.5 m/s (1 minute interval, 5 minutes rest). (C) Tenfold dilution of *B. subtilis* spores.

Application Note:

Automation of the ZymoBIOMICS® 96 MagBead DNA Kits

in Collaboration with Hamilton Robotics

Introduction

The ZymoBIOMICS® 96 MagBead DNA Kit is designed for purifying DNA from a wide array of sample inputs (e.g. feces, soil, water, etc.) that are immediately ready for microbiome or metagenome analyses. The ZymoBIOMICS® innovative lysis system eliminates bias associated with unequal lysis efficiencies of different organisms (e.g., Gram-negative/positive bacteria, fungi, protozoans, and algae), making it ideal for microbiomics studies. Unbiased mechanical lysis of tough microbes is achieved by bead-beating with Zymo Research's proprietary, ultra-high density BashingBeads™ and validated using the ZymoBIOMICS® Microbial Community Standard. The automation friendly workflow integrates the PCR inhibitor removal technology directly into the purification system, removing the need for complex precipitation steps commonly used in other methodologies. The ZymoBIOMICS® 96 MagBead DNA Kit features a simple bind, wash, & elute procedure that is unmatched in providing ultra-pure DNA that is free of PCR inhibitors (e.g. polyphenols, humic acids) in as little as 90 minutes for 96 samples. Purified DNA is ideal for all downstream applications including PCR, arrays, 16S rRNA gene sequencing, and shotgun sequencing.

Materials and Methods

Seventy-two samples of various origin detailed in Table 1 were homogenized using a ZR BashingBead™ Lysis Rack placed on an MP-Biomedicals FastPrep-96™ bead mill. DNA was then extracted from the samples with ZymoBIOMICS® 96 MagBead DNA Kit (Cat. No. D4302) using the extraction workflow shown in Figure 1. All of the samples were processed using the automated Hamilton Microlab® STAR™ liquid handler. In a separate plate, ninety-six samples of 20 mg feces was processed on the Hamilton Microlab® STAR™ in tandem with sixteen samples of 20 mg feces processed manually.

Table 1: Sample input types and amounts used for validation of the ZymoBIOMICS® 96 MagBead DNA Kit workflow, n=8 per sample type

Sample Type	Input Amount
Soil	200 mg
Fecal	80 mg
Blood	200 µl
Listeria Monocytogenes Culture	2 x 10^8 cells
Saccharomyces Cerevisiea	2 x 10^7 cells
Plant	50 mg
Filtered Water	200 µl stream water with 2 x 10^6 *E. coli* cells added
Saliva	200 µl

Figure 1. Workflow for the ZymoBIOMICS® 96 MagBead DNA Kit.

The Microlab® STAR™ used was configured with 8 x 5 ml channels, Autoload , CO-RE 96 MPH, CO-RE Grips, Hamilton Heater Shaker, 96-well Magnetic Stand, as well as required tips and reagent carriers.

The DNA concentration was analyzed using Thermo Scientific NanoDrop 2000 UV-Vis Spectrophotometer and gel electrophoresis using a 1% agarose gel.

Automation Equipment

- Hamilton Microlab® STAR™, 8 channels, Autoload option, CO-RE 96 Probe Head, CO-RE Grip
- Hamilton Heater Shaker (HHS)
- 96-well Magnetic Stand
- All required tip and reagent carriers

Results and Discussion

Consistent Yields and High Quality

DNA concentration and total DNA yields from replicate samples were compared between eight automated processed samples of a variety of sample types. The results are shown in Figures 2 and 3. Results indicate that the kit is capable of purifying DNA from a variety of sample types reliably and consistently.

Conclusions

Samples processed using the ZymoBIOMICS® 96 MagBead DNA Kit procedure with the Hamilton Microlab® STAR™ are capable of being purified with consistency and reliability. This is shown by the successful recovery and excellent reproducibility and consistency in concentration and yield. This innovative method yields high-quality total DNA from microbial communities from a wide array of sample sources providing an efficient solution for reliable high-throughput hands-free DNA purification.

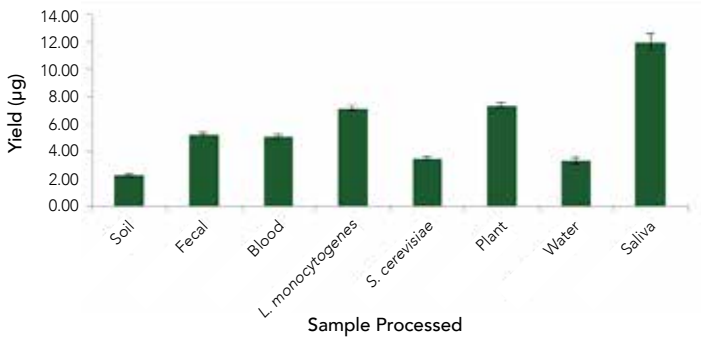


Figure 2. DNA yield recovered after processing each sample type on the Hamilton Microlab® STAR™ liquid handling system with the ZymoBIOMICS® 96 MagBead DNA Kit. (n=8)

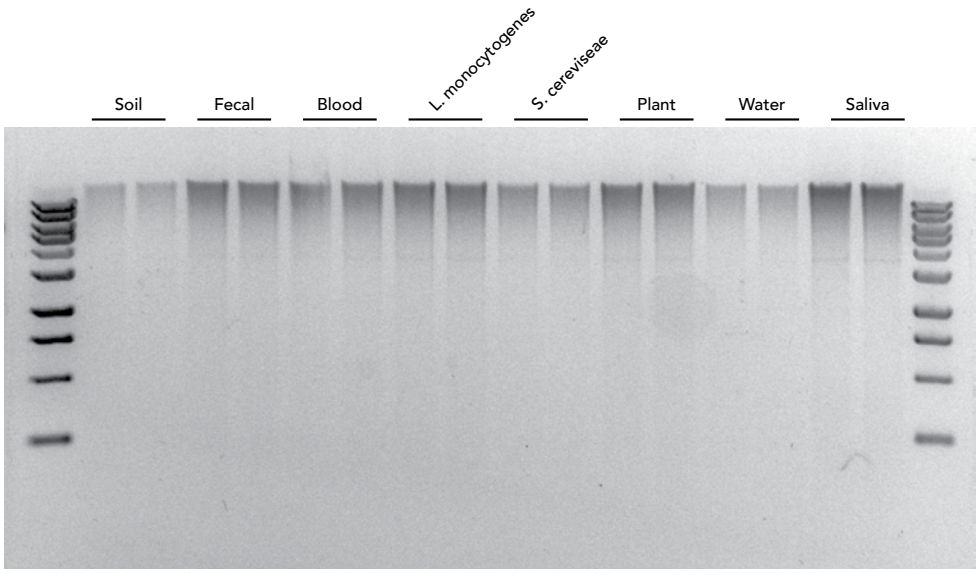


Figure 3. Agarose gel electrophoresis images recovered after processing soil, fecal, blood, *L. monocytogenes*, *S. cereviseae*, plant, water, and saliva samples on the Hamilton Microlab™ STAR liquid handling system with the ZymoBIOMICS® 96 Magbead DNA Kit.

Overcoming Challenges with Automating Microbiomic Workflows

The time consuming nature of repetitive, simple tasks have irked mankind since the dawn of time. Since the advent of the personal computer, we have witnessed the start of an era of rapid technological advancement. From phones to food service to automobiles, every aspect of our lives is becoming automated in ways that make our world a more efficient, productive, and creative environment.

These advances have made their way into the lab. DNA extraction and purification can now be as simple as the cliché, but literal, push of a button. The switch from manual to automated extraction and purification techniques is propelling our ability to produce meaningful and consistent data. Automated methods have enabled the discoveries of trends in larger sample sets, which was previously impossible.

With the recent exponential growth in the field of microbiomics, the demand for higher throughput processing has never been greater. While laboratory automation technology has improved greatly in only a few short years, there are still many challenges that need to be addressed before automation can become a universally viable alternative for DNA extraction. For automation to become the ideal alternative, systems would need to produce consistent, reliable results at a high enough throughput to maintain cost effectiveness. The field of microbiomics makes the automation challenge even more complicated with sample types that are difficult to process. Typical microbial community samples, such as feces and soil, have a high propensity for inhibitory compounds. When analyzing microbial communities, it is essential to have a bias-free purification system and workflow, ensuring an accurate "snapshot" of the microbial community. Before automated workflows dedicated to microbiomics sample processing become reality, these unique challenges must be addressed.

Consistent & Reliable Results

One of the most desirable traits in automated sample processing is the ability to purify DNA with consistency and reliability. When processing a large number of samples, it is paramount that each sample produces consistent yields in order to be easily funneled into downstream applications for further analysis. In addition, maintaining sample purity is vital, as salt contamination can inhibit PCR and prevent accurate quantification and analysis for sensitive downstream applications. Often silica-coated magnetic beads are utilized in sample purification on high-throughput liquid handlers, and it

is commonly seen that this purification technique can produce samples of lower yield and purity than their spin-column counterparts.

At Zymo Research, we've recognized the need for pure, high-quality DNA purification for microbiomics based applications, which led us to develop a rigorous magnetic bead wash system for the ZymoBIOMICS® 96 MagBead DNA Kit (D4302) that provides consistently pure samples, with reliable yields every time. This system has been uniquely designed using proprietary Zymo Research technology to quickly process samples without sacrificing DNA quality and has been validated for sensitive downstream applications such as PCR and Next-Generation Sequencing. The MagBead technology will consistently extract microbial DNA from a wide array of sample types, including feces, soil, biofilms, biological fluids, and tissues with A260/280 and A260/230 ≥ 1.80.

Inhibitory Compounds

The study of microbiomics provides unique challenges not present in other types of sample processing and analysis. Due to the inhibitor-rich samples typically studied in microbiomics, such as feces and soil, there is a need for inhibitor removal during the DNA purification process. These inhibitors - including humic acid, tannic acid, fulvic acid, heme, and bile salts - can significantly affect downstream applications. Any inhibitors present in the sample can affect the ability to accurately portray the microbial community being studied by biasing or completely inhibiting PCR. Additionally, many available methods of purification from these sample types require lengthy and complex pre-processing steps to precipitate or otherwise remove inhibitors from their samples prior to introduction to the automated sample processing platform.

Understanding the needs of the microbiomics community, Zymo Research has developed an innovative buffer system for the ZymoBIOMICS® 96 MagBead DNA Kit (D4302) with built-in inhibitor removal technology, removing the need for off-deck inhibitor removal, precipitation, and centrifugation steps. These technologies have enabled the first fully automatable purification system, streamlining the process for a more reliable, inhibitor-free protocol.

Bias-free Purification

To provide the most accurate portrayal of a microbial community as possible, it is necessary to ensure the entire process from purification to sequencing is bias-free. During purification, bias can be introduced from non-uniform lysis and cross contamination of organisms from outside sources. Of particular importance to this process is the over-representation of Gram-negative bacterial strains in the processing of microbial communities, as they are often completely lysed, while Gram-positive strains show more of a resistance to the homogenization techniques regularly used in the industry.

To combat these systemic problems with microbiomics studies, Zymo Research has released a new product, the ZymoBIOMICS® Microbial Community Standards (D6300), which contain a mock microbial community consisting of bacterial and fungal strains in known quantities. These organisms of differing resistance to mechanical lysis, allow us to evaluate the DNA extraction pipeline for inefficiencies and bias. The ZymoBIOMICS® 96 MagBead DNA Kit (D4302) has been developed and evaluated with the community standards as a benchmark, and has been able to address the problems associated with bias by utilizing our proven ZR BashingBead™ Lysis Tube system (S6012-50). This Lysis Tube system provides unbiased mechanical homogenization of microbial communities to provide the most accurate portrayal of that community. Coupled with Zymo Research's DNA/RNA Shield™ (R1100-50) for sample preservation and the inherent low bioburden capabilities of the kit, the ZymoBIOMICS® pipeline is capable of addressing all the needs required in any microbiomics laboratory.

High-throughput

Lower costs for high-throughput sample processing is a major reason why labs are making the switch to automated liquid handling systems. When purchasing a liquid handler or other robotic sample processing system, the main point of focus is finding a system that provides the largest throughput per dollar spent. However, one of the largest, and often overlooked, bottlenecks in automated sample processing is off-deck handling time, which is necessary to prepare samples for automated processing.

Off-deck handling time means more hands-on work. This introduces a greater chance of cross-contamination due to handling errors, and slows down throughput significantly, leading to greater costs across the board. In order to address these issues, we developed our protocols to maximize throughput and efficiency. We have reduced off-deck handling time of the ZymoBIOMICS® 96 MagBead DNA Kit to the bare minimum, with as little as 15 minutes of pre-processing required prior to the automated portion of the protocol. Our protocols are the fastest in the industry, with scripts capable of processing up to 96 samples every 90 minutes. Furthermore, Zymo Research's team of technical support staff who are dedicated to providing scripting and high-throughput support to ensure you are provided with the fastest, most efficient automated setup for your lab's processing needs.

To stay on the forefront of the rapidly advancing field of automation, Zymo Research is proud to announce an ongoing collaboration with Hamilton Robotics® in automating and supporting the ZymoBIOMICS® 96 MagBead DNA Kit. We have full scripting support and assistance available for our kits from both companies and the kit has been specifically designed to be fully compatible with the Hamilton Microlab® STAR™ line of automated liquid handlers. Our team has collaborated in validating and evaluating the ZymoBIOMICS® 96 MagBead DNA Kit using the Hamilton Microlab® STAR™ system and will work closely with Hamilton to ensure that you are provided with the best solution for your lab's specific needs.

Conclusion

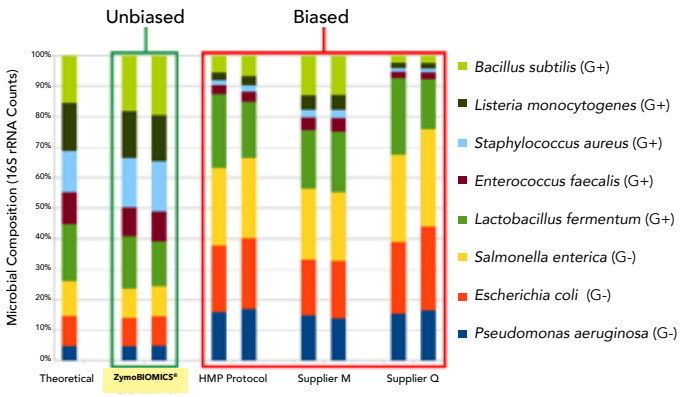
With the ZymoBIOMICS® line of products, Zymo Research has provided a suite of easy-to-use products which provide a complete pipeline from start-to-finish for all your microbiome related needs. The newest addition to the family of ZymoBIOMICS® products, the ZymoBIOMICS® 96 MagBead DNA Kit, continues to further expand the capabilities of this line of products by providing the same principles of unbiased sample lysis, inhibitor-free DNA purification, and low bioburden buffer systems in a new, high throughput, automatable format. In addition, this kit also addresses all of the challenges listed above, providing consistently pure, scalable DNA purification in a high throughput format that is both cost effective and easy to use. With the continuing growth of automation in the lab, Zymo Research continues to develop cutting edge DNA purification technologies that are capable of meeting the demands of the ever-changing workflows of the modern day laboratory.

ZymoBIOMICS® 96 MagBead DNA Kits

For processing feces, soil, water, biofilms, body fluids, etc.

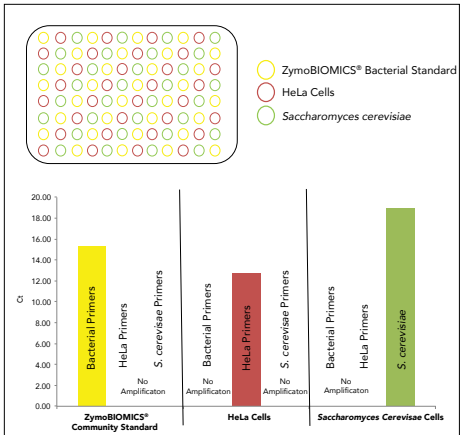
- **Validated Unbiased for Microbiome Profiling:** Unbiased cellular lysis was validated using the ZymoBIOMICS® Community Standard.
- **Inhibitor-free DNA from Any Sample:** Isolate ultra-pure DNA from any sample that is ready for any downstream application.
- **Certified Low Bioburden:** Boost your detection limit for low-abundance microbes.
- **Fully Automatable Workflow:** 96 samples can be processed in 90 minutes. No precipitation. No centrifugation. No lengthy incubations.

Accurate Profiling



The ZymoBIOMICS® 96 MagBead Kit provides accurate representation of the organisms extracted from the ZymoBIOMICS® Microbial Community Standard.

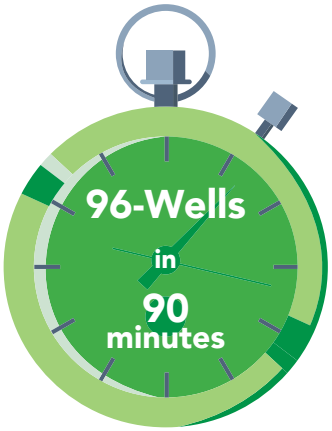
No Cross-Contamination



The ZymoBIOMICS® 96 MagBead DNA Kit provides cross-contamination free samples across a standard 96-well plate purification performed on a liquid handler. Samples were evaluated using quantitative PCR with primer sets targeted at the bacterial 16S rRNA gene, the human LINE gene, and the fungal ITS gene. PCR was performed in technical duplicates.

No Precipitation or Centrifugation Required

Bias-free Lysis → Quick Bind, Wash, Elute Workflow → Superior Yields and Integrity of Ultra-Pure DNA



Product	Cat. No.	Size
ZymoBIOMICS® 96 MagBead DNA Kit (includes ZR BashingBead™ Lysis Rack)	D4302	2 x 96 preps.
ZymoBIOMICS® 96 MagBead DNA Kit (Lysis Matrix Not Included)	D4306	2 x 96 preps.
ZymoBIOMICS® 96 MagBead DNA Kit (includes ZR BashingBead™ Lysis Tubes)	D4308	2 x 96 preps.

Learn more and view additional formats at www.zymoresearch.com/zymobiomics

Depletion of Host DNA To Optimize Results of Microbiome Metagenomics

Next-Generation sequencing, once considered as a luxury service that cost hundreds of thousands of dollars and countless hours of time, is rapidly becoming the go-to technology for sample identification thanks to incredible improvements in cost and efficiency. As the field of microbiomics continues to grow, so too has the appeal of whole genome sequencing (WGS). Because of its increased accessibility, comprehensive coverage of organisms present in a sample, and ability to identify novel genomes, WGS is now a realistic and desirable option for microbial sample identification.

Challenges

An increasingly important application of microbiomics is how the microbes living in and on humans affect us, for better or worse. A major challenge to assessing the human microbiome with shotgun sequencing is the presence of human host DNA that "contaminates" the sample. In clinical samples such as skin swabs or biological fluids, the results of WGS are dominated by sequences from the human genome. Even though the expense of sequencing has decreased significantly, this type of host contamination negates some of this benefit by diminishing the amount of relevant data produced. For this reason, a method to remove the host DNA prior to sequencing is essential.

How to Deplete Host DNA

To overcome the challenge of contaminating host nucleic acids, Zymo Research has enhanced the DNA isolation process with the HostZERO™ Microbial DNA Isolation Kit (D4310). This host depletion kit uses a novel method to reduce the amount of contaminating host DNA by selectively lysing the human cells and degrading this DNA prior to total DNA purification. Paired with the most-accurate purification technology available, the HostZERO™ Microbial DNA Isolation Kit allows for the exclusive capture of DNA from living microbial cells in a biological or environmental sample. This new technology is able to reduce the presence of human DNA in a saliva sample from 64.5% in the untreated sample to just 0.8% in the treated sample (Figure 1). At the same time, bias is minimized by linking the depletion kit to the purification kit (Figure 2). By removing the presence of host DNA and reducing bias in purification, the HostZERO™ Microbial DNA Isolation Kit produces the highest-quality and highest-volume data for microbial samples.

Conclusion

To achieve the highest volume of pertinent microbial metagenomic data, steps must be taken prior to sample processing to remove the host DNA present in the sample. The HostZERO™ Microbial DNA Isolation Kit aims to increase the number of sequences identified to microbial DNA rather than host DNA while maintaining the integrity of the sample composition.

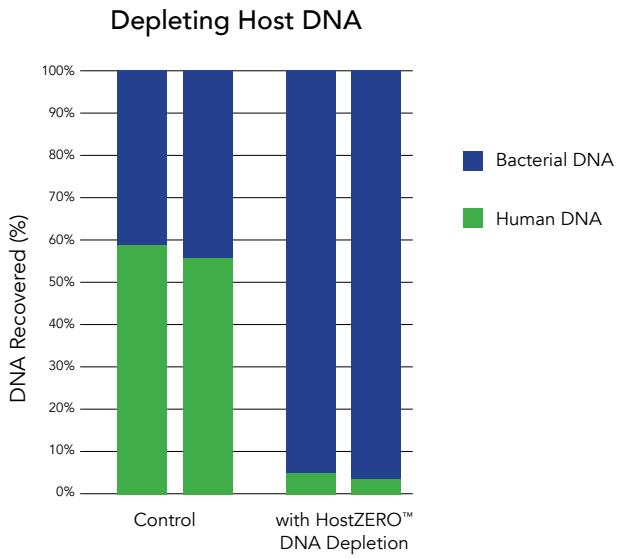


Figure 1. Isolating microbial DNA and depleting host DNA of a saliva sample with the HostZERO™ Microbial DNA Isolation Kit. One human saliva sample was processed using either the control method the ZymoBIOMICS® DNA Microprep Kit, which extracts total DNA from the sample without host DNA depletion or the HostZERO™ Microbial DNA Isolation Kit. The composition of the purified DNA from saliva in terms of bacterial and human DNA abundance. The abundance was determined by quantitative PCR.

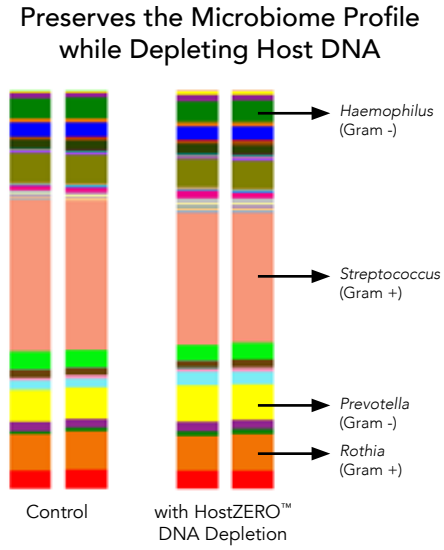


Figure 2. Isolating microbial DNA and depleting host DNA of a saliva sample with the HostZERO™ Microbial DNA Isolation Kit. One human saliva sample was processed using either the control method the ZymoBIOMICS® DNA Microprep Kit, which extracts total DNA from the sample without host DNA depletion or the HostZERO™ Microbial DNA Isolation Kit. The yield of purified microbial DNA as determined by quantitative PCR. The apparent yield of bacterial DNA in samples with HostZERO™ DNA depletion appears higher; we suspect this is because host DNA depletion increased the PCR efficiency.

Accurate, Low Bioburden PCR and Quantification Methods

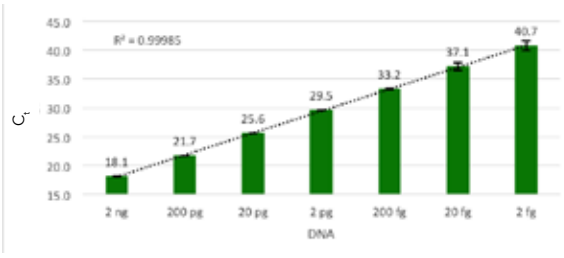
The library preparation process is quite prone to bias and error. The 16S rRNA gene sequencing library preparation process can suffer from potentially significant bias due to the inherent weaknesses of its primary step, PCR. A common source of PCR-related bias includes GC content variation in templates and degeneracy in primers. Amplification of the 16S rRNA gene using broad coverage primers is further challenged by the high similarity of the targets. PCR chimeric sequences - which are a result of the recombination between similar targets/templates - are significant contributors of error and bias in this process (Gohl et al, 2016; Haas, et al, 2014). Library preparation for shotgun metagenomic sequencing can also be prone to some PCR related bias/error. However, PCR-free library preparation is available given there is sufficient DNA input. Besides PCR-related bias, shotgun library preparation can be inaccurate in other ways, such as biased DNA fragmentation. In general, however, shotgun metagenomic sequencing is considered less biased as compared to targeted sequencing such as 16S rRNA sequencing.

Next-Generation sequencing (NGS) is generally thought to introduce little bias to the determination of microbial composition. However, all NGS platforms carry specific patterns of sequencing errors. For example, 454 and Ion Torrent™ sequencing platforms have high error rates in sequencing regions of homo-polynucleotides (Bragg et al., 2013, Gilles et al., 2013). Even with a sophisticated program for read-quality-based trimming, some sequencing errors will survive and potentially cause misleading interpretations. In 16S rRNA sequencing, sequencing errors can result in the assignment of false taxa and the overestimation of alpha diversity.

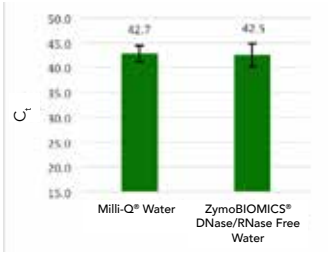
ZymoBIOMICS® PCR PreMix

- Simply add water, DNA, primers and go!
- Certified low bioburden.
- Robust amplification for the detection of low copy DNA.
- Ideal for highly sensitive applications.

Sensitive Detection Range Free of Bacterial DNA



A tenfold serial dilution of *Lactobacillus fermentum* genomic DNA was quantified via real-time PCR, after the addition of 2.5 µM SYTO® 9 to a 50 µl reaction volume. Amplification of the 16S rRNA gene can be quantified down to 2 femtograms of bacterial genomic DNA in a 45 cycle qPCR.

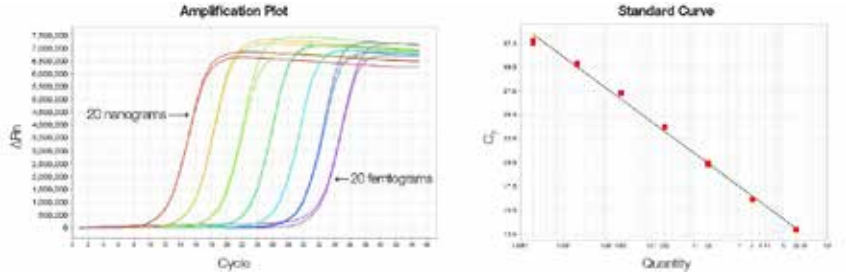


Quantification of no template controls (NTCs) via real-time PCR was determined by amplification of the 16S rRNA gene, after the addition of 2.5 µM SYTO® 9 to a 20 µl reaction volume. Real-time PCR was performed for 45 cycles to determine the amount of bacterial contamination. NTCs include Millipore filtered water and DEPC-treated Millipore filtered water.

Femto™ Bacterial DNA Quantification Kit

- Quantify down to 20 femtograms of DNA in as little as 1 µl of sample.
- High specificity and sensitivity for DNA in a background of non-target DNA.
- Fast and simple: add samples to the PreMix and quantify.

Reliable Quantification



Reliable standards for the qualification of bacterial DNA: Bacterial DNA Standards (measured in duplicates) comprise a tenfold dilution series ranging from 20 ng to 20 fg.

Product	Cat. No.	Size
ZymoBIOMICS® PCR Premix	E2056	50 rxns.
ZymoBIOMICS® PCR Premix	E2057	200 rxns.
Femto™ Bacterial DNA Quantification Kit	E2006	100 rxns.

ZymoBIOMICS® Services

- Zymo Research offers the most comprehensive services for 16S rRNA and shotgun sequencing from any sample type.
- ZymoBIOMICS® Services are validated using the ZymoBIOMICS® Microbial Community Standards for unbiased, publication-quality data.
- Services include low bioburden processing and accurate DNA/RNA isolation using the ZymoBIOMICS® product line for the most accurate taxonomic profiling.

Simply send us your samples and we will handle the rest!

Optimized, Accurate Workflows

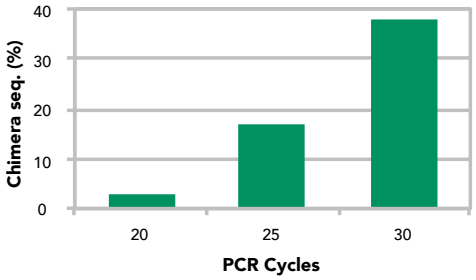
Remove Noise in 16S rRNA Profiling

Regular Workflow

Low bioburden reagents
Ultra-clean processes
Control for PCR chimeras
Excellent MiSeq run
Uchime filtration
Others

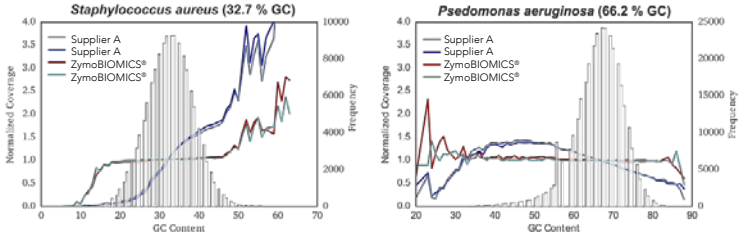
ZymoBIOMICS® Services

Track PCR Chimeras in 16S rRNA Gene Sequencing



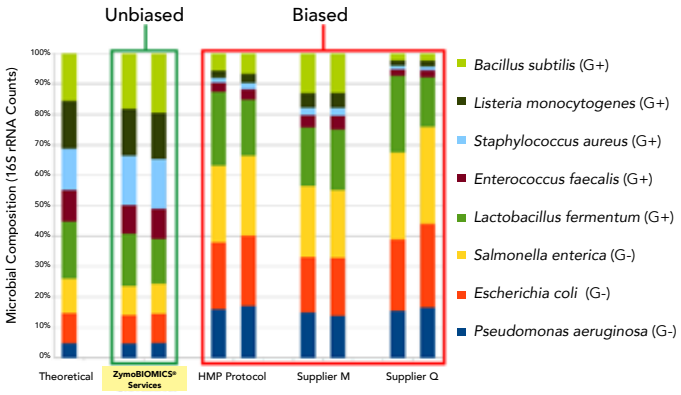
PCR chimeras increase with higher PCR cycle number in the library preparation process of 16S rRNA gene targeted sequencing. 20 ng of the ZymoBIOMICS® Microbial Community DNA Standard was used as a template. The PCR reaction was performed with primers that target V3-4 region of 16S rRNA gene. Chimera rate in percentage was determined with Uchime and using the 16S rRNA gene of the eight bacterial strains in the standard as reference.

Assess GC-Bias in Shotgun Metagenomics



Library preparation for shotgun metagenomic sequencing was performed in two different ways using Supplier A and an in-house method. Shotgun sequencing was performed on MiSeq with paired-end sequencing (2x150 bp). Raw reads were mapped to the 10 microbial genomes to evaluate the potential effect of GC content on sequencing coverage. Normalized coverage was calculated using the average sequencing depth of each genome.

Validated, Non-Biased Workflows from Collection to Analysis

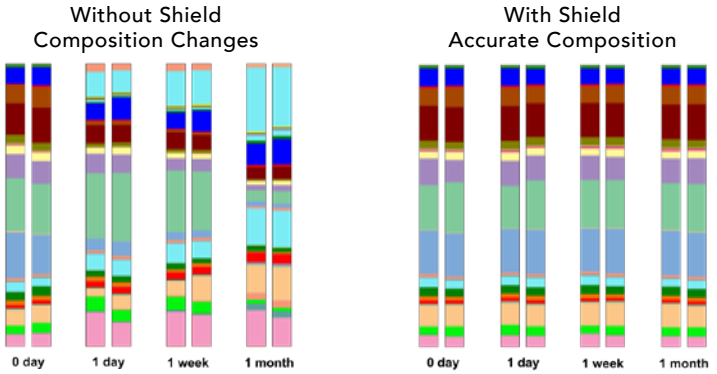


ZymoBIOMICS® Services are validated using the ZymoBIOMICS® Microbial Community Standards for unbiased, accurate community profiling.

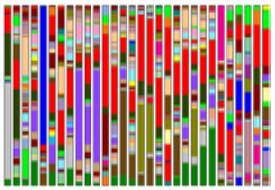
Unbiased Sample Collection & Storage

DNA/RNA Shield™ Preserves Microbial Composition at Ambient Temperature

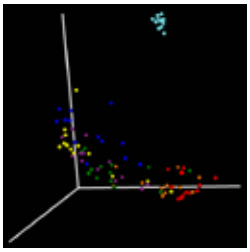
Microbial composition of stool is unchanged after one month at ambient temperature with DNA/RNA Shield™.



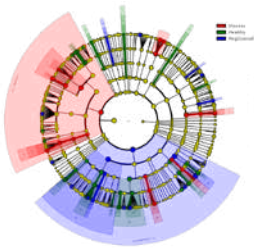
Comprehensive, Customizable Bioinformatics & Data Analysis



Composition Barplots



Beta-Diversity



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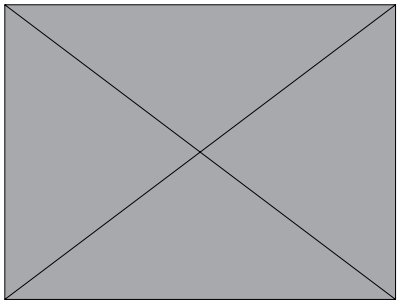


Tips and Tricks to Reduce Bioburden

Bioburden should be a consideration in every microbiomics workflow, as the contamination of microbes not native to the sample can skew profiling results and interpretation. Taking steps to reduce bioburden in any microbiomics workflow can help greatly increase the accuracy of community profiling and interpretation.

Work In Proper Environments

Work in proper environments, such as an enclosed workstation or a clean room. Bacteria exist and persist on a variety of surfaces, and trace amounts of DNA can be present at any time or any place. Remember that you are working in a laboratory environment where amplicons – specifically amplicons targeted for your microbiomics analyses – exist. Ensure that you have a separate workstation for each application and clean the surfaces adequately.

A clean work environment is essential to minimize bioburden. At Zymo Research, we have dedicated an entire wing of our facility for clean practices to ensure low bioburden workflows.

Tips for your Clean Lab

- Adhesive entry mat (sticky mat) or shoe covers upon entry of the clean room
- Disposable or dedicated lab coat for clean room use only
- Protective face mask or procedure mask for facial concealment
- Bouffant cap (hair net)
- Sterile gloves
- HEPA filtered room

Apply 10% bleach on the counter top, and let it sit for approximately 15 minutes before removing. Additionally, clean the rotor, lid, and internal walls of the micro-centrifuge as well as pipette tips with 10% bleach, followed by 70% ethanol, to ensure neutralization of the bleach to prevent downstream interference.

Use designated sterile pipette tips for the extraction, and if possible use filtered tips.

NOTE: If at any time the researcher must leave this clean environment and return, they must reapply fresh clean-room attire, such as bouffant cap, protected face mask, etc. Lab coats should never leave the clean room environment.

DNA Extraction

Utilize certified low bioburden extraction kits, such as the ZymoBIOMICS® DNA Kits, for DNA extraction from a variety of sources such as fecal, soil, water, cultured bacteria/fungi, etc.

All reagents and components used for the purification and subsequent analyses should be fresh and opened only in a clean room or an enclosed workstation.

Use mechanical lysis (ZR BashingBead™ Lysis Tubes (S6012-50) or DNA/RNA Shield™ Lysis Tubes (R1103)) with a strong bead-beating device to ensure accurate lysis of microbes.

Take caution to ensure there is no cross contamination between samples.

Aliquots of the ZymoBIOMICS® DNase/RNase-Free Water can be taken to ensure there is no cross contamination or introduction of contaminants. Alternatively, users can autoclave the entire bottle by loosening the cap and autoclaving at 121 ˚C for 20 minutes to degrade any contaminating DNA.

Did You **Know**

- The ZymoBIOMICS® DNA Kits have been designed for users who work with high-sensitivity, low-biomass samples to ensure minimal contaminating bacterial background DNA. Low bioburden is determined and quantified by 16S rRNA amplification using qPCR.
- All ZymoBIOMICS® reagents are formulated with clean manufacturing procedures (e.g. HEPA filtered environment) and are filtered before aliquoting.
- BashingBead™ tubes are autoclaved while 0.1 mm and 0.5 mm beads are baked at high temperatures (250˚C for 5 hours) to degrade any contaminating bacterial DNA.
- ZymoBIOMICS® DNase/RNase-Free Water is DEPC-treated (diethyl pyrocarbonate), incubated overnight, and then inactivated by autoclaving at 121˚C. The water is then aliquoted into polypropylene bottles, capped loosely, and then autoclaved at 121˚C to eliminate any contaminating DNA that may have been introduced during the aliquoting process. After this treatment the bottle is capped tightly to prevent further contamination.

Good Handling **Practices**

In addition to working in the proper environments, we recommend a few measures to ensure low contamination during the qPCR set up or similar process:

- Ice chest/bucket used for stabilizing any reagent should stay in the clean room, and a second ice chest should be used to transfer the ice to the chest in the clean room.
- Use DNase/RNase-Free Water for ease and reassurance, ZymoBIOMICS® DNase/RNase-Free Water (D4302-5) is available for purchase, which is certified low bioburden.
- Use new filtered pipette tips for each prep; if possible use new pipette tips even for reloading sample to the same column.
- New pipette tips should be used for elution of the DNA. Users are recommended to pipette the ZymoBIOMICS® DNase/RNase-Free Water directly on to the column matrix, so the pipette tip may come into close contact with the column matrix.
- It is necessary for pipettes to be calibrated for accurate quantification of low amounts of DNA present within a sample.
- We recommend changing pipette tips frequently to reduce contamination of reagents.
- When opening any bottle, container, tube, etc. use the cap to shield the mouth of the container. By doing so users can avoid any contaminants falling into the reagent.
- Aliquots of reagent should be made to encompass one or (at most) a couple of experimental set-ups. By doing so, users can minimize the amount of freeze/thaw steps and the reuse of previously contaminated reagents, if contamination is present.
- Between different preparations, clean the tools used as necessary with 10% bleach, followed by 70% ethanol.
- A master mix should be made and aliquoted initially for the assay. Remember, when applying the sample to each well, flush out the pipette tip to ensure accurate quantification. DNA can remain within the pipette tip, and efficiency in pipetting is required when working with low amounts of DNA.
- Take caution where you place reagents and tools to avoid leaning or hovering over reagents. Tilt PCR plates or similar at an angle so that users can avoid hovering while simultaneously being able to see into the plate.
- Lastly, don't forget your no template controls.

Assessing Low Biomass Samples in Microbiomics for Metagenomic Analysis

The value of studying the microbiome of humans and the environment is undeniable, as the number of published articles regarding microbiomics research increases each year. Concurrently, the efficiency and availability of Next-Generation sequencing (NGS) has increased exponentially. These two occurrences are closely entwined as they continue to induce each other's development and advancement - as the field of microbiomics expands and changes, so does the technology of NGS, and vice versa.

The need for a standardized procedure to assess microbiome samples has been expressed in a wide variety of studies. There is presently great variability in results obtained from the same sample through different systems, indicating extended biases, lack of reliable controls, and ultimately results that may be difficult to evaluate. Samples with low biomass are especially difficult to accurately assess, because of low DNA yield and the increased challenge of reducing contamination during processing.

Here we describe how to optimize a workflow that processes low biomass samples for metagenomics analysis, and address many of the common challenges facing such workflows. The practices described below will enable low biomass samples to be analyzed through targeted or shotgun sequencing with reproducible and reliable results.

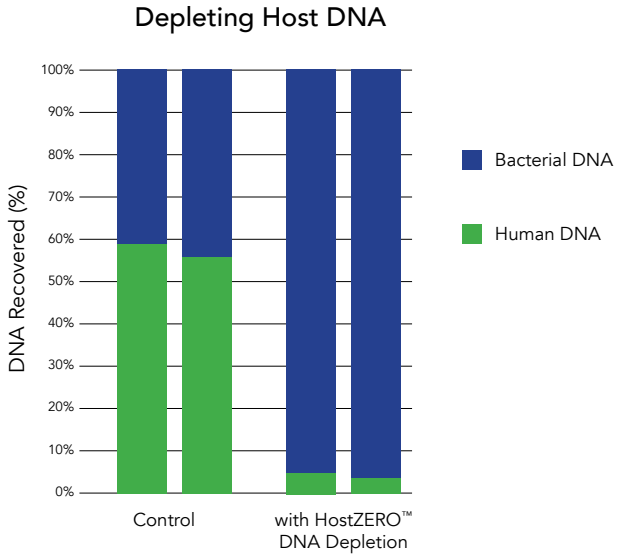
Sample Collection

Low biomass samples are often a challenge for human microbiome studies, in which completely aseptic collection techniques are difficult. Samples obtained from sites where few microbes are present, such as the skin, may be mishandled and introduced to potential contamination and bioburden that impacts the downstream sequencing analysis.

Preservation reagents, such as DNA/RNA Shield™, and related collection devices function to minimize this risk of contamination by reducing the number of hands-on steps as well as to preserve the genetic integrity and expression profiles of samples. For example, the DNA/RNA Shield™ Swab and Collection Tube (R1106) is designed so that the swab tip can be broken off directly into the collection tube and saved for future processing, with no intermediate steps. All samples, including those that are commonly affected by low biomass, will have their entire microbial community preserved for up to one month at room temperature, or over a year at -20°C or lower.

Depletion of Host DNA

The effect of low biomass on pathogen identification and shotgun metagenomics cannot be overemphasized. In human microbiome research, low biomass microbiomes are not only affected by contamination but also by the substantial amount of host DNA present in the collected sample. For shotgun metagenomics, the number of reads mapped to the host DNA overwhelmingly outnumber the reads mapped to microbial reference genomes. For targeted metagenomics, the possibility of amplifying host mitochondrial or chloroplast 16S rRNA undermines the quality of the final data.



Isolating microbial DNA and depleting host DNA of a saliva sample with the HostZERO™ Microbial DNA Isolation Kit. One human saliva sample was processed using either the control method the ZymoBIOMICS® DNA Microprep Kit, which extracts total DNA from the sample without host DNA depletion or the HostZERO™ Microbial DNA Isolation Kit. The composition of the purified DNA from saliva in terms of bacterial and human DNA abundance. The abundance was determined by quantitative PCR.

To overcome these challenges, host DNA must be removed from the original sample source. Zymo Research has developed a protocol that enables a near-total removal of host DNA in just three steps, while selectively isolating intact bacteria from dead bacteria. The treatment depletes host DNA by over 90% in skin, saliva, and blood samples, thus enriching the bacterial DNA content of the sample. By increasing the ratio of bacterial DNA to human DNA in low biomass samples, downstream sequencing analysis data can be reported with higher quality reads mapped to microbial reference genes, providing greater confidence that the reads accurately represent the community of the sample.

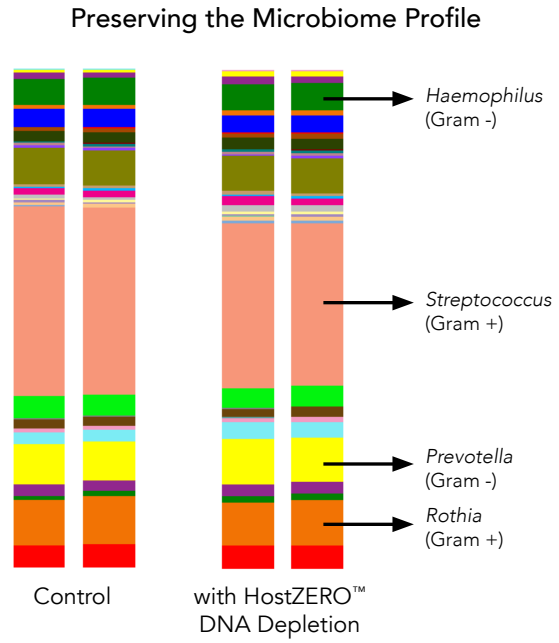
DNA Extraction

Extraction of DNA can be challenging due to the presence of contamination in extraction kits and various laboratory reagents. Zymo Research has historically been a provider of extraction kits which employ workflows that produce ultra-pure, inhibitor-free DNA from various sample types. The ZymoBIOMICS® DNA Miniprep Kit (D4300) produces high-quality DNA from any sample type. This kit is ideal for samples with low biomass because of its ultra-low level of contaminants as well as validated, accurate DNA isolation. The integrity of the composition of the sample is maintained throughout the process, and the data is not affected by common contaminating bacterial sequences.

Beyond utilizing a contaminant-free extraction method with the ZymoBIOMICS® DNA Miniprep Kit, the ZymoBIOMICS® Microbial Community Standard (D6300) can be used to determine if any bias is present in the extraction and downstream processes. Composed of eight species of bacteria and two of yeast, the standard helps ensure that the DNA extraction process is free of bias and contaminants by acting as a positive control with known composition. Negative process controls are also necessary to understand contributions of operators, steps, and reagents.

Conclusion

Zymo Research has developed a comprehensive and streamlined method for ensuring that microbiomics samples with low biomass are processed effectively and with minimal bias for downstream metagenomics applications. Moreover, the amount and quality of the metagenomics data produced from these samples can be greatly improved by using a combination of new products and techniques.



Isolating microbial DNA and depleting host DNA of a saliva sample with the HostZERO™ Microbial DNA Isolation Kit. One human saliva sample was processed using either the control method the ZymoBIOMICS® DNA Microprep Kit, which extracts total DNA from the sample without host DNA depletion or the HostZERO™ Microbial DNA Isolation Kit. The yield of purified microbial DNA was determined by quantitative PCR. The apparent yield of bacterial DNA in samples with HostZERO™ DNA depletion appears higher; we suspect this is because host DNA depletion increased the PCR efficiency.

FEATURED STORY:

Science for Fun: The Microbiome of International Cellphones

Background

There is almost no item more universal and personal than the cellphone. They are our portal to the outside world, with seemingly endless utility. We are constantly poking at them, talking at them, and holding them, so much that they have essentially become an extension of our body. However, that metaphor might actually be literal when it comes to a cellphone's microbiome.

We were curious to find out what might be living on our phones, and how geography might play a role. So that is why at Zymo Research's 2016 global distributor meeting, each attendee swabbed their cellphone to help us analyze the microbiome of cellphones used by individuals around the globe.

Methods

The samples were collected and processed using the entire ZymoBIOMICS® workflow from sample collection to conclusion. Swab samples were collected from the cellphones of users from various countries using the DNA/RNA Shield™ Collection Tube with Swab (Zymo Research, R1106). Tubes were shipped at room temperature before long term storage at -80°C. DNA was extracted from each sample using the ZymoBIOMICS® DNA Microprep Kit (D4301) according to the protocol. Targeted paired-end sequencing was performed against the V3-4 region of the bacterial 16S rRNA ribosomal RNA gene using the ZymoBIOMICS® Target-Specific Primer Set: V3-4. The sequencing library was prepared using the ZymoBIOMICS® Targeted Sequencing method. The amplicon libraries were cleaned with the *Select-a-Size* DNA Clean & Concentrator™ (Zymo Research, D4080) to keep fragments ≥200 bp, quantified, and normalized together. The final library was sequenced on Illumina® MiSeq® with the MiSeq® Reagent Kit V3 (600 cycle). The sequencing was performed with a 15% PhiX control library.

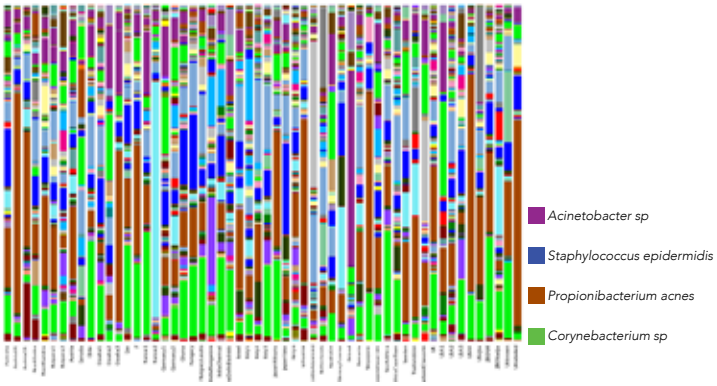


Figure 1. Taxa abundance of bacterial species identified in cellphone swabs using targeted 16S rRNA sequencing.

Results

The bacterial species identified in each cellphone swab sample and their relative abundances varied widely among the fifty-six samples (Figure 1). However, three species of bacteria were identified in every cellphone sample tested: *Propionibacterium acnes*, *Staphylococcus epidermis*, and a single species of the genus *Acinetobacter*. In addition, a single species of the genus *Corynebacterium* was found in every sample, except for a single swab originating in Canada. All four of these species are associated with normal human skin microflora[1].

Cellphone swab samples were collected from people who lived all over the world. The average relative abundance of thirteen genera is shown in Figure 2. While the presence of the genera is similar across the continents, there are notable differences in their relative abundances. For example:

- Bacteria in the genus *Corynebacterium* are identified in similar abundances in samples from Africa and South America; the same trend is seen among samples from Asia and Europe.
- The average relative abundance of bacteria in the genus *Propionibacterium* is much smaller in samples from Africa than any other continent.
- There is a greater average abundance of bacteria from both *Paracoccus* and *Acinetobacter* genera in Asia and

Europe than in any other continents.

- Samples from people who resided in North America had the highest relative abundance of bacteria from the genus *Pseudomonas*.

The bacteria from the genus *Alloiococcus*, of which there is currently only one known species that was first isolated from human middle ear fluid, was originally thought to be a pathogenic organism.

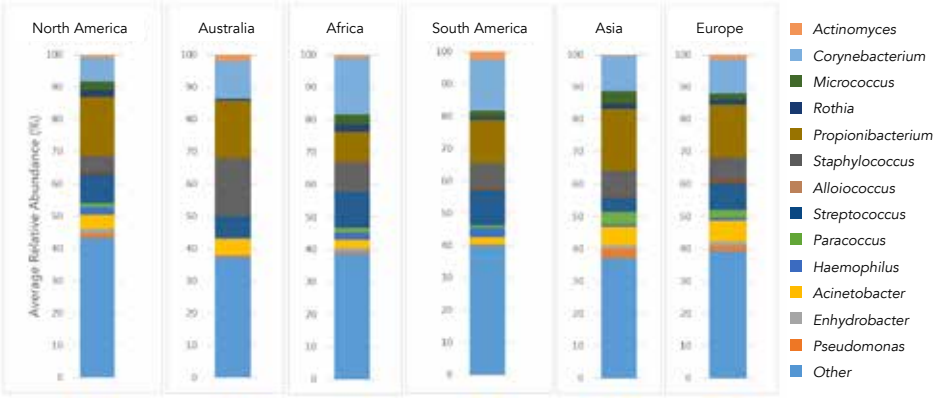


Figure 2. Average relative abundance of thirteen selected bacterial genera identified in cellphone swabs using targeted 16S rRNA sequencing by continents of the world.

Today, new research has shown that it may be a commensal organism in the human microflora[2]. Interestingly, this organism was identified in the majority of samples from the cellphones belonging to people living in countries in North and South America. However, it was only identified in half of the samples from countries in Europe, and was absent in the majority of samples from Australia, Africa, and Asia (Figure 3). These initial results indicate that this species is more prevalent in the normal microflora of people living in countries in the Western Hemisphere.

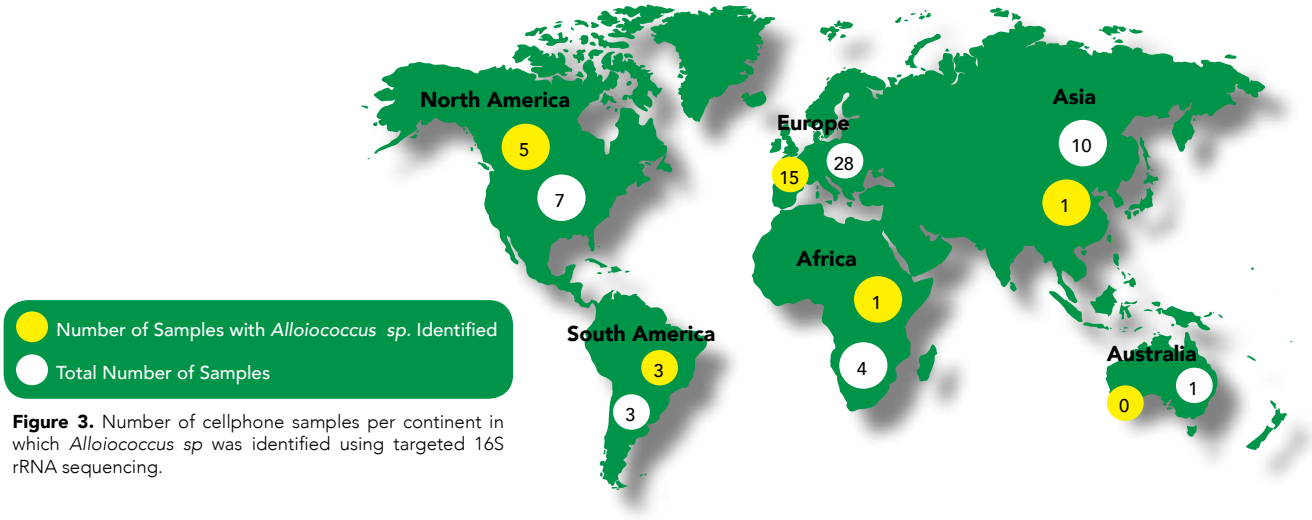
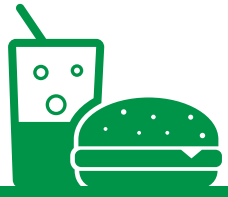


Figure 3. Number of cellphone samples per continent in which *Alloiococcus sp* was identified using targeted 16S rRNA sequencing.



You are what you eat

In addition to the various bacteria identified that are commonly linked to the human microflora, a few organisms stood out. The bacteria *Corynebacterium variabile* was identified in eleven of the swab samples processed, and *Pseudomonas fragi* was identified in seven of the samples processed. *C. variabile* is associated with the microflora of smear-ripened cheese, and *P. fragi* is frequently found as a spoilage bacterium in dairy and meat products. It is possible that when these swab samples were collected from cellphones, these users had just consumed hors d'oeuvres consisting of cheese and meats and inadvertently transferred bacteria present on the food to their cellphones.

References:
1. Davis CP. Normal Flora. In: Baron S, editor. Medical Microbiology. 4th edition. Galveston (TX): University of Texas Medical Branch at Galveston; 1996. Chapter 6. Available from: https://www.ncbi.nlm.nih.gov/books/NBK7617/
2. Tano, Krister et al. "Alloiococcus otitidis- Otitis media pathogen or normal bacterial flora?" Apmis 116 (2008): 785-90. Web. 16 Feb. 2017.

The Future of Microbiomics

by Andrew J. Gasparrini,[a] Gautam Dantas[a,b,c,d]

In the past decade, there has been an explosion of interest in the role of the microbiome in health and disease. Advancements in sequencing and computational capabilities have laid the groundwork for an increased appreciation of the taxonomic, genetic, and metabolic diversity of the microbiome, as well as the role that this critical consortium of microbes plays in host physiology, metabolism, and immune function. Key studies have implicated disruptions in microbiome composition and function to wide-ranging human pathologies, from obesity and malnutrition to Parkinson's disease.

The exciting future of microbiomics will necessarily build on these and other seminal discoveries. Informed by the descriptive microbiome surveys of the past decade, the field of microbiomics is rapidly maturing to a state where basic science hypotheses about the microbiome are beginning to show potential for tangible translation to improve human, animal, plant, and ecosystem health. Success in these ventures will require a few key transitions in the design and execution of next-generation microbiome studies, some of which are already underway by leaders in the field. First, it will require deep mechanistic examination of hypotheses generated in past microbiome studies to identify molecular mechanisms by which the microbiome influences host physiology. These studies will require targeted validation of "omics" predictions by classical biochemical, genetic, and physiological techniques. Second, it will require a re-commitment to culture-based techniques to interrogate the functions of select members of the microbiome, in isolation and in defined microbial communities. Lastly, it will require rigorously controlled animal studies, both in conventional and gnotobiotic model systems, to validate the observations from human studies and establish causal relationships between functional compositions of the microbiome as well as the genetics, immune state, physiology, and health of the host. Integration of these approaches with continued "omics" surveys of the microbiome will help realize the immense translational potential of microbiome based diagnostics and therapeutics in the not too distant future.

Author Affiliations:
a. Center for Genome Sciences and Systems Biology, Washington University School of Medicine, 4515 McKinley Avenue, Campus Box 8510, St. Louis, MO 63110, USA.
b. Department of Pathology & Immunology, Washington University School of Medicine, 660 South Euclid Ave, St. Louis, MO 63110, USA
c. Department of Biomedical Engineering, Washington University in St. Louis, 1 Brookings Drive, St. Louis, MO 63130, USA
d. Department of Molecular Microbiology, Washington University School of Medicine, 660 S. Euclid Ave, St. Louis, MO 63110, USA.

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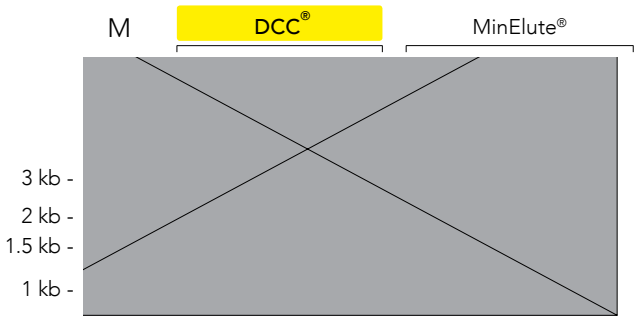
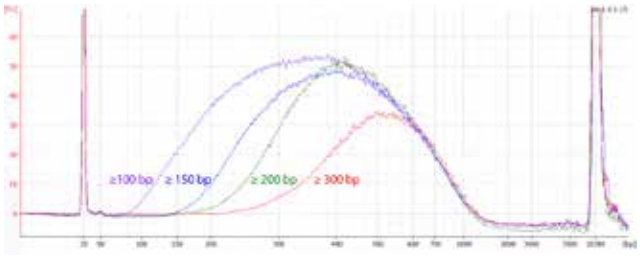


High efficiency DNA recovery using the DCC®-5. Equivalent amounts of DNA were purified using the DCC®-5 or MinElute® Kit and analyzed by electrophoresis in a 0.8% (w/v) agarose/TAE/EtBr gel.

6 µl elution volume, **2** minute procedure, **0** µl retention volume

Product	Size	Cat. No.
DNA Clean & Concentrator ®-5 (uncapped)	50 Preps	D4003
	200 Preps	D4004
DNA Clean & Concentrator ®-5 (capped)	50 Preps	D4013
	200 Preps	D4014
ZR-96 DNA Clean & Concentrator ®-5	2 x 96 Preps	D4023
	4 x 96 Preps	D4024

Select-a-Size DNA Clean & Concentrator®



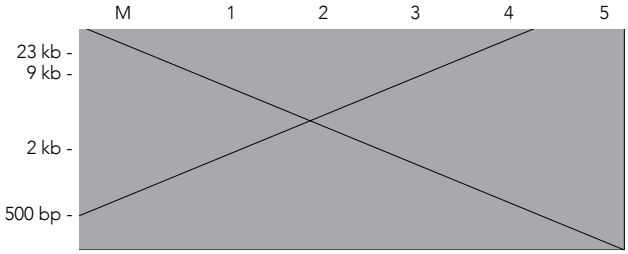
Select-a-Size™ DNA Clean and Concentrator® allows for selection at ≥300 bp, ≥200 bp, ≥150 bp, ≥100 bp and ≥50 bp.

Select for DNA species ≥ 50 bp, ≥ 150 bp, ≥ 200 bp, ≥ 300 bp, ≥ 700 bp or perform double size selection

Product	Size	Cat. No.
Select-a-Size™ DNA Clean & Concentrator®	25 preps	D4080

Zymoclean™ DNA Gel Recovery

Boost recoveries from agarose gels to > 80%



DNA fragments recovered from an agarose gel using the Zymoclean™ Gel DNA Recovery Kit. Lanes: M: DNA Ladder; 1-5: individual ladder DNA fragments.

Product	Size	Cat. No.
Zymoclean™ Gel DNA Recovery Kit (uncapped)	50 preps	D4001
	200 preps	D4002
Zymoclean™ Gel DNA Recovery Kit (capped)	50 preps	D4007
	200 preps	D4008
ZR-96 Zymoclean™ Gel DNA Recovery Kit	2 x 96 preps	D4021
	4 x 96 preps	D4022
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