

Ribosomal RNA depletion for metatranscriptomic studies

Ribo-Zero™ Plus Microbiome
RNA-to-analysis solution
enables deeper insights into
microbial activity

- Optimize RNA sequencing of complex microbial samples, including stool samples
- Access an easy-to-use software solution for metatranscriptomics analysis
- Focus on high-value portions of the transcriptome to understand the role of the microbiome in health and disease

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Introduction

The microbiome can impact multiple aspects of health and disease. Traditional microbiome research uses 16S gene sequencing to look at overall bacterial diversity or shotgun metagenomics to determine the functional potential of microbes at the strain level. In complement to these DNA-based methods, RNA sequencing, or metatranscriptomics, provides direct information about active gene expression to understand the metabolic profile of the microbiome. However, metatranscriptome sequencing is impeded by the high relative abundance of ribosomal RNA (rRNA) in bacterial cells, comprising $\geq 85\%$ of molecules in total RNA.¹ Removal of rRNAs prior to sequencing enables researchers to focus on analyzing high-value, informative portions of the transcriptome.

Illumina offers an RNA-to-analysis solution (Figure 1) for metatranscriptome sequencing of complex microbial samples, such as stool samples. The Ribo-Zero Plus Microbiome Depletion Kit allows for deeper analysis and insight into microbial activity by reducing unwanted reads and increasing coverage of expressed bacterial RNA. Ribo-Zero Plus Microbiome Depletion Kit includes depletion oligonucleotide pools that remove rRNA from bacteria common in the adult and infant human gut, as well as host RNA from human, mouse, and rat. The kit also includes access to a complementary BaseSpace™ Microbiome Metatranscriptomics app, that runs pipelines to analyze complex metatranscriptomes with an easy-to-use graphical interface.

This technical note demonstrates rRNA depletion performance on stool samples with the Illumina Stranded Total RNA Prep, Ligation with Ribo-Zero Plus Microbiome Depletion Kit, including some highlight outputs from the BaseSpace Microbiome Metatranscriptomics app.

Methods

Sample preparation

Stool samples from healthy adults and infants were collected using the OMNIgene•GUT DNA and RNA collection tool (DNA Genotek, Catalog no. OMR-205). Total RNA was extracted from 100 ng of the combined adult and infant samples using the RNeasy PowerMicrobiome Kit (QIAGEN, Catalog no. 26000-50) following the manufacturer's protocol. These stool RNA samples are referred to as "G" samples. Control stool samples were purchased from ZymoBIOMICS Fecal Reference with TruMatrix Technology (ZymoBIOMICS, Catalog no. D6323). Total RNA was extracted from 100 ng control sample using the ZymoBIOMICS RNA mini kit (ZymoBIOMICS, Catalog no. R2001). These stool RNA samples are referred to as "Z" samples.

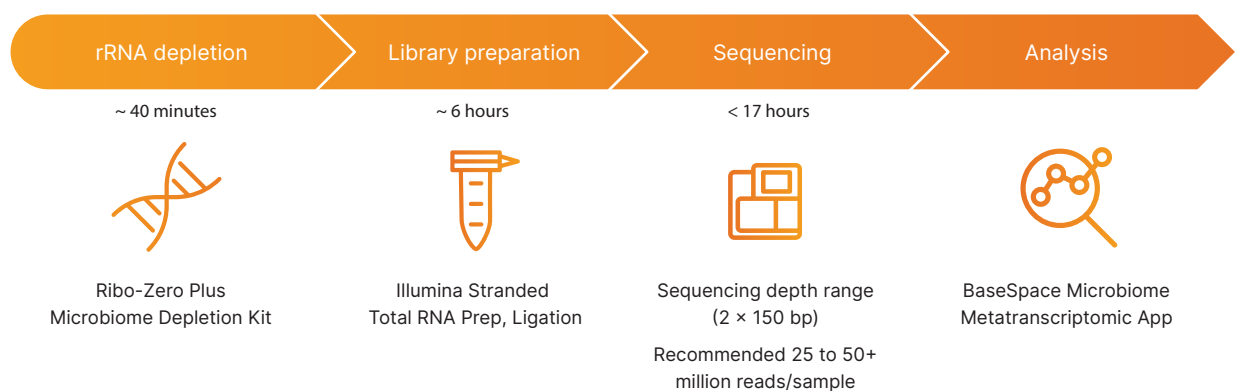


Figure 1: Ribo-Zero Plus Microbiome Depletion Kit in NGS-based RNA-sequencing (RNA-Seq) workflows—The Ribo-Zero Plus Microbiome Depletion Kit can be incorporated into existing workflows for RNA-Seq to effectively eliminate undesirable rRNA from total RNA samples in ~40 minutes.

Library preparation

For rRNA depletion, RNA is hybridized with DNA probes complementary to rRNA. These probes enable targeted digestion of rRNA molecules by RNase H. The DNA probes are then digested with DNase I, followed by a bead purification of the remaining, intact RNA.² Ribodepleted RNA samples then undergo library preparation. The Illumina Stranded Total RNA Library Prep, Ligation with Ribo-Zero Plus Microbiome (Illumina, Catalog no. 20072063) includes both DP1 and DPM depletion probe sets. DP1 is the depletion probe set found in the Ribo-Zero Plus Depletion Kit (Illumina, Catalog no. 20037135), while DPM is the newest microbiome-targeting rRNA depletion probe set. The DPM probe set was developed as described in Tan et al.³ Briefly, probes were designed to deplete rRNA found in healthy human stool samples, including adults and infants, and all 38 bacterial species in the American Type Culture Collection (ATCC) mock community samples (MSA-2002, MSA-2005, and MSA-2006).

Libraries were prepared as described in the Illumina Stranded Total RNA Prep, Ligation with Ribo-Zero Plus, Ribo-Zero Plus Microbiome Reference Guide⁴ with either no depletion, Ribo-Zero Plus (DP1 only) depletion, or Ribo-Zero Plus Microbiome (DP1 + DPM) depletion. A third-party rRNA depletion kit was also tested on the same samples using the standard procedure. PCR cycles were increased to 15 from the recommended 13 in the protocol for library preparation.

Sequencing

Libraries were pooled and sequenced on the NextSeq™ 2000 System using a 2 × 150 bp read length. Loading concentration was 750 pM with 1% PhiX spike-in on a NextSeq 1000/2000 P2 flow cell (Illumina, Catalog no. 20046813). While samples will vary, 50M+ reads/sample is expected to be sufficient to obtain most of the uniquely expressed gene counts and capture full gene function diversity, including rarely expressed genes.

Data analysis

All sequence filtering, alignment, and transcript coverage were performed in BaseSpace Sequence Hub using the BaseSpace Microbiome Metatranscriptomics app (included with the Illumina Stranded Total RNA Prep with Ribo-Zero Plus Microbiome Depletion Kit) with default settings (Figure 2).

Results

The BaseSpace Microbiome Metatranscriptomics app provides the necessary outputs and figures to determine which bacteria are present in a sample and the functional pathways those bacteria are using in their environment. The BaseSpace Microbiome Metatranscriptomics app workflow includes BBduk, HUMAnN 3.0, and Kaiju bioinformatics pipelines (Figure 2).⁵⁻⁷

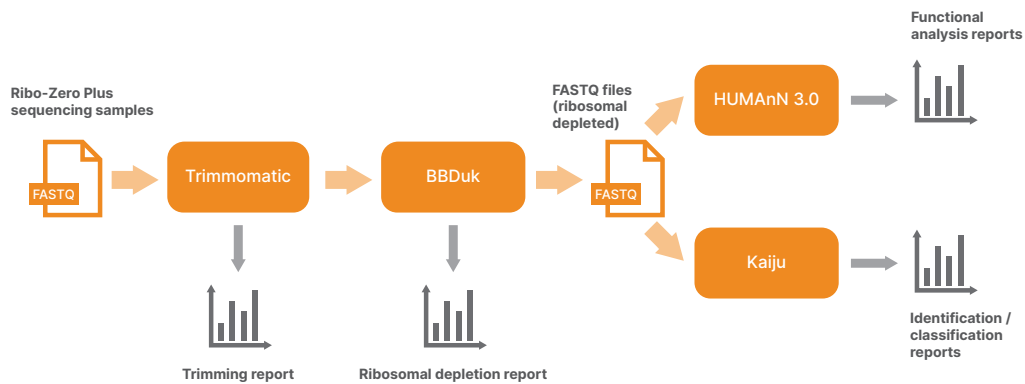


Figure 2: BaseSpace Microbiome Metatranscriptomics app workflow—The BaseSpace Microbiome Metatranscriptomics app is included with the Ribo-Zero Plus Microbiome Depletion Kit to offer bioinformatics tools needed for comprehensive metatranscriptome analysis.

The BBDuk pipeline⁵ reports rRNA and host RNA that was bioinformatically filtered (Figure 3 and Figure 4). Retained reads are the remaining RNA. The HUMAnN 3.0 pipeline⁶ assigns relative abundance by gene function and pathways (Figure 5). The Kaiju pipeline⁷ assigns taxonomy, which is displayed in an interactive Krona plot (Figure 6).

RNA sequencing libraries from two mixed stool samples, sample G and sample Z, were prepared without rRNA depletion or with one of three rRNA depletion kits:

Ribo-Zero Plus (which was designed for single-species rRNA depletion), Ribo-Zero Plus Microbiome Depletion Kit, or a third-party kit. The BBDuk pipeline provided detailed information regarding which rRNA genes or host RNA remain after depletion (Figure 3). As expected, greater than 98% of nondepleted libraries were rRNA. The Ribo-Zero Plus Microbiome Depletion Kit effectively depleted unwanted rRNA and host RNA and resulted in less rRNA than either Ribo-Zero Plus or the third-party rRNA depletion kit (Figure 4).

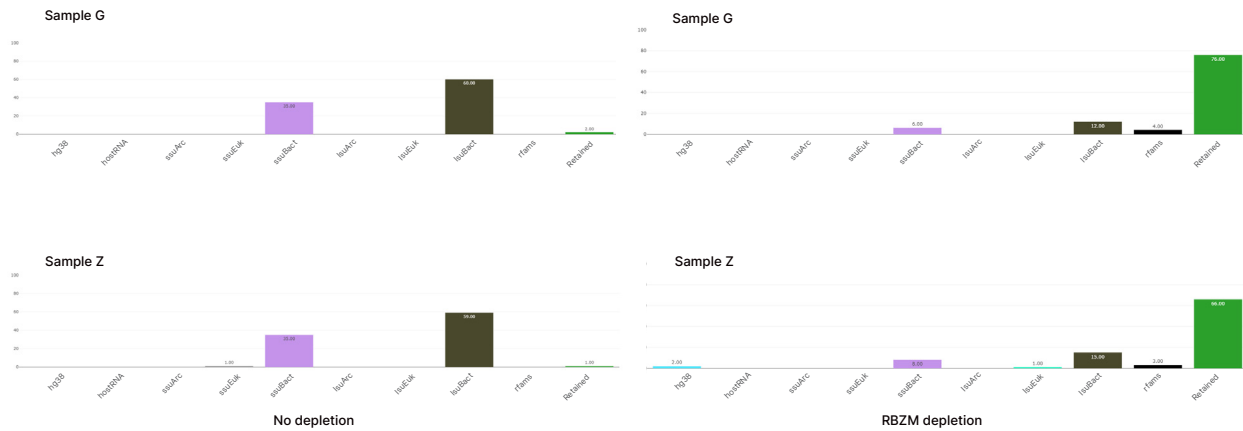


Figure 3: BBDuk output for percent rRNA remaining after depletion—Representative BBDuk output using the BaseSpace Microbiome Metatranscriptomics app for sample G and sample Z, both processed with the Ribo-Zero Plus Microbiome Depletion Kit. The green bar represents the RNA of interest to the microbial research community. The other categories are unwanted and/or host RNA that is filtered out. RBZM, Ribo-Zero Plus Microbiome Depletion Kit.

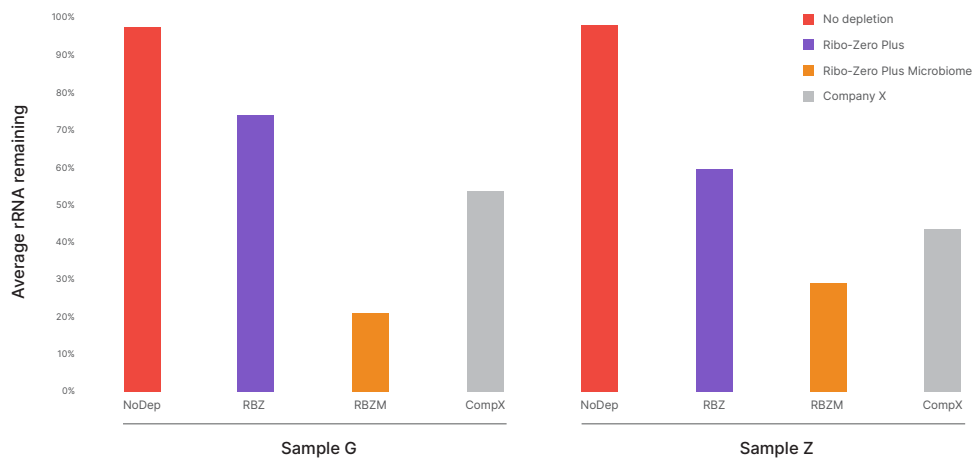


Figure 4: Percent rRNA remaining after depletion—Robust ribodepletion with Ribo-Zero Plus Microbiome Depletion Kit (pumpkin bars). Less rRNA is desirable. Results are compared to Ribo-Zero Plus rRNA Depletion Kit (purple bars) and a commercially available third-party ribodepletion kit (gray bars). NoDep, no depletion; RBZ, Ribo-Zero Plus only depletion; RBZM, Ribo-Zero Plus Microbiome depletion; CompX, third-party depletion kit.

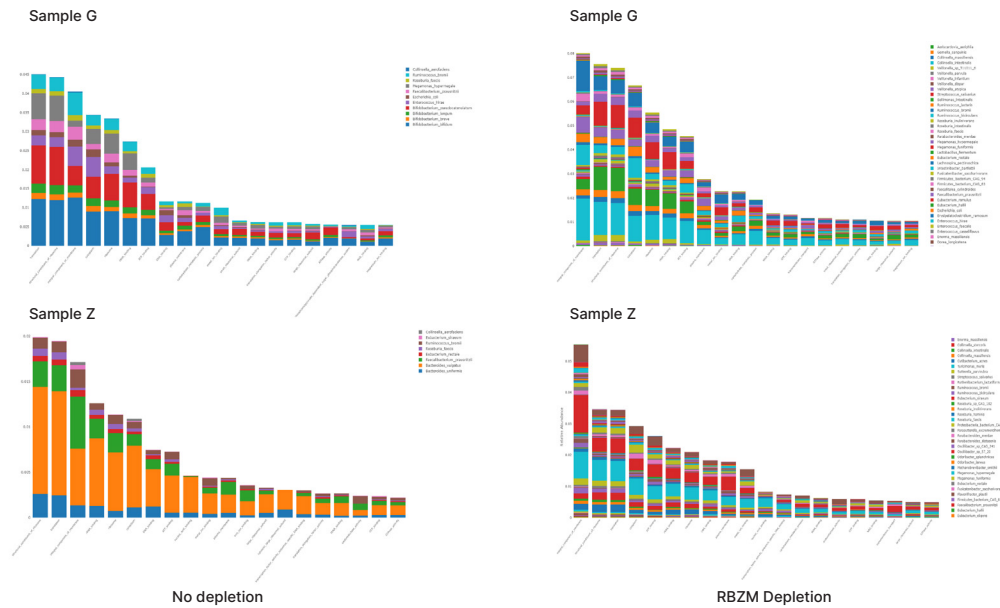


Figure 5: With rRNA depletion, more gene families can be sequenced at finer resolution—Results from HUMAnN 3.0 pipeline graphed according to gene families for the top 20 gene ontology (GO) terms with predicted bacterial contributions. Each color in the bar graphs represents individual microbial species contributing to the metatranscriptome. Sample G and sample Z processed with Ribo-Zero Plus Microbiome (right panels) show higher resolution of individual microbial contribution for each gene than the samples without depletion.

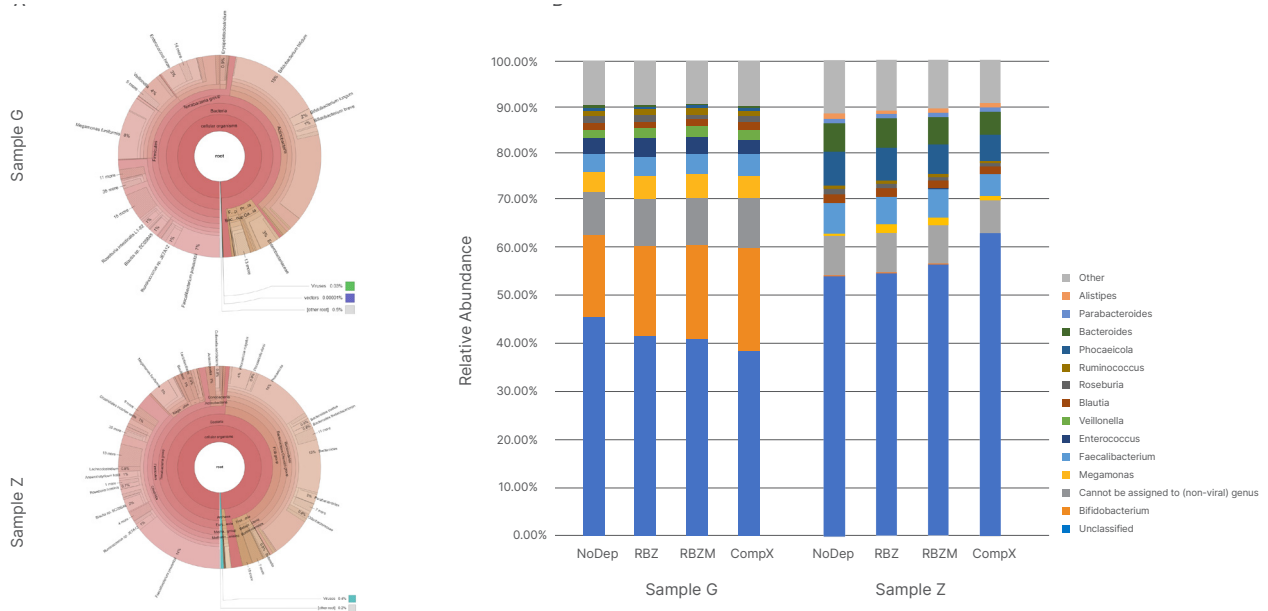


Figure 6: Taxonomic profiles in a format that is easy to visualize and download—Kaiju pipeline results from the Microbiome Metatranscriptomics BaseSpace App. (A) Krone plots from two different samples processed with Ribo-Zero Plus Microbiome depletion. The Microbiome Metatranscriptomics BaseSpace App Krone graph is dynamic and allows for further exploration of the microbial diversity in the samples, including viruses and nonbacterial taxa. (B) A TSV file that can be graphed in Excel is available in the outputs. Taxonomy is consistent across the various methods. Note that similar taxa may have homologous RNA and thus this method is only as accurate as the databases.

Data analysis from HUMAnN 3.0 pipeline showed how rRNA depletion is critical to reduce undesired reads and optimize sequencing for expressed genes. Data from libraries prepared with the Ribo-Zero Plus Microbiome Depletion Kit revealed greater diversity of gene families at finer resolution, identifying which species were contributing to gene expression, compared to nondepleted libraries (Figure 5). The BaseSpace Microbiome Metatranscriptomics app aids with additional metatranscriptomic analysis, such as taxonomy, presented in interactive Krona graphs and Excel-compatible file formats (Figure 6).

Summary

The Ribo-Zero Plus Microbiome Depletion Kit offers an RNA-to-analysis workflow solution for metatranscriptomic research. The Ribo-Zero Plus Microbiome depletion probe sets reduce unwanted rRNA reads from stool samples and increase RNA reads of interest. In addition, the complementary analysis software democratizes this method for both experienced and new microbiome users. The focus on analyzing high-value portions of the transcriptome allows deeper insight into active gene function in the microbiome. Understanding how the microbiome responds to treatments, diets, or disease states can inform biomedical research and potentially lead to better and more targeted treatments.

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Illumina Stranded Total RNA Prep with Ribo-Zero Plus Microbiome Depletion Kit, [illumina.com/products/by-type/sequencing-kits/library-prep-kits/stranded-total-rna-prep](https://www.illumina.com/products/by-type/sequencing-kits/library-prep-kits/stranded-total-rna-prep)

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