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Microbial RNA sequencing enabled with the Illumina Ribo-Zero™ Plus rRNA Depletion Kit

Focus on analyzing high-value portions of the transcriptome and save on sequencing costs.

Introduction

Microbial transcriptomic analysis by RNA sequencing is impeded by the high relative abundance of ribosomal RNA (rRNA) in bacterial cells, comprising ≥ 85% of molecules in total RNA.[1] Removal of rRNA prior to RNA-Seq enables researchers to focus on analyzing high-value, informative portions of the transcriptome while lowering sequencing costs. To assist with rRNA removal, Illumina offers the Ribo-Zero Plus rRNA Depletion Kit.

How it works

This new methodology facilitates rich transcriptome analyses of microbial isolates. The single-tube enzymatic ribodepletion method is compatible with low inputs (≤ 100 ng) and reduces rRNA in both prokaryotic and eukaryotic samples. Abundant rRNA is removed from total RNA by targeted hybridization to DNA probes and subsequent RNase H-mediated cleavage. Ribodepleted samples then undergo library preparation, sequencing, and data analysis.

This technical note demonstrates rRNA depletion performance on microbial isolate and community samples with the Illumina Ribo-Zero Plus rRNA Depletion Kit, as compared to the Ribo-Zero kit (previously available with TruSeq™ Stranded Total RNA and TruSeq Stranded mRNA).

Methods

Sample preparation

Several microbial isolates and mock community standards were obtained from ATCC for evaluation (Table 1). Total RNA was extracted using the RNeasy PowerMicrobiome Kit (QIAGEN, Catalog no. 26000-50), following the manufacturer's protocol. Total RNA integrity and quantity were measured using the Agilent Bioanalyzer (Agilent, Catalog no. G2939BA).

Table 1: Microbial samples tested in this study

Sample type	Microbial species
Microbial isolates	*B. cereus, B. subtilis, E. cloacae, E. coli, S. epidermis*
ATCC MSA-2002 (20-strain even mix)	*A. baumannii, A. odontolyticus, B. adolescentis, B. cereus, B. vulgatus, C. acnes, C. beijerinckii, D. radiodurans, E. coli, E. faecalis, H. pylori, L. gasseri, N. meningitidis, P. aeruginosa, P. gingivalis, R. sphaeroides, S.agalactiae, S. aureus, S. epidermidis, S. mutans*
ATCC MSA-2006 (human gut mix)	*B. adolescentis, B. fragilis, B. vulgatus, C. difficile, E. cloacae, E. coli, E. faecalis, F. nucleatum, H. pylori, L. plantarum, S. enterica, Y. enterococolitica,*

Ribosomal RNA depletion

10–250 ng of total RNA from each sample were processed for enzymatic ribodepletion with the Illumina Ribo-Zero Plus rRNA Depletion Kit (Illumina, Catalog no. 20037135) (Figure 1). Briefly, RNA is hybridized with DNA probes complementary to rRNA. These probes target RNase H to digest rRNA species. The DNA probes are then digested with DNase I, followed by a bead purification of the remaining, intact RNA. For comparison, total RNA from each sample was also processed with the Ribo-Zero rRNA Removal Kit.[2]

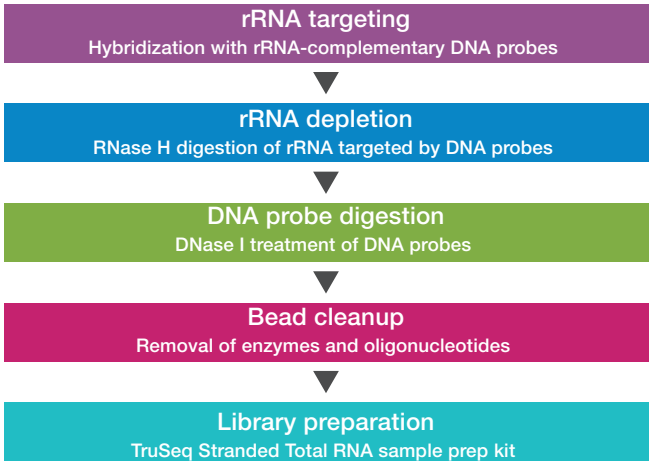

Figure 1: Illumina Ribo-Zero Plus ribodepletion workflow.

Library preparation and sequencing

Ribodepleted and nondepleted RNA (control) were prepared for sequencing using the TruSeq Stranded Total RNA LIbrary Prep kit*.[3] Libraries were pooled and sequenced on a MiSeq™ or NextSeq™ 550 System, using a 2 × 76 bp paired-end read length.

Data analysis

All sequence filtering, alignment, and transcript coverage were performed in BaseSpace™ Sequence Hub using a custom workflow (Figure 2). This analysis workflow is applicable to any microbe with an available annotated genome. Transcript counts (> 0.1 transcripts per million (TPM) generated by the RNA-Seq Alignment App were used for pairwise linear regression and correlation analysis. To quantify rRNA from multiple strains within microbial community samples, the respective rRNA sequences (from NCBI annotated genomes) were used as inputs for the custom analysis workflow.

* Indicates recommended library preparation kit; protocol is compatible with other kits.

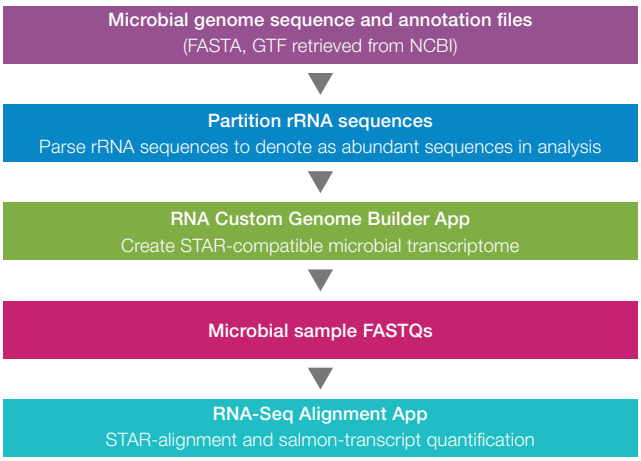

Figure 2: Microbial RNA-Seq custom analysis workflow—The custom analysis workflow involves using the RNA Custom Genome Builder App to create a STAR-compatible microbial transcriptome. The RNA-Seq Alignment App is used for STAR-alignment[4] and salmon-transcript[5] quantification.

Results

Effective rRNA removal with the Illumina Ribo-Zero Plus kit across various microbial species

Transcriptome sequencing results of five microbial isolates prepared with the Illumina Ribo-Zero Plus kit, Ribo-Zero kit, or no ribodepletion were compared. Ribodepletion with the Illumina Ribo-Zero Plus kit was highly effective and outperformed the Ribo-Zero kit in all species tested, as measured by percent rRNA reads (Figure 3). The improvement was most significant on a low-input sample (*E. coli* 10 ng RNA), with < 0.5% vs 13% average rRNA reads for Illumina Ribo-Zero Plus and Ribo-Zero, respectively.

Significant reduction in minimum sequencing depth for transcript detection with the Illumina Ribo-Zero Plus kit

To assess the enrichment of biologically important RNA reads with the Illumina Ribo-Zero Plus kit, the number of transcripts detected at different read depths was compared with and without ribodepletion (Figure 4). In general, a 20–50× reduction in read depth was needed to detect an equivalent number of transcripts with the Illumina Ribo-Zero Plus kit. The lower read depth requirement greatly reduces the cost of sequencing for microbial transcriptome profiling.

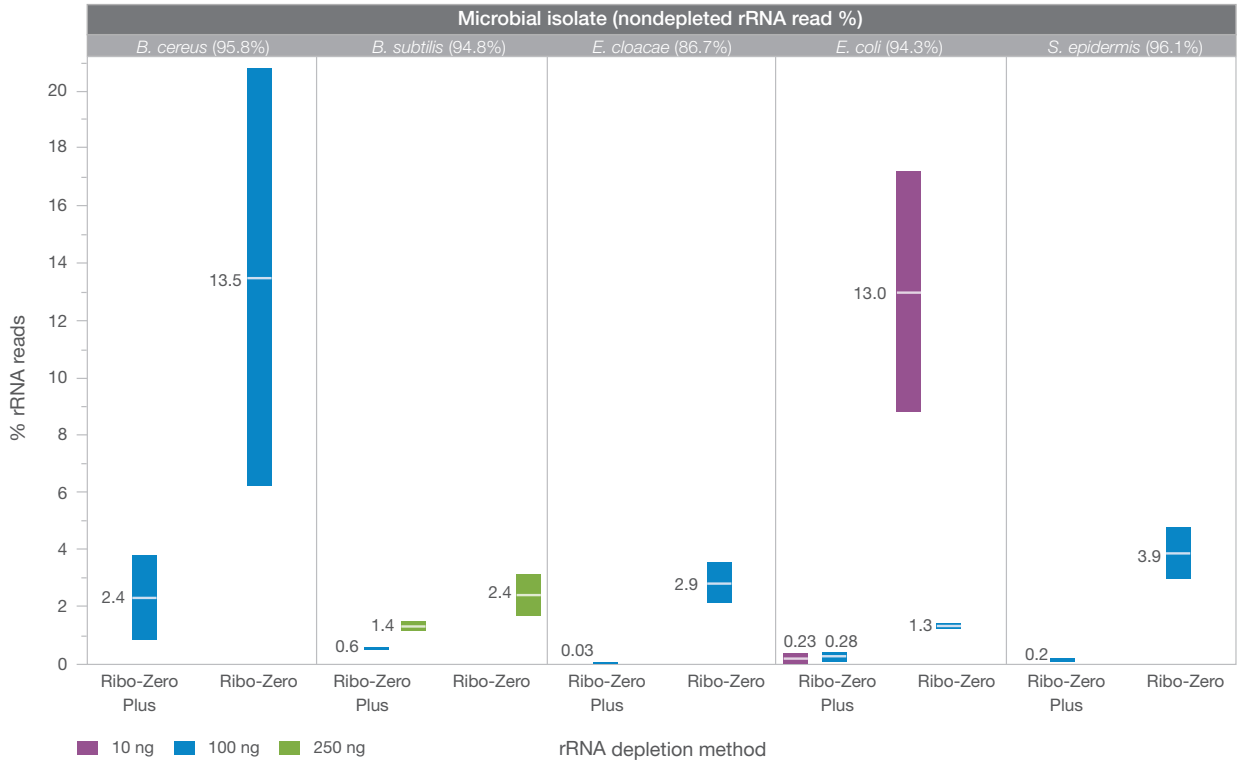

Figure 3: Comparison of ribodepletion methods—Enzymatic ribodepletion was compared against the Ribo-Zero rRNA Removal Kit for rRNA depletion from five microbial species. For each species, all sample read depths were normalized and the mean percent rRNA reads from two replicates is shown.

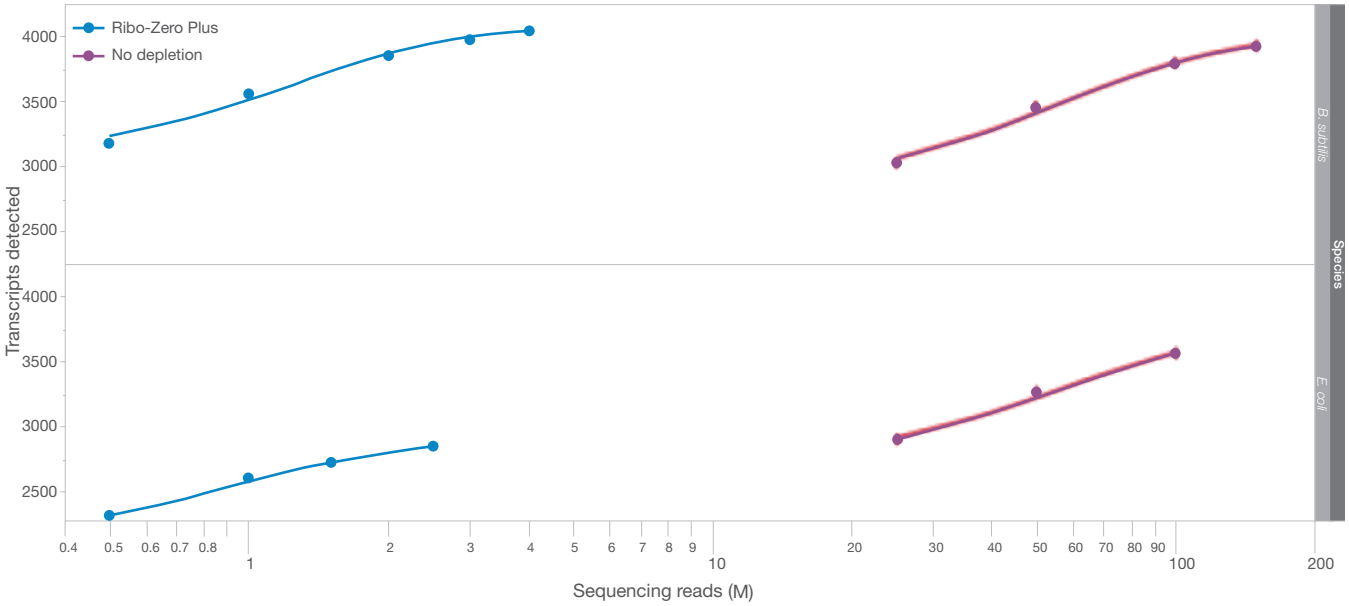

Figure 4: Transcript detection at various read depths—The number of transcripts detected with and without enzymatic ribodepletion was compared at various read depths for *B. subtilis* and *E. coli*. Enzymatic ribodepletion resulted in a 20×–50× reduction in read depth needed to detect an equivalent number of transcripts, as compared to nondepleted sequencing results.

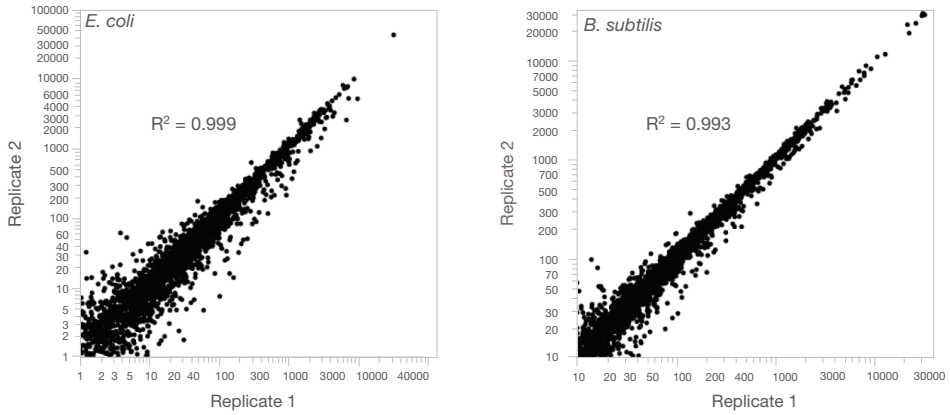

Figure 5: Reproducible transcriptome analysis—A pairwise linear regression of gene expression levels (determined as TPMs) between technical replicates was performed. High correlation ($R^2 > 0.99$) was observed for two different microbial isolates, E. coli and *B. subtilis*.

Reproducible transcriptome sequencing of microbial species enabled with the Illumina Ribo-Zero Plus kit

To demonstrate the reproducibility of RNA-Seq data enabled with the Illumina Ribo-Zero Plus kit, a pairwise linear regression of gene expression levels (determined as TPMs) between technical replicates was performed. High correlation ($R^2 > 0.99$) was observed for two different microbial isolates, *E. coli* and *B. subtilis* (Figure 5).

Effective rRNA removal in metatranscriptomic samples with the Illumina Ribo-Zero Plus kit

After demonstrating the effectiveness of the Illumina Ribo-Zero Plus kit with microbial isolates, ribodepletion was evaluated with metatranscriptomic samples. Two mock community samples from ATCC (Table 1) were tested with inputs of 10 ng and 80 ng total RNA. The Illumina Ribo-Zero Plus kit outperformed the Ribo-Zero kit, reducing the percent rRNA reads from an undepleted level of ~83% to < 2%, and from ~95% to < 13%, for samples MSA2002 and MSA2006, respectively (Figure 6). The Ribo-Zero kit resulted in less effective rRNA removal and also greater variability between replicates (Figure 6).

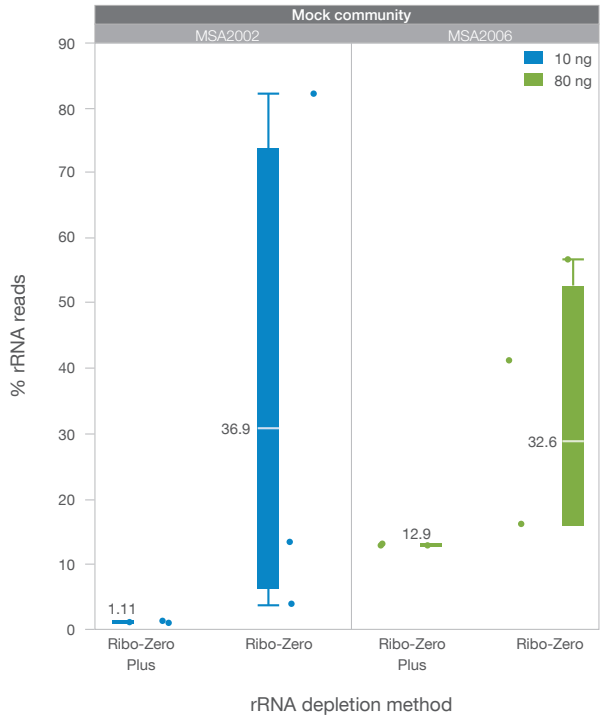

Figure 6: Effective ribodepletion in mock communities—The Illumina Ribo-Zero Plus kit outperformed the Ribo-Zero kit with lower, less variable amounts of rRNA present after depletion, as measured by percent rRNA reads for two different microbial mock communities with different input amounts.

Summary

The Illumina Ribo-Zero Plus rRNA Depletion Kit is a robust, effective, and simple method to reduce rRNA content for high-quality microbial transcriptome analysis. The kit meets or exceeds Ribo-Zero performance metrics in all the tested microbial isolates and "microbiome" mock community samples. The Illumina Ribo-Zero Plus kit is uniquely compatible with low inputs, facilitating metatranscriptomic analysis of low biomass samples.

Learn more

Learn more about the Illumina Ribo-Zero Plus rRNA Depletion Kit, visit www.illumina.com/products/by-type/accessory-products/ribo-zero-plus-rrna-depletion.html

References

1. Karpinets TV, Greenwood DJ, Sans CE, Ammons JT. RNA: protein ratio of the unicellular organism as a characteristic of phosphorous and nitrogen stoichiometry and of the cellular requirement of ribosomes for protein synthesis. *BMC Biol*. 2006;4:30.
2. Illumina (2016).Ribo Zero rRNA Removal Kit Reference Guide (15066012 v02) Accessed March 16, 2020.
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4. Dobin A, Daves CA, Schlesinger F, et al. STAR: ultrafast universal RNA seq aligner. *Bioinformatics*. 2013;29(1):15–21.
5. Patro R, Duggal G, Love MI, Irizarry RA, Kingsford C. Salmon provides fast and bias-aware quantification of transcript expression. *Nat Methods*. 2017;14(4):417–419.

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