JUMPCODE GENOMICS

APP NOTE

# Increase bacterial species detection in microbiome stool samples

## Performance comparison study of CRISPRclean® Plus and Illumina® Ribo-Zero™ Plus for microbiome analysis

Next-Generation Sequencing (NGS) metagenomics and metatranscriptomics approaches offer unlimited possibilities for analyzing complex microbial communities because of their unbiased nature. They enable researchers to uncover the complex interactions between the microbiome and humans, making them effective tools for understanding disease pathogenesis and developing novel therapeutics. Metagenomics (DNA sequencing) studies all genes in the microbiome, profiling the composition and genetic diversity of complex multispecies samples. In contrast, metatranscriptomics (RNA sequencing) informs on the dynamics of gene expression patterns of microbial communities as a whole under changing environmental conditions or treatments. Metatranscriptomics sequencing also enables researchers to better understand metabolically active microbes by providing vital information on active gene pathways and for characterizing the taxonomic composition of the microbiome in greater detail.

### CRISPRclean Plus: The Benefits of Eliminating Noise

CRISPRclean Plus Stranded Total RNA Prep with rRNA Depletion is an optimized workflow that simultaneously detects viral genomic data, microbiome composition, co-infections, and host gene expression. Complex biological samples like fecal, saliva, and nasopharyngeal swabs frequently contain a mixture of human host and bacterial cells. Typically, total RNA comprises 90% ribosomal RNA (rRNA) that hinders detection of low expressing transcripts.[1]

Metatranscriptomic sequencing and microbiome profiling without removing these noisy rRNA sequences reduces sequencing capacity and makes detecting lower expressing, biologically relevant transcripts harder. Combining rRNA depletion technology with NGS library construction has improved the discovery process in metatranscriptomic sequencing approaches by increasing the number of bacterial species detected compared to undepleted samples, thereby enabling comprehensive and accurate profiling of bacterial composition and diversity in complex biological samples.

### The CRISPRclean Plus Workflow

CRISPRclean Plus harnesses the specificity of CRISPR systems to remove rRNA sequences from human, and more than 200 bacterial species, to help researchers identify rare, biologically relevant sequences. This kit is

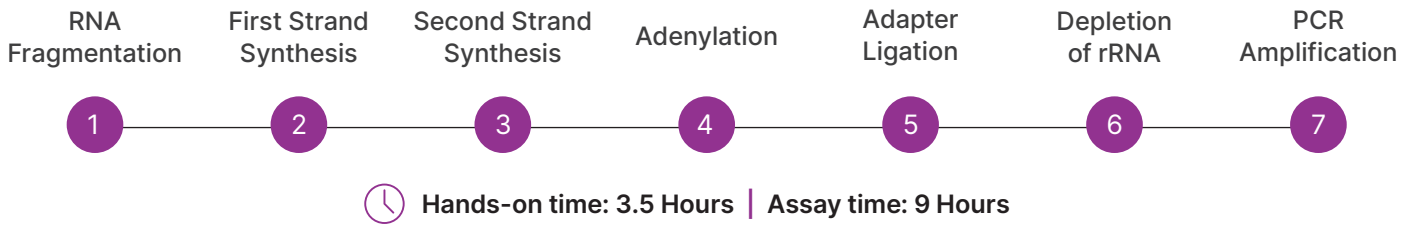


**Figure 1:** CRISPRclean Plus Stranded Total RNA Prep with rRNA Depletion (Human, Mouse, Rat, Pan Bacteria) is a streamlined workflow from total RNA to sequencing-ready, strand-specific libraries in 7 steps with multiple safe stopping points. Depletion is performed after library construction.

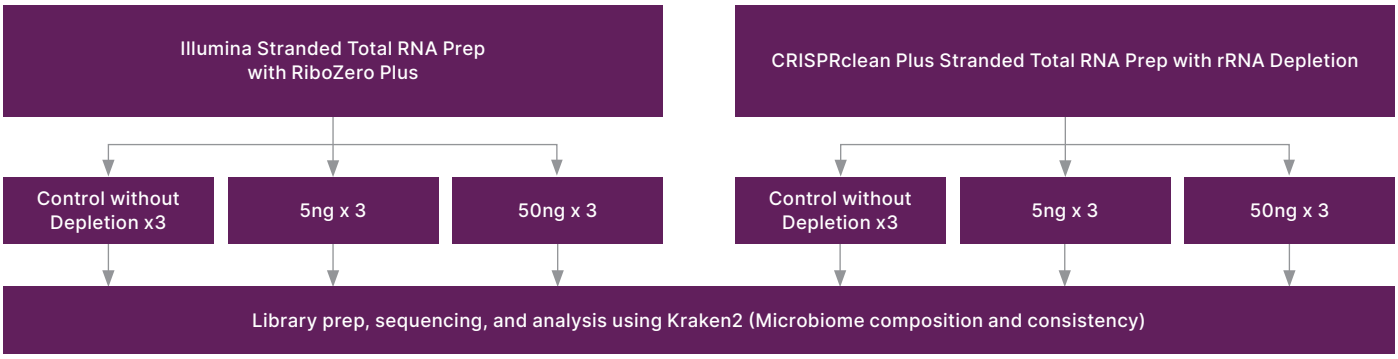


**Figure 2:** Experimental overview of comparison between CRISPRclean Plus and Illumina Ribo-Zero Plus kits.

ideal for analyzing samples containing complex mixtures of eukaryotic and bacterial rRNA. CRISPRclean Plus Stranded Total RNA Prep with rRNA Depletion (Human, Mouse, Rat, Pan Bacteria) is a streamlined workflow from total RNA to sequencing-ready, strand-specific NGS libraries (Figure 1).

This workflow is unique compared to most commercially available rRNA depletion kits in that depletion is performed after library construction. The post-library construction rRNA depletion strategy helps to mitigate against bias that may occur if depletion is performed before library construction because rRNA molecules can act as carrier molecules to help stabilize the library preparation against biasing. With CRISPRclean Plus, researchers can increase the sensitivity to detect lower expressing, biologically relevant transcripts by removing uninformative reads.

This application note presents a comparison study between the CRISPRclean Plus Stranded Total RNA Prep with rRNA Depletion kit (from here referred to as CRISPRclean Plus) and the Illumina Stranded Total RNA Prep with Ribo-Zero Plus kit (from here referred to as Illumina Ribo-Zero Plus) using microbial reference material composed of stool from healthy donors; the ZymoBIOMICS Fecal Reference control (ZymoBIOMICS™ Fecal Reference with TruMatrix™ Technology, from here referred to as Zymo control).[2]

## Study design

CRISPRclean Plus and Illumina Ribo-Zero Plus kits were tested under three conditions and with three replicates each. A total of 18 libraries were prepared, sequenced on a NextSeq 2000, and processed through Kraken2 to analyze microbiome composition and consistency compared to the Zymo control. Data were down sampled to 64M paired-end reads (Figure 2).

The Zymo control was used as a common reference material to compare the CRISPRclean Plus and Illumina Ribo-Zero Plus methods. The Zymo control is a microbial reference material composed of stool from healthy donors. Bacterial read counts for the Zymo control method were provided by Zymo Research and derived from the following Zymo method: 100 µl of the product was extracted using the ZymoBIOMICS™ RNA Miniprep (R2001). The RNA library was prepared using the Zymo-Seq RiboFree® Total RNA Library Kit (R3003). Sequencing was performed on Illumina® HiSeq™. Bioinformatics analysis was performed using the ZymoBIOMICS™ Metatranscriptomics Service Pipeline, which uses Centrifuge (v1.0.4) for taxonomy identification.

## Performance

The overabundance of rRNA in stool samples can interfere with the efficient identification of biologically significant transcripts, meaning genes or bacterial species with low expression are often barely detectable. In addition, much of the transcriptomic data captured is uninformative, thus hindering discovery. While fecal samples comprise a mixture of both human and bacterial cells, they typically have a higher bacterial to human ratio. By eliminating the noise and enabling greater sensitivity for low-expressing genes, rRNA depletion increases the number of bacterial species that can be detected compared to non-depleted fecal samples.

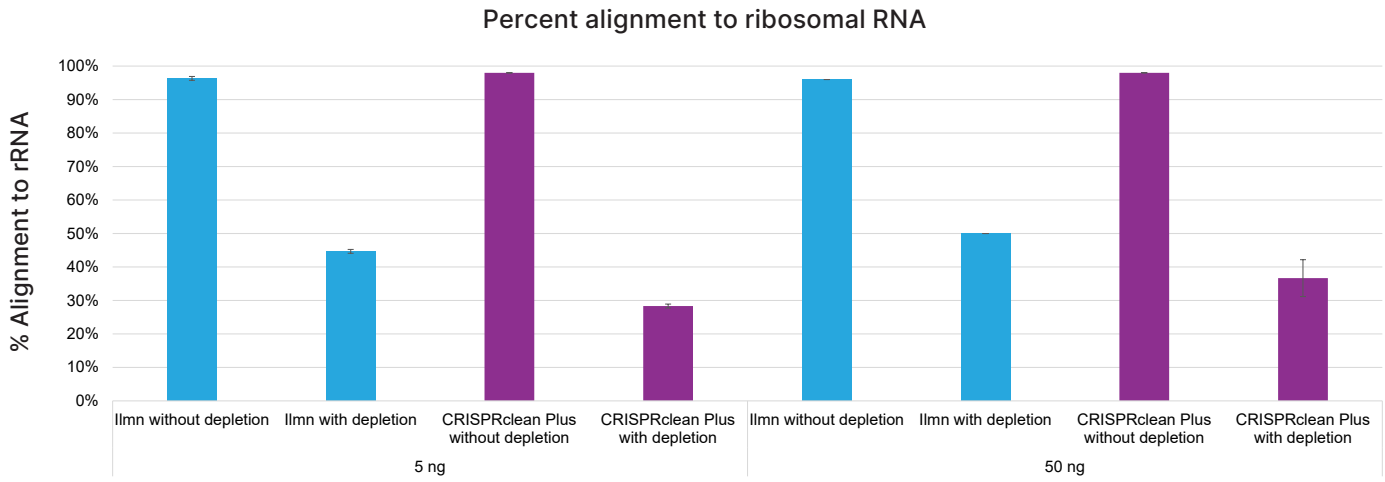


**Figure 3: CRISPRclean Plus is more efficient at removing rRNA**. Percent alignment to rRNA comparison between CRISPRclean Plus and Illumina Ribo-Zero Plus kits at 5 ng and 50 ng input.

## CRISPRclean Plus is more efficient at removing rRNA

To compare the rRNA depletion efficiencies of CRISPRclean Plus with Illumina Ribo-Zero Plus, the percent alignment to rRNA at 5 ng and 50 ng total RNA input was assessed. CRISPRclean Plus was more efficient at rRNA depletion with a lower percent alignment to rRNA after depletion compared to both non-depleted control and Illumina Ribo-Zero Plus (Figure 3).

## CRISPRclean Plus depletes more bacterial rRNA and provides deeper insight into the taxonomic composition of the microbiome

We surmised that assessing the depletion rate alone did not provide a comprehensive picture and that further insight could be gained from a more in-depth analysis of the depleted library content. Thus, we set out to evaluate the taxonomic composition of the CRISPRclean Plus and Illumina Ribo-Zero Plus depleted libraries and compared these data to the taxonomic composition present in non-depleted libraries.

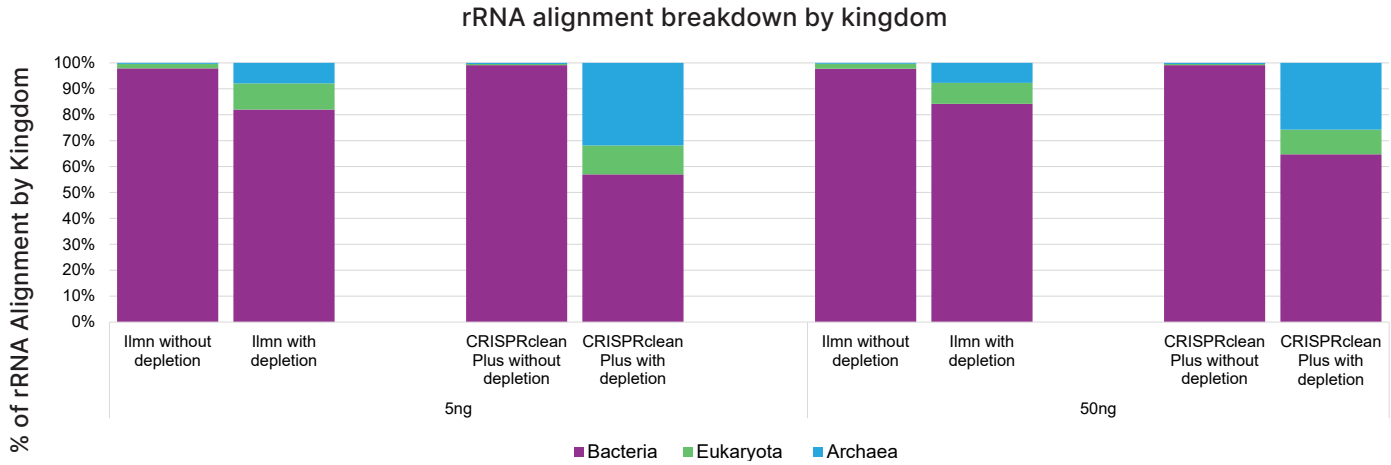


**Figure 4:** CRISPRclean Plus depletes more bacterial rRNA and provides deeper insight into the taxonomic composition of complex microbial samples. Stacked bar chart comparison of percent rRNA alignment by taxonomic composition between CRISPRclean Plus and Illumina Ribo-Zero Plus after depletion of rRNA at 5 ng and 50 ng input. Percent rRNA alignment by bacterial (purple), eukaryota (green), and archaeal (blue) taxonomic kingdoms are indicated.

A closer examination of the taxonomic composition of all three kingdoms of life revealed that while bacterial rRNA accounted for most of the overall remaining coverage following depletion (Figure 4, purple), the presence of relatively more eukaryota rRNA (Figure 4, green) and archaeal rRNA transcripts (Figure 4, blue) were observed in the CRISPRclean Plus depleted libraries compared to Illumina Ribo-Zero Plus.

CRISPRclean Plus is designed to deplete rRNA from 212 bacterial species, covering all phyla in a single depletion reaction. By comparison, Illumina Ribo-Zero Plus is only designed to deplete E. coli and B. subtilis rRNA. Indeed, analysis of the depletion content composition revealed that more bacterial rRNA was depleted using the CRISPRclean Plus method compared to Illumina Ribo-Zero Plus, producing a lower percent alignment to bacterial rRNA after depletion compared to both non-depleted control and Illumina Ribo-Zero Plus (Figure 4, purple). These data highlight the utility of CRISPRclean Plus depletion in enabling researchers to gain deeper insight into the taxonomic composition and diversity of complex microbial samples.

## CRISPRclean Plus depletion provides increased sensitivity for greater bacterial species detection

We next set out to evaluate the bacterial detection sensitivity of CRISPRclean Plus compared to Illumina Ribo-Zero Plus. The data revealed that CRISPRclean

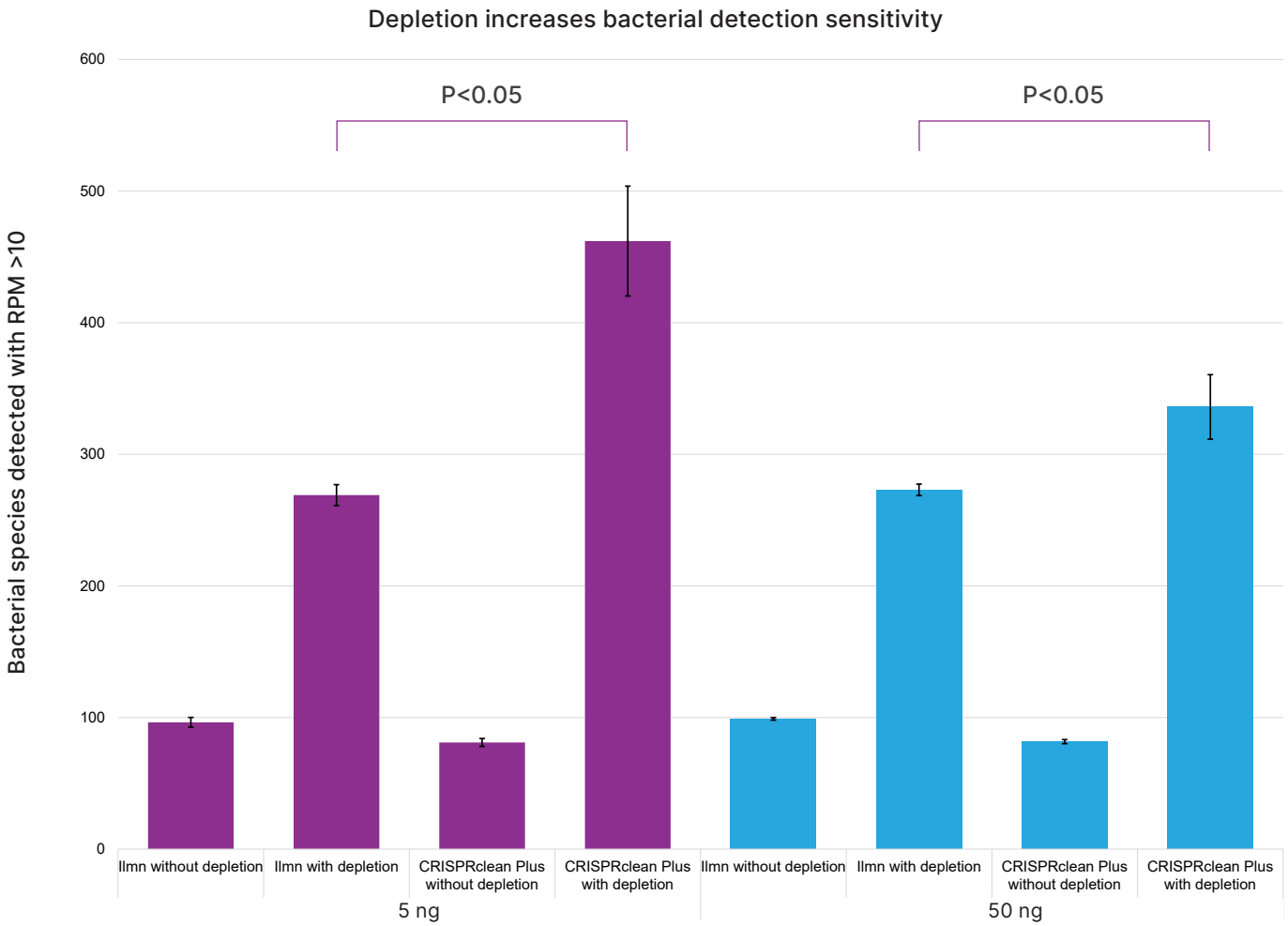


**Figure 5: CRISPRclean Plus yields increased bacterial detection sensitivity.** Observed 4–5 fold increase in bacterial species identified using CRISPRclean Plus compared to Illumina Ribo-Zero Plus after depletion of rRNA at 5 ng and 50 ng input. The cut-off is set at greater than or equal to 10 RPM (reads per million).

Plus produced a 4–5 fold increase in bacterial species detection compared to Illumina Ribo-Zero Plus following depletion (Figure 5). These data highlight the superior rRNA depletion utility of CRISPRclean Plus, thus enabling researchers to gain greater detection sensitivity into the quantity and composition of bacterial species in complex microbial samples.

## CRISPRclean Plus retains more bacterial species read counts

To further assess bacterial detection performance following depletion, we evaluated the impact of the CRISPRclean Plus and Illumina Ribo-Zero Plus methods on the retention of bacterial species in depleted vs. non-depleted samples. The data revealed that at 5 ng input, the CRISPRclean Plus method retained more bacterial species read counts than the Illumina Ribo-Zero Plus method (50 vs. 9-fold increased sensitivity, respectively; Figure 6). These data provide researchers with further confidence in the performance of CRISPRclean Plus rRNA depletion for providing increased bacterial detection.

## CRISPRclean Plus depletion does not perturb microbial composition

The inclusion of appropriate reference controls is an important consideration in metatranscriptomic microbiome profiling for method validation and comparing consistency across replicates. We evaluated the concordance of the data sets obtained from CRISPRclean Plus and Illumina Ribo-Zero Plus methods in depleted fecal samples. We used the Zymo control as a positive control.[2] Read counts for the top 10 bacterial species in the Zymo control were

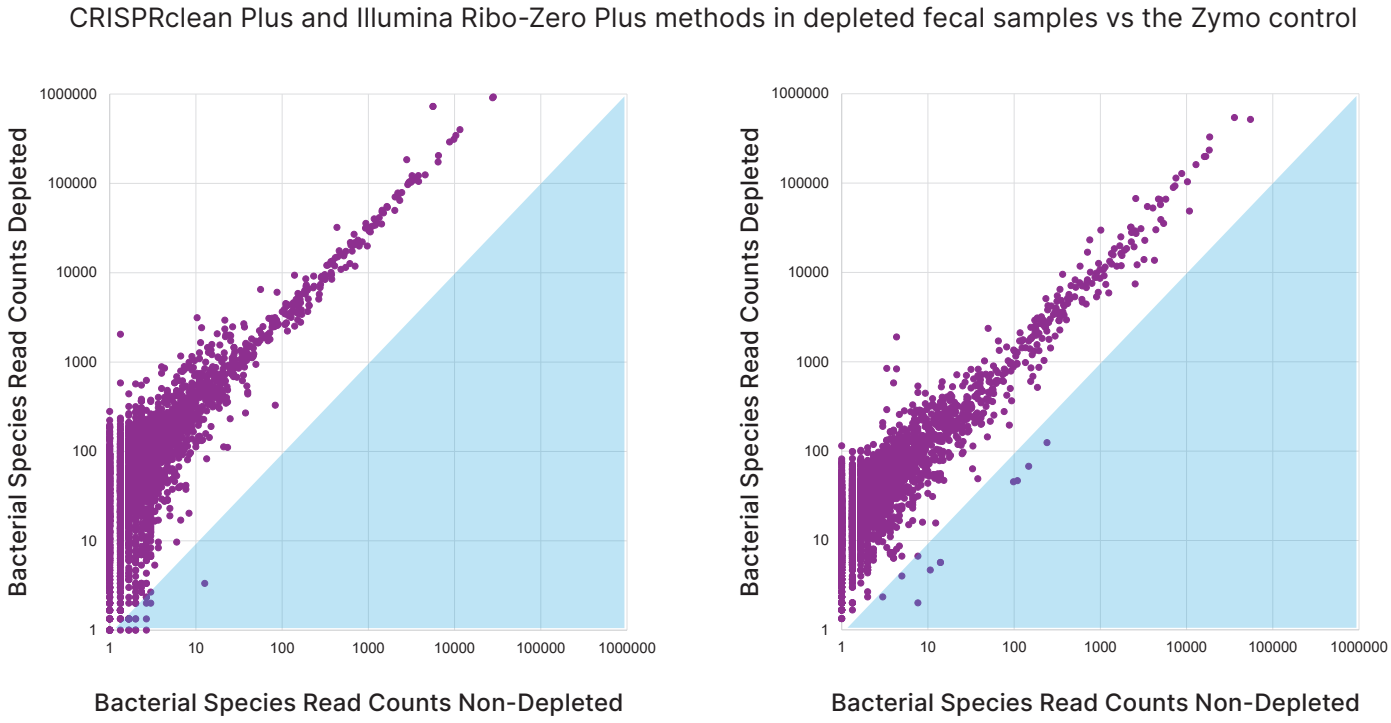


**Figure 6: CRISPRclean Plus retains more bacterial species read counts for depleted compared to non-depleted samples at 5 ng.** Read counts from replicates before and after depletion were higher for CRISPRclean Plus depleted samples (50-fold) compared to Ribo-Zero Plus for depleted samples (9-fold). Note that the y=x line of the shaded area indicates how the read counts would line up if the bacterial species read counts between the depleted and the non-depleted samples were balanced. A shift to the left shows that bacterial species read counts are higher for the CRISPRclean Plus methods compared to the Ribo-Zero Plus method (right-shifted correlation compared to CRISPRclean Plus).

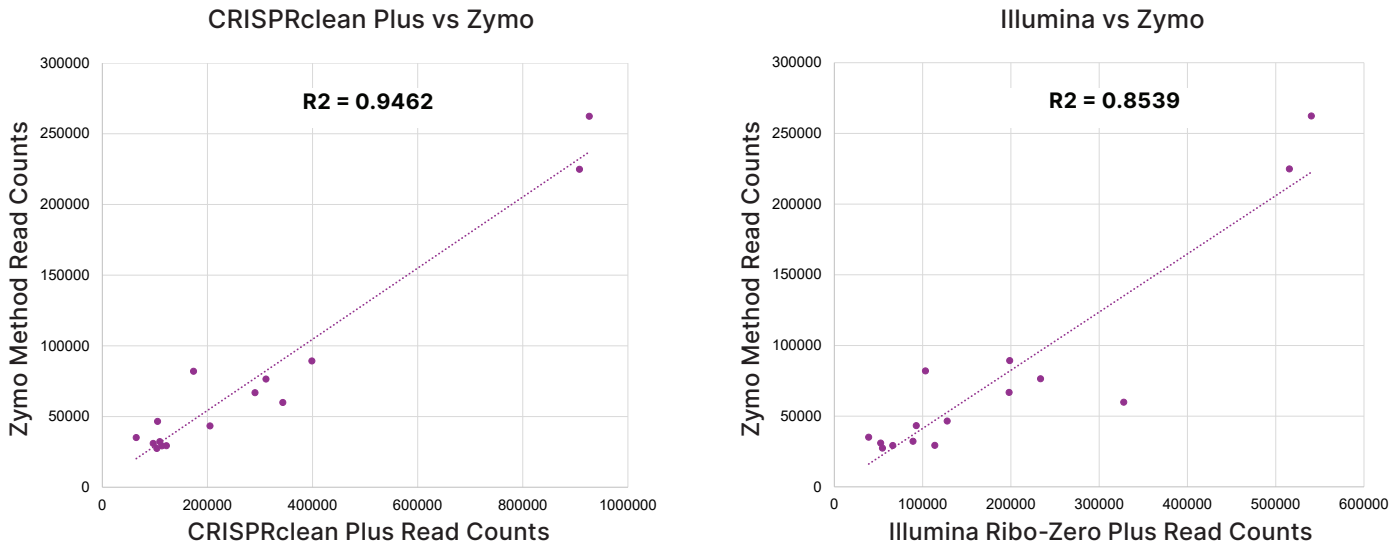


**Figure 7: CRISPRclean Plus depletion does not perturb microbial composition.** Read counts for the top 10 bacterial species in the Zymo method (y-axis) were compared to counts from the CRISPRclean Plus (x-axis) and Illumina Ribo-Zero Plus methods, at 5 ng total RNA input. Zymo Research provided read counts for the control sample. CRISPRclean Plus read counts were more highly correlated (i.e., R2 closer to 1.0) with the Zymo control, and produced extremely low library bias, compared to Illumina Ribo-Zero Plus.

compared to counts from the Illumina Ribo-Zero Plus and CRISPRclean Plus kits. Zymo Research provided read counts for the positive control sample. Our analysis revealed that CRISPRclean Plus read counts were more highly correlated with the Zymo control, and produced extremely low library bias, compared to Illumina Ribo-Zero Plus (Figure 7). Thus, the CRISPRclean Plus method provides a more accurate and unbiased representation of the top bacterial species in the Zymo control.

## Conclusion

CRISPRclean Plus offers more comprehensive and accurate insights into complex microbial communities. CRISPRclean Plus applied to fecal samples increases the number of identified bacterial species by 4-5 fold, providing researchers with greater biological insight.

**When compared to Illumina Ribo-Zero Plus:**

- CRISPRclean Plus outperforms Illumina Ribo-Zero Plus in depletion rates.
- CRISPRclean Plus is a more accurate method for detecting bacterial species in fecal samples compared to Illumina Ribo-Zero Plus.
- CRISPRclean Plus provides deeper insight into the taxonomic composition of the microbiome in fecal samples than Illumina Ribo-Zero Plus.
- CRISPRclean Plus retains more bacterial species after depletion than Illumina Ribo-Zero Plus.
- CRISPRclean Plus read counts are more highly correlated with the Zymo control and produced extremely low library bias.

**To learn more, visit jumpcodegenomics.com/products/stranded-rna-prep-hmr-bacteria-rrna/**

## References

1. O'Neil, D., Glowatz, H. and Schlumpberger, M. (2013), Ribosomal RNA Depletion for Efficient Use of RNA-Seq Capacity. Current Protocols in Molecular Biology, 103: 4.19.1-4.19.8.
2. Zymo Research (2021) ZymoBIOMICS Fecal Reference datasheet (ds1714). Accessed March 10, 2022