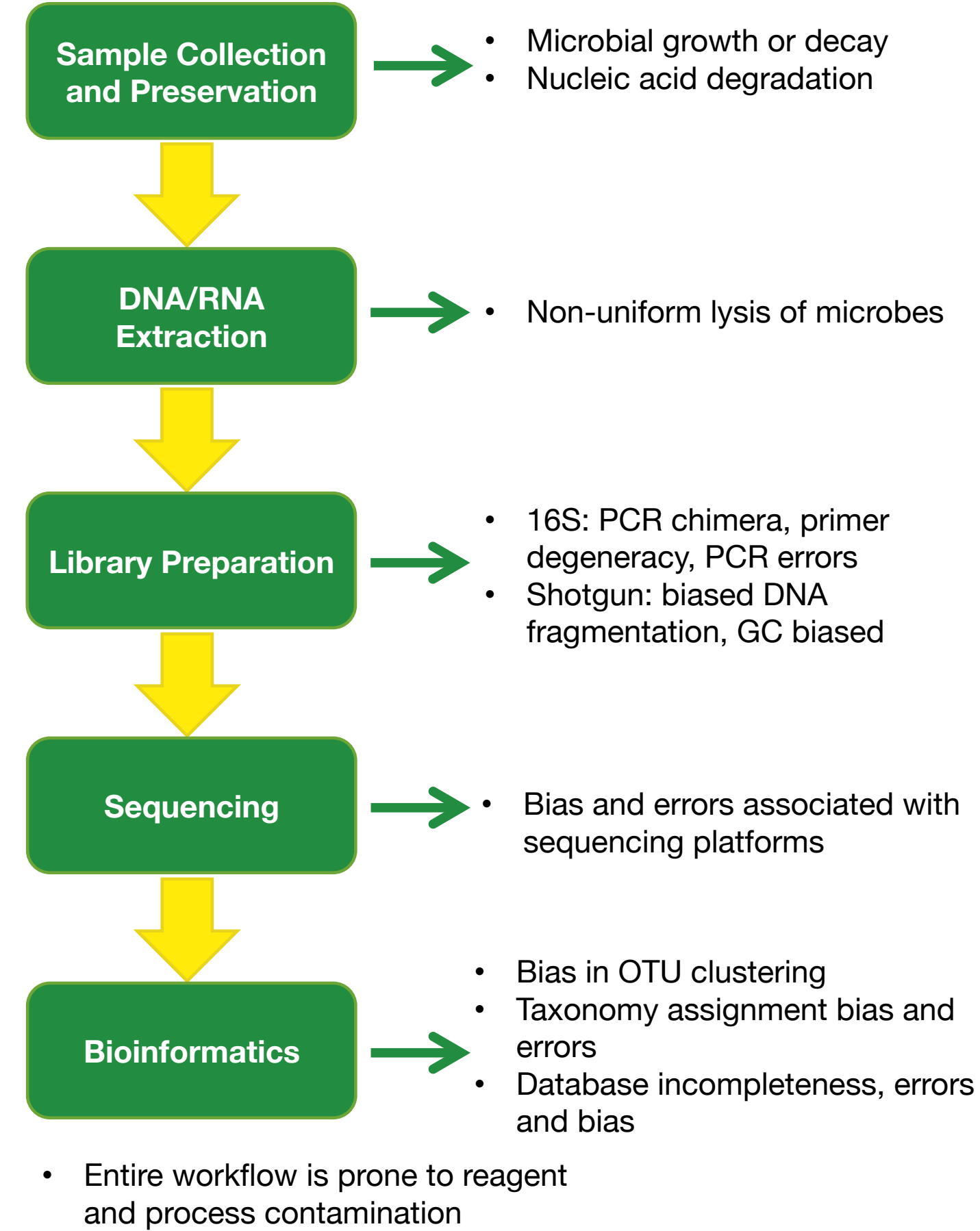


## Introduction

### The Need For Microbiomics Reference Materials and Standards

Microbial composition profiling techniques powered by Next-Generation sequencing (NGS) are becoming routine studies. However, it is well known that results are prone to bias and errors in every step of the workflow, including sample collection, DNA /RNA extraction, library preparation, sequencing and bioinformatics analysis. Therefore, standardization of these NGS workflows is necessary to minimize bias and improve data quality and reproducibility in microbiomics studies. The figure on the right shows challenges associated with each step.

At Zymo Research, we have constructed a comprehensive product line, coined ZymoBIOMICS®, designed for unbiased, accurate microbiomics and metagenomics analyses and validated using the ZymoBIOMICS® Microbial Community/DNA Standards.



### ZymoBIOMICS® is a Total Solution for your Microbiomics Workflows



## Methods & Results

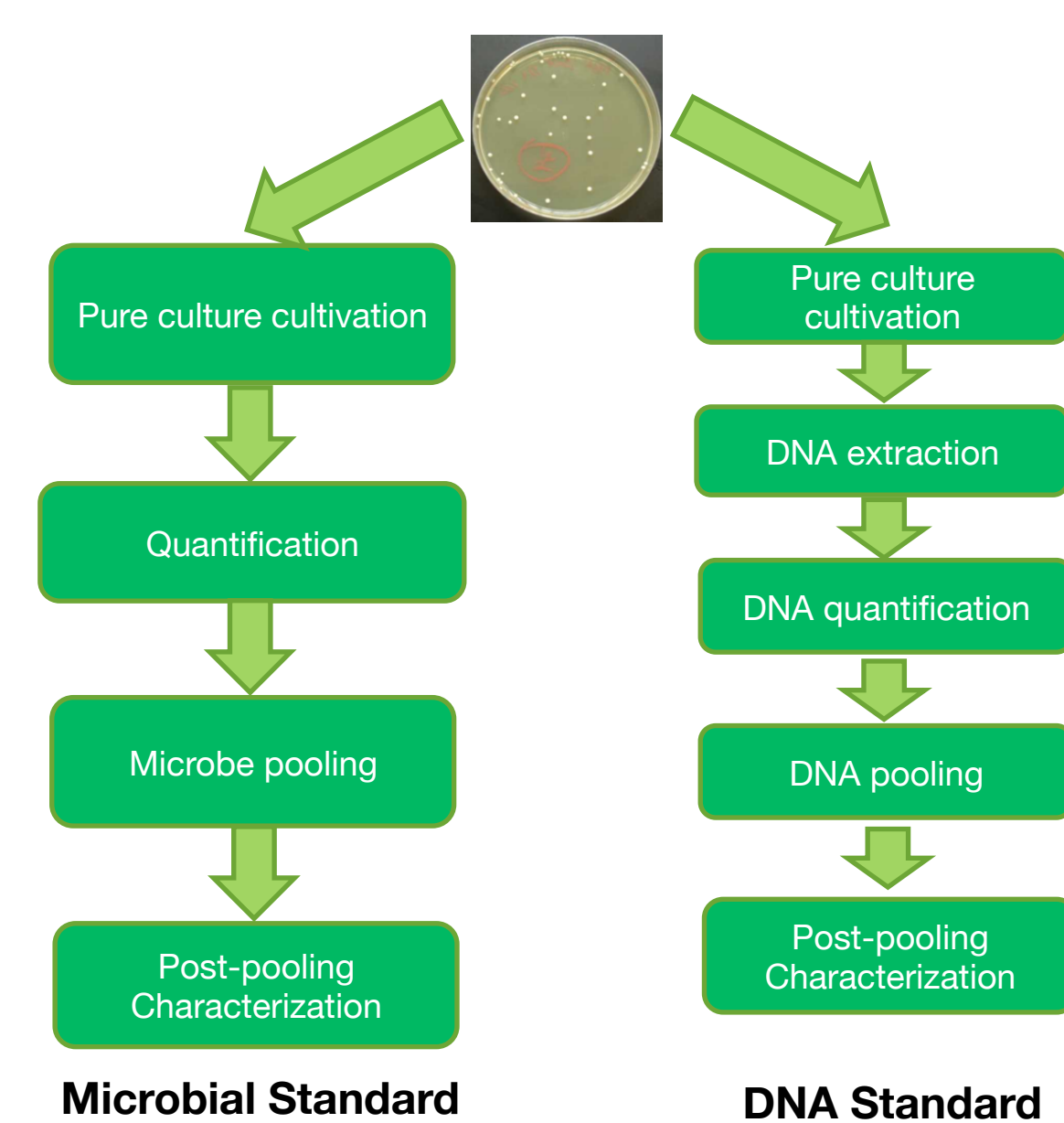
### Construction and Characterization of the ZymoBIOMICS® Microbial Community Standards

In order to assess bias and errors in NGS-based microbial composition profiling workflows, reference materials that mimic a mixed microbial community of well-defined composition are necessary. The ZymoBIOMICS® Microbial Community/DNA Standards contain both tough-to-lyse and easy-to-lyse microbes – both prokaryotes and eukaryotes – that span a wide GC range (15% - 85%; Table 1) and contain negligible impurities (<0.01%, Table 2) making it ideal for optimizing 16S rRNA gene seq. and metagenomic seq. workflows.

**Table 1. Microbial Composition of ZymoBIOMICS® Standards**

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

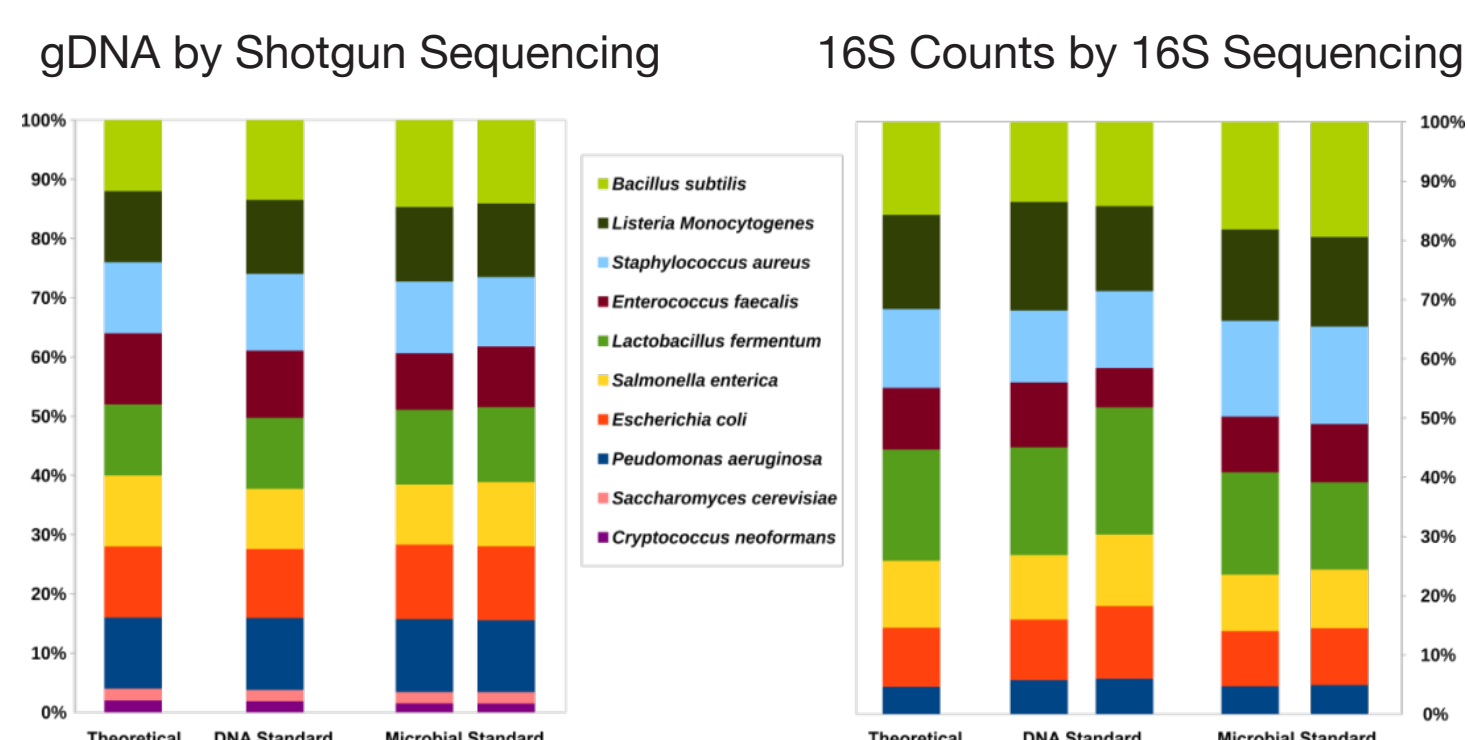
**Figure 1. Construction of ZymoBIOMICS® Standards**



### Negligible Impurities (<0.01%)

**Table 2. Assessing impurities in ZymoBIOMICS® Microbial Community Standard using shotgun metagenomic sequencing.** Genomic DNA was extracted using ZymoBIOMICS® DNA Mini kit. Shotgun library was prepared with an internal method. The library was sequenced on HiSeq, resulting in ~178 million reads (100 bp). Taxonomy identification was performed with mOTU (<http://www.bork.embl.de/software/mOTU>).

| Species                        | mOTU Counts | mOTU Abun. (%) |
|--------------------------------|-------------|----------------|
| <i>Bacillus subtilis</i>       | 9048        | 11.86          |
| <i>Listeria monocytogenes</i>  | 11454       | 15.01          |
| <i>Staphylococcus aureus</i>   | 7960        | 10.43          |
| <i>Enterococcus faecalis</i>   | 11322       | 14.84          |
| <i>Lactobacillus fermentum</i> | 17081       | 22.39          |
| <i>Escherichia coli</i>        | 6994        | 9.17           |
| <i>Salmonella enterica</i>     | 7939        | 10.41          |
| <i>Pseudomonas aeruginosa</i>  | 4484        | 5.88           |
| <i>Propionibacterium acnes</i> | 1           | 0.0013         |

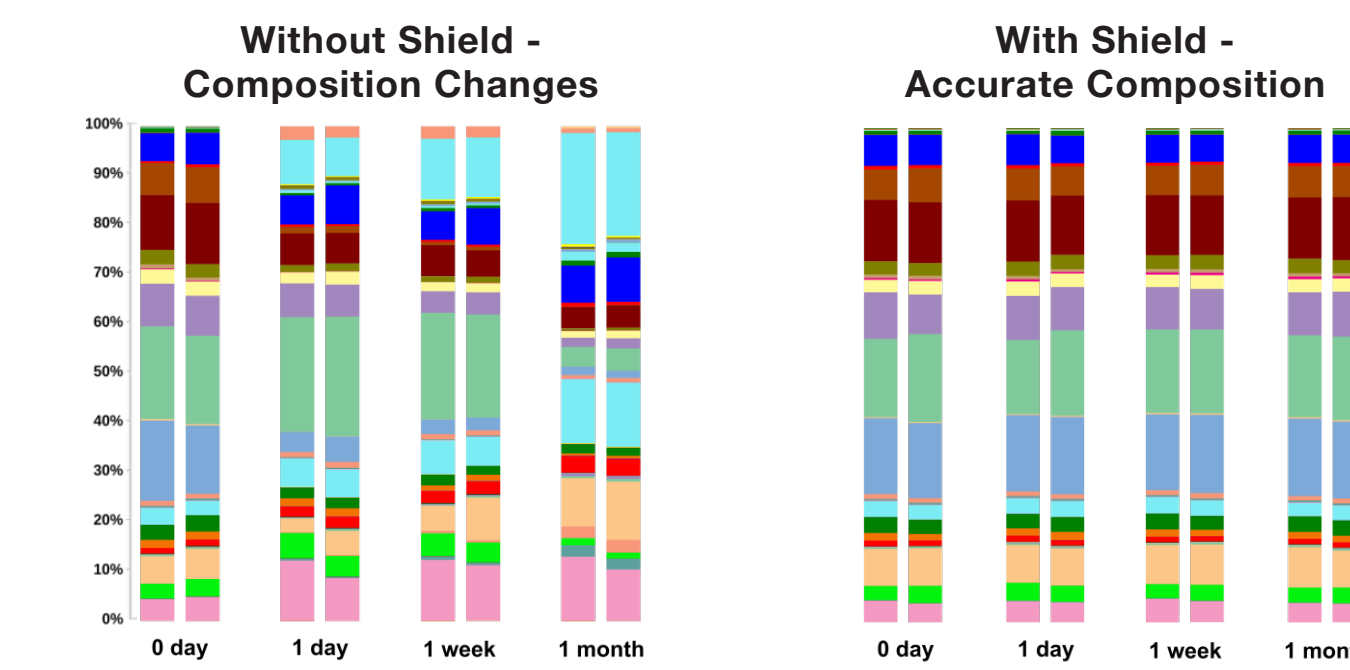


### Accurate composition

**Figure 2. Assessing microbial composition of ZymoBIOMICS® standards using shotgun metagenomic sequencing and 16S rRNA gene targeted sequencing.** Genomic DNA from the microbial standard was extracted using ZymoBIOMICS® DNA Mini Prep Kit. Shotgun library was prepared with an internal method and sequenced on MiSeq. Microbial composition was determined by mapping raw reads to the 10 microbial genomes contained in the standard. The library of 16S rRNA targeted sequencing was prepared with primers that amplify 16S v3-4 region and sequenced on MiSeq (2x250 bp). Paired-end reads were joined to construct complete amplicon sequences, which were mapped to the 8 bacterial 16S sequences contained in the standard to determine relative abundance.

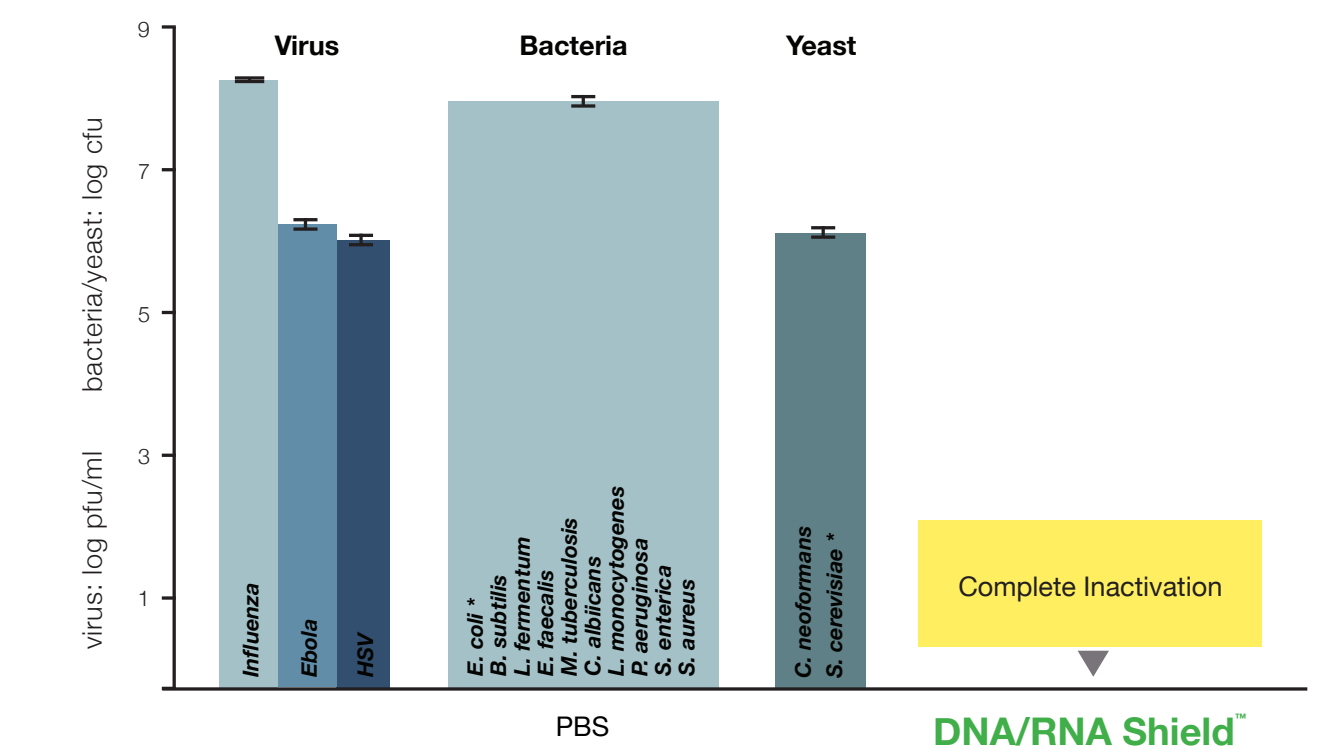
## Eliminating Bias in Sample Collection

Sample collection can cause significant bias due to microbial growth, decay or nucleic acid degradation. DNA/RNA Shield™ is a reagent that can preserve both DNA and RNA composition in almost any samples at ambient temperature for at least 30 days.



### Preserve Microbial Composition

**Figure 4. Microbial composition of stool is unchanged after one month at ambient temperature with DNA/RNA Shield™.** Stool samples suspended in DNA/RNA Shield™ and stored at room temperature were compared to stool without preservative for one month. They were sampled at the indicated time points and processed with ZymoBIOMICS® DNA Mini Prep Kit. 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.

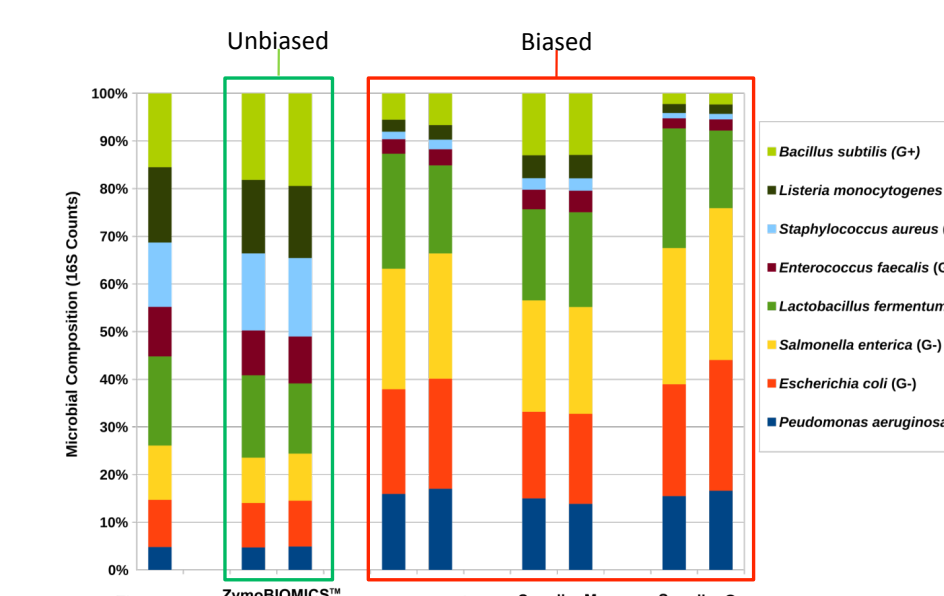


### Microbial Inactivation

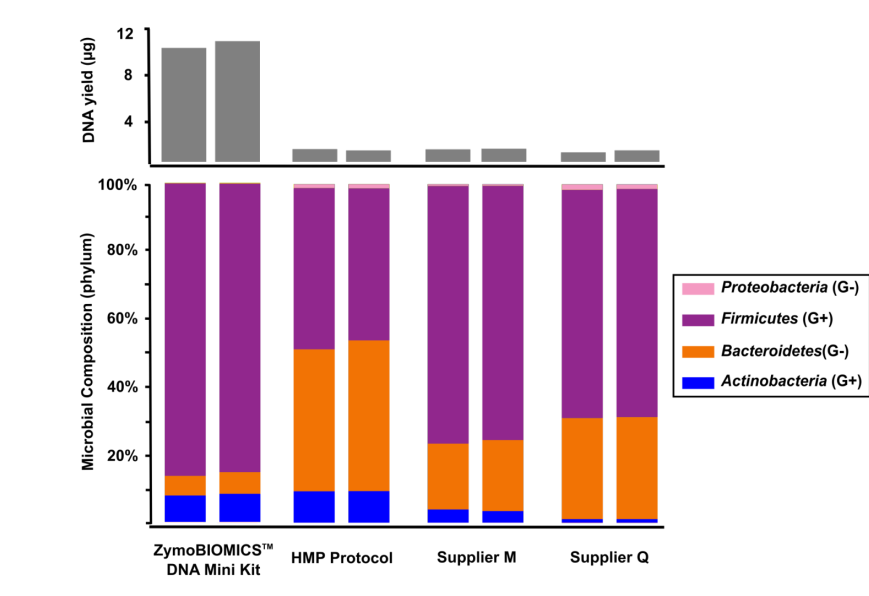
**Figure 5. Viruses, bacteria and yeast are effectively inactivated by DNA/RNA Shield™.** Samples containing the infectious agent (virus, bacteria, yeast) were treated with DNA/RNA Shield™ or mock (PBS) treated for 5 minutes. Titer (PFU) was subsequently determined by plaque assay. Validated by: Influenza A - D. Poole and Prof. A. Melnik, 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).

## Eliminating Bias in DNA Extraction Using the ZymoBIOMICS® DNA Miniprep Kit and Standards

A significant proportion of bias in microbiomics workflow stems from non-uniform lysis of microbes in the step of DNA/RNA extraction. Using ZymoBIOMICS® Microbial Community Standard, we found that most kits commonly used in microbiomics workflows are dramatically biased. ZymoBIOMICS® DNA Miniprep is the only kit on the market that is specifically optimized to eliminate bias in the step of DNA extraction.



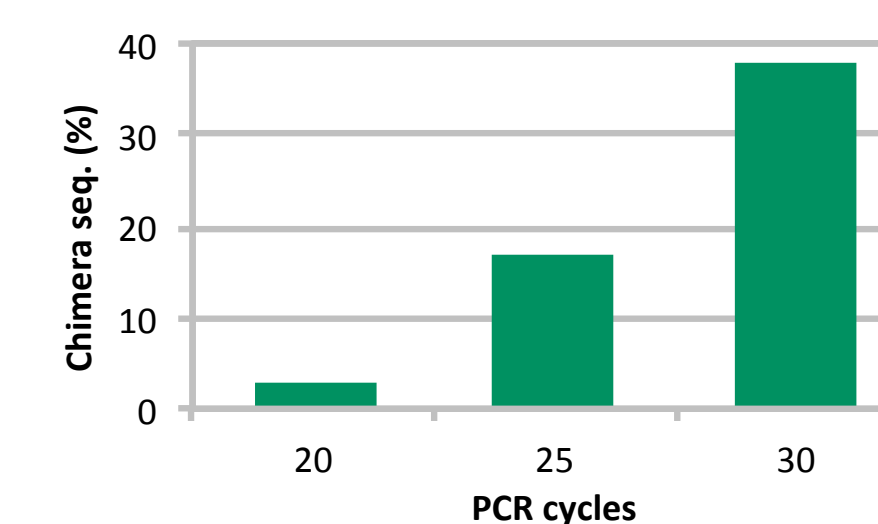
**Figure 6. Assessing bias in DNA extraction protocols using ZymoBIOMICS® Microbial Community Standard.** Genomic DNA was extracted from 75 µL of the microbial standard by strictly following each protocol. The microbial composition was profiled using 16S rRNA gene targeted sequencing in the same way as described in Figure 2.



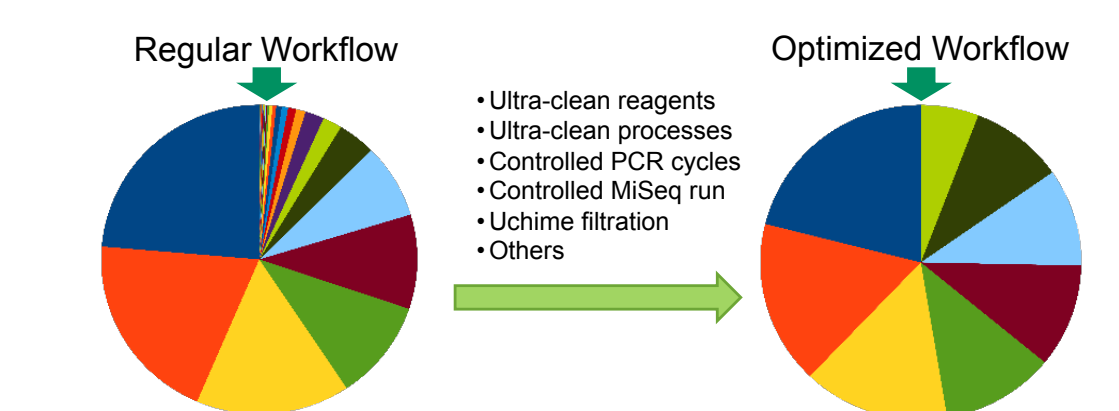
**Figure 7. Comparing DNA extraction protocols using human fecal samples.** Genomic DNA was extracted from 200 µL, 10% (w/v) fecal sample suspended in PBS by strictly following each protocol. The microbial composition was profiled using 16S rRNA gene targeted sequencing in the same way as described in Figure 4. DNA concentration was determined with Qubit.

## Detecting and Eliminating Artifacts in Library Preparation

### Eliminate artifacts in 16S rRNA gene targeted sequencing

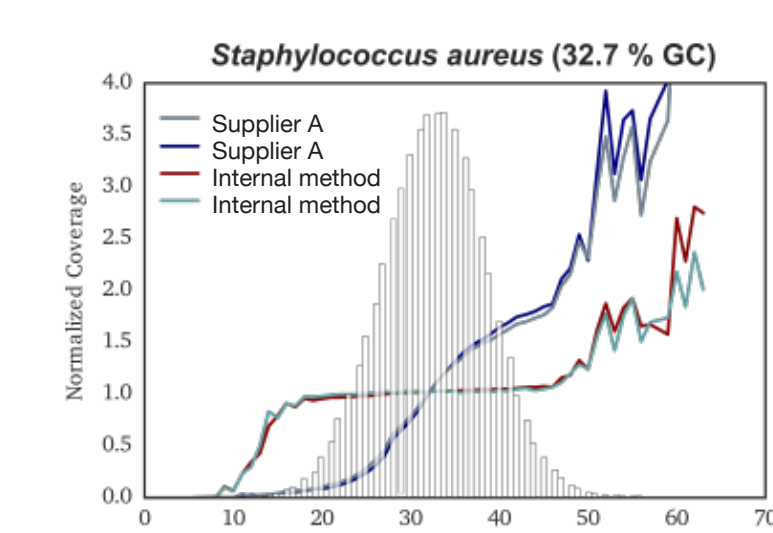


**Figure 8. Assessing PCR chimera formation in library preparation of 16S rRNA gene targeted sequencing using ZymoBIOMICS® Microbial Community DNA Standard.** Each PCR contained 10 ng genomic DNA from ZymoBIOMICS® Microbial Community DNA Standard as template. Primers targeting 16S v3-4 region were used. The libraries were sequenced on MiSeq (2x250bp). Paired-end reads were assembled into complete amplicons. Chimeric sequences were identified with Uchime and using the rRNA genes sequences present in the standard as references.

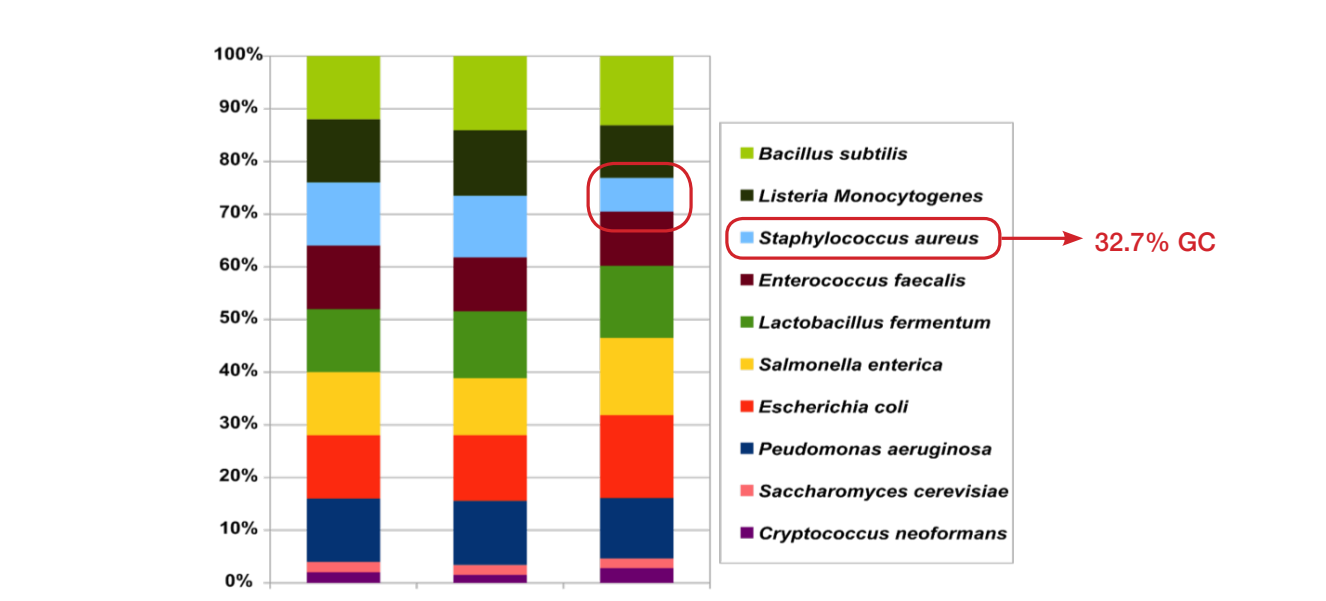


**Figure 9. Comparing the performance of two different workflows of 16S rRNA gene targeted sequencing using ZymoBIOMICS® Microbial Community DNA Standard.** The pie chart on the left shows the genus-level microbial composition of the standard sequenced and analyzed using a regular workflow. The pie chart on the right shows the corresponding profile sequenced and analyzed using an optimized workflow. While the pie chart on the left shows the presence of 43 bacterial genera, most of which we believe were caused by artifacts like PCR chimera, process contaminations, the pie chart on the right only shows the presence of 8 bacterial genera, which agrees with the real composition of the standard.

### Eliminate GC bias in shotgun metagenomic sequencing



**Figure 10. Our internal library preparation process has no GC bias.** Shotgun metagenomics sequencing with two different library preparation processes, Supplier A and an internal method, was conducted. Barplots depict the GC content distribution in each genome. Normalized coverage was defined as the sequencing coverage divided by the average sequencing coverage of the genome. The two genomes from the ZymoBIOMICS® standard was chosen to present the cases of low GC content and high GC content. Sequencing was performed on MiSeq (2x150 bp).



**Figure 11. Assessing bias of two different library preparation processes in shotgun metagenomic sequencing using ZymoBIOMICS® Microbial Community Standard.** Compared to our internal method, the Supplier A kit from Illumina has some bias due to GC content variation. Sequencing was performed on MiSeq (2x150 bp).

## Conclusion

- The use of a reference material is necessary to develop a workflow that is unbiased, reproducible, and accurate from sample collection to analyses.
- The ZymoBIOMICS® product line offers a complete and unbiased solution to microbiomics and metagenomics studies that was validated using the ZymoBIOMICS® Standards.