HHS Public Access

Author manuscript

Nat Protoc. Author manuscript; available in PMC 2022 April 01.

Published in final edited form as:

Nat Protoc. 2021 October ; 16(10): 4676–4691. doi:10.1038/s41596-021-00592-4.

Simultaneous ribosome profiling of hundreds of microbes from the human microbiome

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Abstract

Ribosome profiling enables sequencing of ribosome-bound fragments of RNA, revealing which transcripts are being translated as well as the position of ribosomes along mRNAs. Although ribosome profiling has been applied to cultured bacterial isolates, its application to uncultured, mixed communities has been challenging. We present MetaRibo-Seq, a protocol that enables the application of ribosome profiling directly to the human fecal microbiome. MetaRibo-Seq is a benchmarked method that includes several modifications to existing ribosome profiling protocols, specifically addressing challenges involving fecal sample storage, purity and input requirements. We also provide a computational workflow to quality control and trim reads, de novo assemble a reference metagenome with metagenomic reads, align MetaRibo-Seq reads to the reference, and assess MetaRibo-Seq library quality (https://github.com/bhattlab/bhattlab_workflows/tree/master/metariboseq). This MetaRibo-Seq protocol enables researchers in standard molecular biology laboratories to study translation in the fecal microbiome in ~5 d.

Introduction

Regulation of gene expression at the level of translation is essential for the adaptability and survival of bacteria. For example, genes involved in translation itself are regulated at a translational level via feedback mechanisms[1–5]. Translational regulation is critical for generating proteins at the correct stoichiometry for many protein complexes[6]. This regulation even extends to pathway-specific enzyme stoichiometry in which protein synthesis levels across taxa remain correlated even as transcript abundance and architecture diverges over evolutionary time[7]. Transcript abundance and protein synthesis rates can differ by orders of magnitude[6]. Moreover, specific translational regulation has been extensively observed upon a variety of perturbations to bacteria[8–14]. Taken together, the ability to study

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Author contributions

B.J.F. and A.S.B. conceived the study. B.J.F performed all experiments and data analysis. C.N. wrote the Nextflow computational pipeline. B.J.F. and A.S.B. wrote the paper with input from all authors. All authors read and approved the final manuscript.

Competing interests

The authors declare no competing interests.

translation in bacteria is critical for our understanding of their functions—yet, methods that enable the comprehensive study of translation in microbiomes have been lacking until now.

Existing methods for profiling bacterial translation

During translation, ribosomes protect small fragments of RNA from nuclease digestion. Capitalizing on this observation, ribosome profiling (Ribo-Seq) is an approach that sequences ribosome-protected fragments of mRNA after degrading unprotected mRNA with nuclease. First developed by Ingolia and Weissman for application in yeast, Ribo-Seq enables the study of transcriptome-wide translation and its regulation in vivo[15]. Many modifications and extensions to the protocol have been implemented since this initial application, enabling the application of this approach to a range of other cell types[16].

Ribo-Seq was later applied to bacteria for the first time by Weissman and Bukau[17–19]. While the method is conceptually similar to the original Ribo-Seq protocol, unique challenges arose upon application of Ribo-Seq to bacteria, such as determining which methods to employ to stop elongation of ribosomes and choices of nucleases to generate ribosome-protected footprints[17–19]. New applications and modifications are being constantly implemented in bacterial Ribo-Seq, some of which are inspired by advances first discovered in eukaryotic Ribo-Seq. For example, while traditional Ribo-Seq involves enrichment of monosomes, which are single ribosomes bound to RNA, with a sucrose density gradient, protocols using size-exclusion columns have emerged as a replacement for sucrose density gradients. Size-exclusion columns are faster, require less equipment and produce comparable results[20]. Size-exclusion columns and monosome recovery were later implemented in bacterial Ribo-Seq protocols and demonstrated performance characteristics similar to those observed in eukaryotic Ribo-Seq[21].

In addition to adaptations of methods for ribosome recovery, researchers have also experimented with various antibiotics and chemicals that inhibit translation by different mechanisms. Traditionally, bacterial Ribo-Seq was performed after the addition of chloramphenicol, a ribosome-targeting antibiotic that halts translation[17–19]. Subsequent adaptations have focused on enriching start sites and stop sites, and trying to improve single-codon resolution in bacterial Ribo-Seq signal. For example, antibiotics such as tetracycline[22] and, more recently, retapamulin[23] have been used to identify translation start sites; similarly, apidaecin, an antimicrobial peptide from bees, has been used to identify translation stop sites[24]. Unlike Ribo-Seq in eukaryotes, bacterial Ribo-Seq often struggles to achieve single-nucleotide resolution, in part due to the use of chloramphenicol to halt translation[25,26]. It has been shown that using the endonuclease RelE improves triplet periodicity, or the enrichment of in-frame signal, in bacterial Ribo-Seq[25]. More recently, high-salt buffers have been shown to halt translation better than chloramphenicol and improve resolution of triplet periodicity and pausing[26]. Overall, bacterial Ribo-Seq has a number of unique challenges that are continuing to be addressed by the research community.

Development of MetaRibo-Seq

Historically, Ribo-Seq has been performed on one cultured organism at a time. Consequently, our ability to study translation in high throughput has largely been limited

to a small group of mostly model organisms in somewhat artificial culturing conditions. It would be advantageous to perform Ribo-Seq on a broader range of organisms, culture-free and on many organisms at once. By modifying existing Ribo-Seq protocols, we have enabled the direct study of translation in microbiomes. Previously, Ribo-Seq had never been implemented on uncultured mixtures of bacteria for three reasons: first, standard Ribo-Seq protocols are implemented on freshly grown bacteria in which culturing and growth conditions can be designed appropriately. It is often unrealistic to control the timing of fecal sample collection, and thus, storage methods for these fecal samples are needed; second, standard initial Ribo-Seq protocols require pure, concentrated bacterial cells. These protocols isolate bacterial cells by centrifugation and/or filtration of pure, cultured cells. Obtaining concentrated and pure bacteria from fecal samples is a challenge; third, standard Ribo-Seq protocols have high input requirements that require large quantities of bacteria. Culturing up to a liter of bacteria is typically necessary to satisfy input requirements.

We developed MetaRibo-Seq to solve these three problems. First, our method is compatible for use on frozen fecal samples stored in RNA preservative, not requiring fresh samples[27]. Second, we modified standard Ribo-Seq protocols to crudely isolate nucleic acids and ribonuclear complexes using ethanol precipitation instead of isolating bacterial cells. Thus, both the way we purify and the stage at which we purify ribonuclear complexes are different using MetaRibo-Seq. Third, we scaled down the input requirements and the micrococcal nuclease (MNase) reaction (to create ribosome-protected footprints). MetaRibo-Seq, with these modifications, demonstrates strong correlation with standard Ribo-Seq protocols and correlates more strongly to proteomics than RNA-Seq correlates to proteomics when benchmarked in a mock bacterial community[27].

Of note, some of these revisions are conceptually similar to revised Ribo-Seq protocols that were independently developed simultaneously by Buskirk[26]. In their protocol, the authors freeze whole cultures of 100 mL of bacteria into pellets without centrifuging or filtering bacteria first[28]. These pellets are mechanically lysed, and $MgCl_2$ is used to inhibit translation instead of chloramphenicol. Sucrose cushions are then used to purify ribosomes instead of purifying bacteria initially, which is conceptually similar to MetaRibo-Seq using an ethanol precipitation to more crudely purify nucleic acids and ribonuclear complexes. While Buskirk's systematically revised protocol would not be directly applicable to fecal samples, the conceptual advances of freezing bacteria first and inhibiting translation and purifying ribosomes later are similar to those implemented in MetaRibo-Seq.

Advantages and applications of MetaRibo-Seq

Traditional Ribo-Seq is limited to studying translation only in organisms that are cultured and assayed in vitro. With MetaRibo-Seq, researchers can now study translation in fecal microbiomes. Beyond studying regulation of translation, there is substantial interest in detecting and measuring the proteome in microbiome research. As a surrogate for proteomics, MetaRibo-Seq can provide information on the open reading frames that are translated. Translational confirmation is particularly valuable for validating predicted small open reading frames, whose protein products are difficult to detect experimentally. Because proteomics methods are limited in their ability to detect small proteins and poorly abundant

proteins, we rarely detect small proteins and often struggle to detect many larger proteins unless they are of relatively high abundance[29]. The ability to study protein synthesis is thus a great potential asset to microbiome researchers.

In addition to being a potential surrogate for proteomics, Ribo-Seq is a useful method to reannotate genes and identify new genes, especially small genes[30,31]. Ribo-Seq signal distribution across a genomic region can provide evidence that it likely encodes a protein. Previous work from our laboratory predicted thousands of novel families of small genes in microbiomes using comparative genomics[32]. Building on this framework, MetaRibo-Seq verified translation of hundreds of these novel families in the fecal microbiome and predicted thousands of additional small protein families in the human gut[27]. Many more unannotated small proteins likely exist in this space. MetaRibo-Seq has the potential to be instrumental in identifying additional small protein families in microbiomes. In fact, recent work used machine learning approaches to predict additional small genes and used MetaRibo-Seq to help validate predictions[33]. We anticipate more computational tools to predict small proteins by using MetaRibo-Seq signal distribution will be developed. The ability to study translation in microbiomes thus opens several new avenues of investigation for microbiome researchers.

MetaRibo-Seq can be used for several applications; therefore, the exact experimental design employed will vary based on the research objective of the study. If, for example, the goal is to study translational regulation in fecal samples over time, one could track specific strains of interest over time in people. This could be accomplished by performing metatranscriptomics and MetaRibo-Seq at time points of interest. In the design of such a study, we recommend careful planning and consideration of batch effects and replicates. Another potential study design of interest may be evaluating the impact of acute stresses, such as antibiotics, on a mixed microbial community. In this case, a researcher might choose to expose fecal samples to acute stresses ex vivo in short-term fecal cultures, and study translational regulation[34]. If the goal is to search for translation of genes for open reading frame prediction in individual samples, then metatranscriptomics may not be necessary and the relative timing of obtaining the different samples may require fewer considerations. However, it might be desirable to introduce an agent such as retapamulin, which enriches for start site positions and would thus enable accurate prediction of gene starts in bacteria whose ribosomes are sensitive to retapamulin-based inhibition. In addition to these applications, we expect that additional modifications will enable the study of translation in other types of microbiomes, such as environmental communities.

Overview of the procedure

We describe the MetaRibo-Seq protocol to perform Ribo-Seq on human fecal microbiomes (Fig. 1) and the computational workflow to create a de novo reference and assess library quality (Fig. 2). First, we describe lysis of microbes from fecal samples and the extraction of ribonuclear complexes (Steps 1–12), MNase digestion and purification of monosome footprints (Steps 13–28), and creation and sequencing of MetaRibo-Seq libraries (Steps 29–39). Second, we describe the computational workflow to assess MetaRibo-Seq library

quality, including quality trimming of reads, metagenomic assembly, MetaRibo-Seq read alignment to the assembly, and visualizations (Steps 40–43).

Limitations of MetaRibo-Seq

While MetaRibo-Seq benchmarks well against gold-standard Ribo-Seq, there are several limitations to consider. Although MetaRibo-Seq correlates more strongly to protein abundance in mock communities than RNA-Seq, standard Ribo-Seq still correlates more strongly to protein abundance than MetaRibo-Seq. MetaRibo-Seq is also a snapshot of translation; therefore, the absence of signal does not imply that a protein is not real or that a protein does not exist in the human gut. MetaRibo-Seq must also be coupled with metagenomic assemblies to create a suitable reference for mapping MetaRibo-Seq reads, which requires additional bench and computational work, and also increases experimental costs.

MetaRibo-Seq also inherits many of the limitations of standard Ribo-Seq, such as contamination by other RNA–protein complexes or structured RNAs[35]. Because MetaRibo-Seq does not directly enrich for ribosomes (which could be achieved with sucrose density gradients), the potential for contaminating signal from nonribosomal complexes is higher. Additionally, the resolution of MetaRibo-Seq is compromised by several factors. First, using chloramphenicol to halt ribosomes can alter the ribosome pausing landscape[36,37]. Second, the resolution of ribosome pausing is also likely diminished by the broad distribution of footprint lengths and lack of stringent size selection (which could be achieved with PAGE gels, for example) in the MetaRibo-Seq protocol. Ribo-Seq footprint lengths are more variable in bacteria than in eukaryotes. It is not fully clear what causes this variability; however, this difference seems to be related to inherent properties of bacterial ribosomes as opposed to technical differences such as nucleases[26]. Third, nuclease-mediated depletion of ribosomal RNA (rRNA) (as performed in Ribo-Zero Plus) can obscure the position of ribosomes[38]. Fourth, ribosomes can continue to progress along a transcript during sample collection and processing, which takes longer for fecal samples than for laboratory cultured bacteria. While not yet rigorously evaluated, a future improvement may involve using a cocktail of RNAses to increase resolution of MetaRibo-Seq. Finally, we caution that rRNA depletion will not equally deplete rRNAs across different species.

Level of expertise needed to implement the protocol

This protocol can be performed by researchers with experience in molecular biology techniques and bioinformatics. Specifically, researchers must be familiar with fecal sample processing protocols and next-generation sequencing. Because some of the expensive equipment, such as a Bioanalyzer and Illumina sequencing platforms, can be outsourced, this protocol can be established in most laboratories.

Experimental design

Storage of fecal samples (Steps 1–5)—We recommend preparing fecal samples into aliquots and freezing them in RNAlater quickly after collection. We have not tried preservatives other than RNAlater, such as DNA/RNA Shield (Zymo Research, R1100) or ethanol; however, other preservatives may also be useful. Note that certain taxa may die,

grow and alter their gene regulation in the time between collection and storage. We have successfully performed MetaRibo-Seq on samples that have been frozen for up to 1.5 years.

Obtaining monosome footprints from fecal samples (Steps 6–25)—We recommend using biopsy punches with plungers to divide fecal samples into aliquots in screw-top tubes for lysis. When handling biopsy punches, use twisting instead of jabbing motions and avoid holding the tube by hand. This minimizes the chances of injury. Biomass of fecal samples should also be a consideration. Samples of watery consistency and also those from patients being administered antibiotics typically require more total fecal mass to achieve the downstream RNA input requirements for MetaRibo-Seq.

Researchers may choose to perform RNA-Seq in addition to MetaRibo-Seq for comparison purposes, for example, to distinguish transcriptional changes from translational changes over time. We recommend performing the two methods at the same time to minimize batch effects. Additionally, we recommend performing technical duplicates within the same batch if the goal is to compare between technologies or monitor changes over time. MetaRibo-Seq was designed with timing in mind and contains modifications, such as using size-exclusion columns instead of sucrose density gradients, to simplify and shorten the protocol[21,27]. As a result, RNA-Seq and MetaRibo-Seq follow similar timelines and contain enough incubation periods in between steps to comfortably perform simultaneously[27].

Bacterial cells are mechanically lysed using a bead beater. The samples are centrifuged to remove the fecal debris pellet. The supernatant is subjected to ethanol precipitation. After centrifugation, the pellet is a crude purification of nucleic acids and ribonuclear complexes. Sometimes, these pellets can be difficult to resuspend in MNase buffer. In such cases, gently breaking the pellet apart with a pipette tip is effective. Additionally, multiple extractions can be combined at this resuspension step. The target RNA input for MNase digestion is 80 μg, which is substantially lower than existing bacterial Ribo-Seq protocols. For this reason, we perform a scaled-down MNase reaction. It is also possible to perform this reaction with 40 μg of RNA and half the amount of MNase; however, this will make downstream steps more challenging as input will be limited. We highly recommend doing more extractions to achieve 80 μg if possible; however, we have also found that it can be very difficult to obtain 80 μg from samples collected from patients undergoing antibiotic treatment, even if many extractions are performed. In these scenarios, it is acceptable to carefully proceed with 40 μg. Size-exclusion columns are used for monosome recovery; however, the samples are split across two columns to avoid overloading. The samples are recombined upon purification with the miRNeasy Mini Kit.

rRNA depletion (Steps 26–28)—After the size-exclusion step, there should be between 1 and 5 μg of RNA. To deplete rRNA contaminants, we recommend using the Illumina Ribo-Zero Plus rRNA Depletion Kit and proceeding forward with depletion on 1 μg of RNA. Illumina continues to improve rRNA depletion from microbiome samples by adding additional rRNA probes to their kit. There are other options that we have not thoroughly compared with Ribo-Zero Plus for rRNA depletion, including NEBNext rRNA Depletion Kit (NEB, E7850) and MICROBExpress Bacterial mRNA Enrichment Kit (Invitrogen, AM1905). We prefer cleanup using the RNeasy MinElute Kit for greater purification

through the silica membrane; however, ethanol precipitation has been a successful substitute for this step as a cheaper alternative.

Ligation preparation (Steps 29–34)—At this point, there should be at least 100 ng of RNA for downstream applications. To prepare samples for sequencing library construction, we subject them to T4 polynucleotide kinase to facilitate 5′ phosphorylation of RNA and 3′ phosphoryl removal. Again, we prefer cleanup using the RNeasy MinElute Kit; however, ethanol precipitation can be substituted as a cheaper alternative.

Library preparation (Steps 35–38)—Using 100 ng of input, we create MetaRibo-Seq libraries using the NEBNext Small RNA Library Prep. We recommend following the protocol at https://www.neb.com/protocols/2018/03/27/protocol-for-use-with-nebnext-small-rna-library-prep-set-for-illumina-e7300-e7580-e7560-e7330, with one exception: add 1.5 μL of SR Primer for Illumina and 1.5 μL Index Primer instead of the recommended 2.5 μL before library amplification. This modification was also recommended in a previous Ribo-Seq protocol[21]. We also found that using too much primer results in a substantial primer dimer peak in the final libraries. We have had limited success removing primer dimers using several rounds of a 1.3× ratio of AMPure XP Beads; however, the best solution is to avoid the formation of primer dimers. These primer dimers will not cluster on the flow cell when sequenced (unlike adapter dimers), meaning that libraries with primer dimers can still be sequenced effectively, but the primer dimers can make quantification of the library and pooling of samples more challenging than necessary. A peak around 150–155 bp, representing the MetaRibo-Seq library, should be present on a Bioanalyzer profile (Fig. 3a). For reference, the adapters within the fragments are 127 bp long; therefore, the position at 155 bp, for example, corresponds to a footprint of length of 28 bp.

Sequencing (Step 39)—We recommend pooling all MetaRibo-Seq libraries to minimize batch effects and sequencing these libraries single-end. We sequence libraries using 50 bp reads. While the large majority of fragments will be <50 bp and require no more than 50 bp read lengths, some monosomes can reach a length >50 bp[39], suggesting that 75 bp read lengths may be preferable. The sequencing depth depends on a number of factors including the diversity of the samples being tested and the effectiveness of rRNA depletion for those samples. To determine the sequencing depth needed and also get a sense of the quality of the libraries, it may be desirable to perform low-pass sequencing first by only sequencing several million reads per sample.

Processing and analysis of sequencing reads (Steps 40–43)—Many different analyses of the data can be performed depending on experimental design and research goals. The first computational challenge with MetaRibo-Seq analysis is that these very short reads can be difficult to analyze without representative reference genomes, and existing references are typically not specific to the strains present in a given fecal sample. Additionally, direct taxonomic classification of very short reads can be challenging without longer contigs to associate them to. To be confident in assessments of MetaRibo-Seq signal, it is important to first create a reference genome specific to the sample[40]. After metagenomic and MetaRibo-Seq reads are quality trimmed[41], we use these high-quality metagenomic reads to create

a de novo reference metagenome using metaSPAdes[40]. Depending on the sequencing depth of the samples, this assembly step can take up to 2 d to finish and is typically the rate-limiting step of the workflow (by default, the pipeline uses four threads to run metaSPAdes, though this can be increased to reduce time needed to run). The MetaRibo-Seq reads are aligned to this de novo reference using Bowtie[42]. Visualizations such as aligned footprint lengths, footprint length-specific triplet periodicity and footprint-specific metagene plots are created using riboseqR[43] (Fig. 3). All of these steps are implemented by the provided pipeline: https://github.com/bhattlab/bhattlab_workflows/tree/master/metariboseq. This pipeline not only provides information on quality but also serves as a useful starting point for downstream analyses, by providing an assembly and MetaRibo-Seq read alignment files.

Materials

Biological samples

- Stool sample, stored indefinitely at −80 °C in RNAlater **! CAUTION** All samples should be obtained with informed consent and in accordance with relevant guidelines.

Reagents

- RNeasy MinElute Cleanup Kit (Qiagen, cat. no. 74204)
- Phenol/chloroform pH 8.0 (Sigma-Aldrich, cat. no. 77617–100ML) **! CAUTION** Phenol and chloroform are toxic. Wear protective gloves and clothing to prevent contact.
- 3 M sodium acetate (Fisher Scientific, cat. no. BP333–500)
- Absolute ethanol (Fisher Scientific, cat. no. BP2818500) **! CAUTION** Ethanol is flammable. Store appropriately.
- Nuclease-free water (Invitrogen, cat. no. AM9937)
- 1 M Tris-HCl pH 8.0 (Fisher Scientific, cat. no. 15-567-027)
- 0.5 M egtazic acid (EGTA; Fisher Scientific, cat. no. 50-255-956)
- 5 M sodium chloride (Fisher Scientific, cat. no. S640-3)
- Superase-In 20 U/μL (Fisher Scientific, AM2694)
- Calcium chloride, anhydrous (Fisher Scientific, cat. no. S25223)
- Chloramphenicol (Fisher Scientific, cat. no. BP904-100)
- Igepal CA-630 (Fisher Scientific, cat. no. ICN19859650)
- $MgCl_2$ (Fisher Scientific, cat. no. AC223211000)
- Magnesium acetate (MgOAc), 1 M (Sigma-Aldrich, cat. no. 63052)
- NH_4Cl (Fisher Scientific, cat. no. A661-500)

- RNase-free DNase I, 10 U/μL (Sigma-Aldrich, cat. no. 4716728001)
- Qubit RNA HS Assay Kit (Life Technologies, cat. no. Q32852)
- MNase, 500 Kunitz units/μL (High Concentration special order from New England Biolabs)
- miRNeasy Mini Kit (Qiagen, cat. no. 217004)
- Illumina Ribo-Zero Plus rRNA Depletion Kit (Illumina, cat. no. 20040526)
- ATP, 10 mM (New England Biolabs, cat. no. P0756S)
- T4 polynucleotide kinase (T4 PNK, New England Biolabs, cat. no. M0201S)
- NEBNext Small RNA Library Prep Set for Illumina (New England Biolabs, cat. no. E7330S)
- Qubit DNA HS Assay Kit (Life Technologies, cat. no. Q32851)
- QIAquick PCR Purification Kit (Qiagen, cat. no. 28104)
- Bioanalyzer High Sensitivity DNA Assay (Agilent, cat. no. 5067-4626)

Equipment

- Class II A2 Biosafety Cabinets (Fisher Scientific, cat. no. 302610101)
- 1 mm zirconia/silica beads (Fisher Scientific, cat. no. NC9847287)
- Biopsy punch (Fisher Scientific, cat. no. 12-460-410) **! CAUTION** Biopsy punches are sharp and should be handled with care.
- Sephacryl S400 MicroSpin Columns (Sigma-Aldrich, cat. no. GE27-5140-01)
- LowBind tubes, 1.5 mL (Fisher Scientific, cat. no. 13-698-791)
- LowBind tubes, 2 mL (Fisher Scientific, cat. no. 13-698-792)
- PCR tubes, 0.2 mL (Fisher Scientific, cat. no. AM12230)
- Pipettors (Fisher Scientific, cat. nos. 07-764-700, 07-764-701, 07-764-702, 07-764-704, 07-764-705)
- Aerosol barrier pipette tips (Fisher Scientific, cat. nos. 02-707-439, 02-707-432, 02-707-430, 02-707-404)
- Dry ice
- Ice
- Mini-beatbeater-16 (Biospec, cat. no. 607)
- Analytical scale (Fisher Scientific, cat. no. S72710)
- Microcentrifuge (Fisher Scientific, cat. no. 07-203-954)
- Multi-head benchtop vortex (Benchmark Scientific, cat. no. BV1005)
- Benchtop centrifuge (Beckman Coulter, cat. no. 392244)

- Qubit fluorometer (Thermo Fisher Scientific, cat. no. Q33327)
- Heat block (Fisher Scientific, cat. no. 88-870-001)
- Thermal cycler (Thermo Fisher Scientific, cat. no. A37835)
- Magnetic microcentrifuge tube rack (Thermo Fisher Scientific, cat. no. 12321D)
- Bioanalyzer (Agilent, cat. no. G2939BA)

Software and hardware

- Computer with Windows 7+; OSX Sierra, High Sierra or Mojave; or Linux Ubuntu 16.04 or 18.04; with 100 GB of storage
- The only software that needs to be installed are NextFlow[44] and Singularity[45] with miniconda3 (https://docs.conda.io/en/latest/miniconda.html) being used to manage their installation. The Singularity image (https://github.com/orgs/bhattlab/packages/container/package/bhattlab-metariboseq) includes all other dependencies for the user, including metaSPAdes[40], bowtie[42] and riboseqR[43]

Reagent setup

! CAUTION Ensure that solutions are kept RNAse-free.

Stock solutions

- 5 M NaCl in RNase-free H_2O. Store at 4 °C for up to 1 year
- 1 M pH 8.0 Tris-HCl in RNase-free H_2O. Store at room temperature (RT, 20–25 °C) for up to 3 years
- 1 M $MgCl_2$ in RNase-free H_2O. Store at 4 °C for up to 1 year
- 0.5 M EGTA in RNase-free H_2O. Store at RT for up to 3 years
- 500 mM $CaCl_2$ in RNase-free H_2O. Store at 4 °C for up to a few months **! CAUTION** Calcium chloride releases heat when dissolved in water.
- 1 M MgOAc in RNase-free H_2O. Store at RT for up to 1 year
- 1 M NH_4Cl in RNase-free H_2O. Store at RT for up to 3 years
- 50 mg/mL chloramphenicol in dimethyl sulfoxide. Make this fresh for every experiment
- 10 mM ATP in RNase-free H_2O. Store at −20 °C for up to 1 year
- Recipes **▲ CRITICAL** All recipes should be freshly prepared.

Recipe 1:

lysis buffer

Components	Volume
RLT buffer (Qiagen)	965 μL
β-Mercaptoethanol	10 μL

Components	Volume
Superase-In, 20 U/uL	15 μL
Chloramphenicol, 50 mg/mL	10 μL
Total	1 mL (scale as needed)

Recipe 2:

MNase buffer

Components	Volume
Tris-HCl pH 8.0, 1 M	25 μL
NH_4Cl, 1 M	25 μL
MgOAc, 1 M	10 μL
Chloramphenicol, 50 mg/mL	10 μL
RNase-free water	To 1 mL (930 μL)

Recipe 3:

polysome binding buffer

Component	Volume
Igepal CA-630	100 μL
$MgCl_2$, 1 M	500 μL
EGTA, 0.5 M	500 μL
NaCl, 5 M	500 μL
Tris-HCl, pH 8.0, 1 M	500 μL
RNase-free water	7.9 mL
Total	10 mL

Note: this recipe is identical to the polysome buffer used in Latif et al.[21].

Procedure

Storage of fecal samples ● Timing 15 min

1. Store collected fecal samples at 4 °C for up to 24 h before processing. It is preferable to process as soon as possible.

   ! CAUTION All samples are obtained with informed consent and in accordance with relevant guidelines.

   ! CAUTION All work with human stool samples is performed in a biosafety cabinet until bacteria are lysed. Protective laboratory coat, eyewear and gloves should be worn.

2. Prepare aliquots of 700 μL of RNAlater (Ambion) into 2 mL cryovials. It is helpful to store multiple tubes for each sample, especially if technical replicates are desirable.

3. Homogenize the fecal sample.

▲ **CRITICAL STEP** Try to avoid sampling from one specific region of the fecal sample as this may bias results and create technical differences. If the sample is liquid, pipette 700 μL into the vial containing RNAlater. Use a wide bore pipette tip or a pipette with trimmed tip to avoid difficulties with transferring the sample. If the sample is solid, transfer ~20 g of stool to one corner of a small plastic, zipper reclosable bag. Taking care to keep stool localized to a single corner of the bag, massage the contents to thoroughly homogenize the sample. Cut an opening in the clean corner of the bag ~0.5 mm wide. Transfer ~1.0–1.3 g of stool sample to the tube with RNAlater by squeezing the base of the bag gently.

4. Add ~1.0–1.3 g of fecal sample to cryovials with RNAlater. Mix by inverting and gently vortexing to ensure that the sample is submerged in RNAlater.

5. Freeze the samples at −80 °C, and store until ready to perform MetaRibo-Seq.

■ **PAUSE POINT** Successful libraries have been created from samples stored at −80 °C for as long as 1.5 years.

Obtaining monosome footprints from fecal samples ● Timing 5 h

6. Obtain two screw-top tubes for each sample.

▲ **CRITICAL STEP** For low microbial biomass samples with less extractable RNA (such as from patients on antibiotics), more tubes may be necessary. Additionally, technical replicates should be treated as different samples and would require two additional tubes.

7. Add beads, lysis buffer and frozen stool to each screw-top tube. Keep the stool samples on dry ice the entire time they are out of the freezer. We recommend using a biopsy punch on frozen stool to prepare aliquots. Add the following to each screw-top tube:

Reagent	Quantity
1.0 mm zirconia/silica beads	~20 beads
Lysis buffer (recipe 1)	600 μL
Frozen stool (in RNAlater)	150 mg

▲ **CRITICAL STEP** Prepare lysis buffer (recipe 1) and MNase buffer (recipe 2) ahead of time, and keep on ice until use.

! CAUTION Biopsy punches are sharp objects. Take precautions: avoid holding sample tubes by hand, and use twisting motions instead of forceful, jabbing motions to divide stool into aliquots.

8. Bead beat the tubes for 3 min at RT.

9. Centrifuge at RT for 3 min at 21,000*g*, and transfer 500 μL of supernatant to new 2 mL tubes; discard pellet.

10. Add 0.1 volumes 3 M sodium acetate and 2.5 volumes absolute ethanol.

11. Incubate for 30 min on ice.

12. Centrifuge at 21,000*g* for 30 min at 4 °C; discard supernatant.

13. Resuspend pellet in 100 μL MNase buffer (recipe 2), and combine the two resuspensions for each fecal sample. Keep on ice.

    ▲ **CRITICAL STEP** Recall that, for each fecal sample, two extractions were performed (starting with two screw-top tubes); therefore, two pellets are present, representing the same sample at this step. Resuspend these pellets in MNase buffer, and combine resuspensions into one sample with a combined volume of 200 μL. The resuspension volume may vary if more extractions were performed per sample. In the case of low-biomass fecal samples that require starting with four tubes, for example, resuspend each pellet in 50 μL and combine those four resuspensions.

14. Use Qubit RNA HS Assay Kit to assess RNA concentration. We recommend first taking 1 μL of sample and performing a ~1:50 dilution in nuclease-free water to bring it into detectable range for quantification. Then perform Qubit quantification on the diluted sample using fresh preparations of RNA standards, which are provided with the kit, to create the curve.

15. Prepare MNase treatment reaction as follows:

Reagent	Volume
Lysate (from Step 13)	*X* μL (to 80 μg RNA total)
$CaCl_2$, 500 mM	2 μL
Superase-In, 20 U/μL	2 μL
NEB MNase, 500 U/μL	1 μL
MNase buffer	To 200 μL

16. Incubate for 2 h at RT.

17. Quench the reaction by adding 2.5 μL EGTA (500 mM stock).

18. Obtain two Sephacryl S400 MicroSpin columns for each sample.

19. Invert columns multiple times vigorously to resuspend resin.

20. Centrifuge columns at 600*g* for 1 min (all column centrifugation steps are performed at 4 °C); discard flow through.

21. Wash columns two times with 500 μL polysome binding buffer (recipe 3); centrifuge each time for 1 min at 600*g*.

22. Wash the column by adding 500 μL polysome binding buffer (recipe 3) and centrifuging for 4 min at 600*g*. Place each column into a clean collection tube.

23. Apply 100 μL of MNase Treatment reaction (from Step 17) to each column.

24. Centrifuge for 2 min at 600*g* to collect flow through.

25. Proceed using the flow through with miRNeasy Mini Kit (https://www.qiagen.com/us/resources/resourcedetail?id=da6c8d17-58c4-411c-a334-bc1754876db3&lang=en). Briefly, add 700 μL Qiazol and 140 μL chloroform to each flow through. Shake for 15 s. Incubate at RT for 3 min. Centrifuge at 21,000*g* for 15 min at 4 °C. Extract the aqueous (top) phase, and add 1.5× the volume of 100% ethanol and mix. Apply this to an RNeasy mini spin column (700 μL at a time), and centrifuge at 21,000*g* for 1 min at RT. Discard the flow through. Wash the column with RWT and RPE. Elute in 15 μL RNase-free water.

    ▲ **CRITICAL STEP** Please note that, at this step, the sample that was previously split into two upon loading onto Sephacryl S400 MicroSpin columns is combined. Apply both of these onto the same RNeasy column. Do not combine technical replicates if also being prepared, as these will be barcoded separately and pooled at the end of the protocol.

    ■ **PAUSE POINT** The purified RNA can now be stored at −80 °C for up to 30 d.

rRNA depletion ● Timing 2 h

26. Quantify RNA with Qubit RNA HS Assay Kit.

27. Treat 1 μg of RNA with Illumina Ribo-Zero Plus rRNA Depletion Kit using half reactions (https://www.illumina.com/content/dam/illumina-marketing/documents/products/technotes/ribo-zero-plus-data-concordance-tech-note-1270-2020-003.pdf).

28. Purify with RNeasy MinElute Cleanup, and elute in 19 μL RNase-free water.

    ▲ **CRITICAL STEP** 18 μL is needed for the downstream steps. For troubleshooting purposes, it can be helpful to save 1 μL of this elution in a separate tube for quantification later.

    ■ **PAUSE POINT** The rRNA-depleted RNA can be stored at −80 °C for up to 30 d.

Ligation preparation ● Timing 2 h

29. Prepare the following reaction:

Reagent	Volume
RNA footprints (from Step 28)	18 μL

Reagent	Volume
10X T4 PNK buffer	2.2 μL
Superase-In, 20 U/μL	1 μL
T4 PNK, 10 U/μL	1 μL
Total volume	22.2 μL

30. Incubate the reaction at 37 °C for 30 min.

31. Add 1 μL 10 mM ATP to the reaction, and incubate for 30 min at 37 °C.

32. Add 27 μL of RNase-free water, 350 μL RLT buffer and 600 μL absolute ethanol.

33. Apply to RNA MinElute column, wash with 500 μL of Buffer RPE, then wash with 500 μL of 80% (vol/vol) ethanol, and finally elute with 10 μL RNase-free water (https://www.qiagen.com/us/resources/resourcedetail?id=0acfc1c7-a1f8-4425-9aaf-b0d98b81bd1f&lang=en).

34. Check concentration with Qubit RNA HS Assay Kit.

Library preparation ● Timing 10 h

35. Perform NEBNext Small RNA Library Prep using recommendations for 100 ng input (https://www.neb.com/protocols/2018/03/27/protocol-for-use-with-nebnext-small-rna-library-prep-set-forillumina-e7300-e7580-e7560-e7330). The only exception to the manufacturer's protocols is to add 1.5 μL of SR Primer for Illumina and the Index Primer instead of 2.5 μL before library amplification. This protocol involves the ligation of the 3′ adaptor, hybridization of the reverse transcription primer, ligation of the 5′ SR adaptor, reverse transcription, and library amplification.

36. Purify the reaction with QIAquick PCR purification kit (https://www.qiagen.com/us/resources/resourcedetail?id=95f10677-aa29-453d-a222-0e19f01ebe17&lang=en).

37. Quantify DNA with Qubit DNA HS Assay Kit.

38. Check distribution of the library on HS dsDNA Bioanalyzer (https://www.agilent.com/cs/library/usermanuals/public/G2938-90322_HighSensitivityDNAKit_QSG.pdf).

Sequencing ● Timing 1 d

39. Pool and sequence the libraries using an Illumina sequencing platform, for example, NextSeq or HiSeq, as single-end with at least 50 bp reads.

Processing and analysis of sequencing reads ● Timing 2 d

40. Install miniconda3 (https://docs.conda.io/en/latest/miniconda.html), Nextflow and Singularity. Clone the bhattlab_workflows repository from https://github.com/bhattlab/bhattlab_workflows, and navigate to the metariboseq subdirectory.

41. Create a parameter file with the desired parameters using example settings found in the workflows/params subdirectory. This is described in detail within the GitHub repository, found at https://github.com/bhattlab/bhattlab_workflows/tree/master/metariboseq. An example can also be found below:

```
inputPrefix: /oak/stanford/scg/lab_asbhatt/cosn/metariboseq/
samples/resultsPrefix: /oak/stanford/scg/lab_asbhatt/cosn/
metariboseq/results/
trimGaloreOptions: “--cores 4 -q 30 --illumina”
spadesOptions: “--threads=4 --memory=96”
alignmentMemory: “96 GB”
assemblyMemory: “96 GB”
bowtieIndexOptions: “--threads 4”
bowtieAlignmentOptions: “--threads 4”
analysisScript: “/oak/stanford/scg/lab_asbhatt/cosn/
bhattlab_workflows/metariboseq/workflows/analysis/
plotVisualsRibo.R”
sampleSpecs:
- name: sample
metagenomic: SampleBDNA
metariboseq: SampleBRibo
```

Parameters that likely require changing include the inputPrefix, resultsPrefix, analysisScript and sampleSpecs because these all depend on the user’s specific data structure and sample names. The user should replace the example values with the file system paths that contain their input data (inputPrefix), the file system location where they wish their results to be written (outputPrefix), the location of the analysis script where they cloned the repository to (analysisScript) and, finally, the specification for the analysis they wish to run (sampleSpec), which specifies the original sample (the name field) and the analysis (metagenomic or metariboseq) to be performed on a given processed sample. The user can also control many aspects of the pipeline, including threads and memory usage of individual tools.

42. Run the Nextflow workflow (using nextflow run), and provide relevant parameters, including the parameter file you created and the Singularity image provided. More details are available on the GitHub repository, and NextFlow itself is also well documented[44]. The Github also includes an example script named-nextflow.sh, which can be used as a starting point for customization to match the local system configuration of the user.

43. Go to the results folder to view trimming statistics and alignments in BAM format. Assemblies can be found in the results/$SampleName-assembly folder in FASTA format. Visualizations of aligned footprint lengths, triplet periodicity

and metagene plots at various footprint lengths can be found in the results/$SampleName-plots folder in PDF format.

Troubleshooting

Troubleshooting advice can be found in Table 1.

Timing

Steps 1–5, preparation of fecal samples for storage: 15 min

Steps 6–25, obtaining monosome footprints from fecal samples: 5 h

Steps 26–28, rRNA depletion: 2 h

Steps 29–34, ligation preparation: 2 h

Steps 35–38, library preparation: 10 h

Step 39, sequencing: 1 d

Steps 40–43, processing and analysis of sequencing reads: 2 d

Anticipated results

We find great variability in the extraction yield. From individuals who have not received antibiotics, we expect 40–200 μg of RNA from 150 mg of fecal sample. From lower-biomass samples, often from individuals receiving antibiotics, we expect 1–40 μg of RNA from 150 mg of fecal samples. Due to variability, these ranges may change as more samples are analyzed. These are just rough estimates to help inform how many extractions to perform. After MNase treatment and monosome recovery, before rRNA depletion, we expect 1–5 μg of RNA. After rRNA depletion, we expect 100–500 ng of RNA. The final library should contain a large peak in fragment length around 150–155 bp. Additionally, we expect to see local enrichment in MetaRibo-Seq signal within coding regions, relatively stronger signal across start and stop sites of genes on average, and, in many cases, weak triplet periodicity.

Data availability

The data presented in Fig. 3 were generated as part of ref.[27] and can be accessed under BioProject accession PRJNA510123.

Code availability

The pipeline implemented in this paper is available at https://github.com/bhattlab/bhattlab_workflows/tree/master/metariboseq (https://zenodo.org/record/4638134#.YGDevkhKgcg)[46]. This pipeline creates a de novo assembly from metagenomic reads, maps MetaRibo-Seq reads to the assembly, calls open reading frames across the assembly and creates visualizations such as metagene plots, triplet periodicity histograms and fragment length distribution for aligned MetaRibo-Seq reads. This serves as a baseline assessment of library quality and a starting point for future analysis. Though specifically

designed for MetaRibo-Seq, this pipeline would also be generally useful for any situation in which both DNA-sequencing and Ribo-Seq data for an organism with no existing reference genome are available.

Acknowledgements

We thank D. Maghini, E. Brooks and S. Vance for helpful comments on the manuscript. This work was funded by the Damon Runyon Clinical Investigator Award, grant nos. NIH R01AI148623 and NIH R01AI143757 as well as grant no. NIH P30 AG047366, which supports the Stanford ADRC. Computational work was supported by NIH S10 Shared Instrumentation grant no. 1S10OD02014101 and by NIH grant no. P30 CA124435.

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Related links

Key references using this protocol

Fremin BJ et al. Nat. Commun 11, 3268 (2020): 10.1038/s41467-020-17081-z [PubMed: 32601270]
Durrant MG & Bhatt AS Cell Host Microbe 29, 121–131.e4 (2021): 10.1016/j.chom.2020.11.002 [PubMed: 33290720]

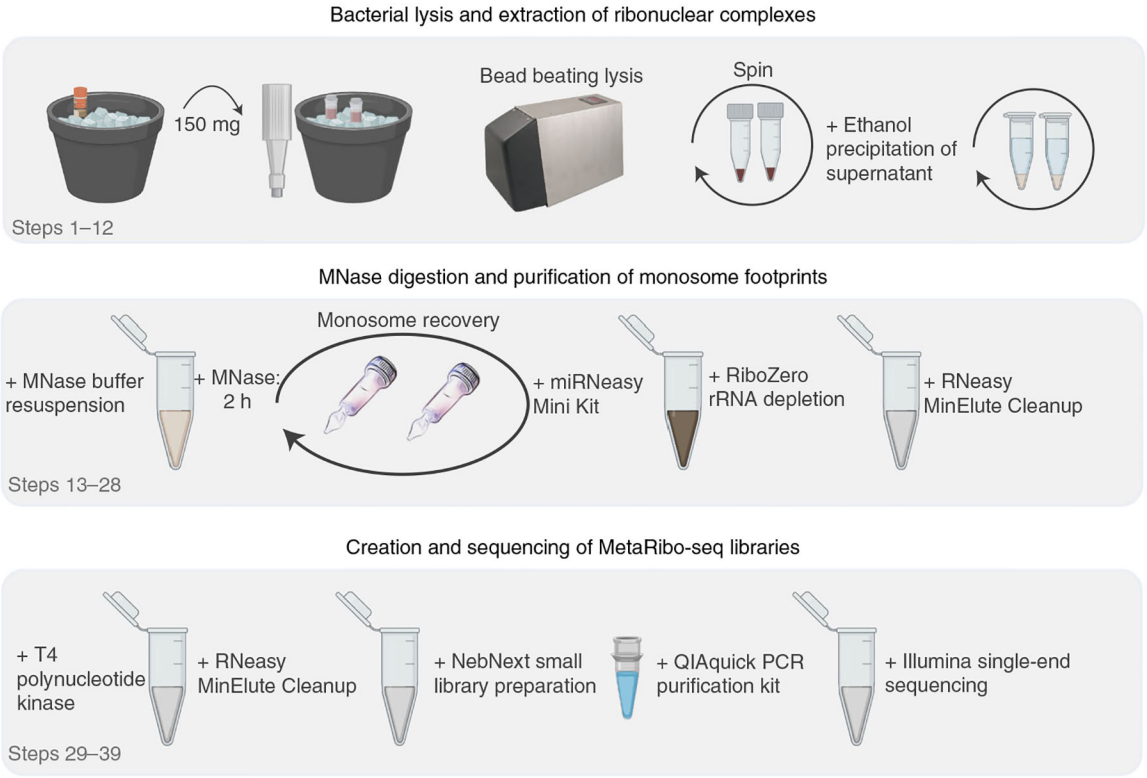

Fig. 1 |. MetaRibo-Seq protocol workflow.

A biopsy punch with a plunger is used to prepare 300 mg aliquots of stool into two screw-top tubes (150 mg per tube) with lysis buffer (recipe 1). Bacterial cells are lysed using a bead beater and centrifuged to pellet fecal debris, and an ethanol precipitation is performed on the supernatant. Precipitate is resuspended in MNase buffer (recipe 2) and digested with MNase. Monosomes are recovered using size-exclusion columns, purified, and subjected to rRNA depletion. MetaRibo-Seq libraries are created from these footprints and sequenced.

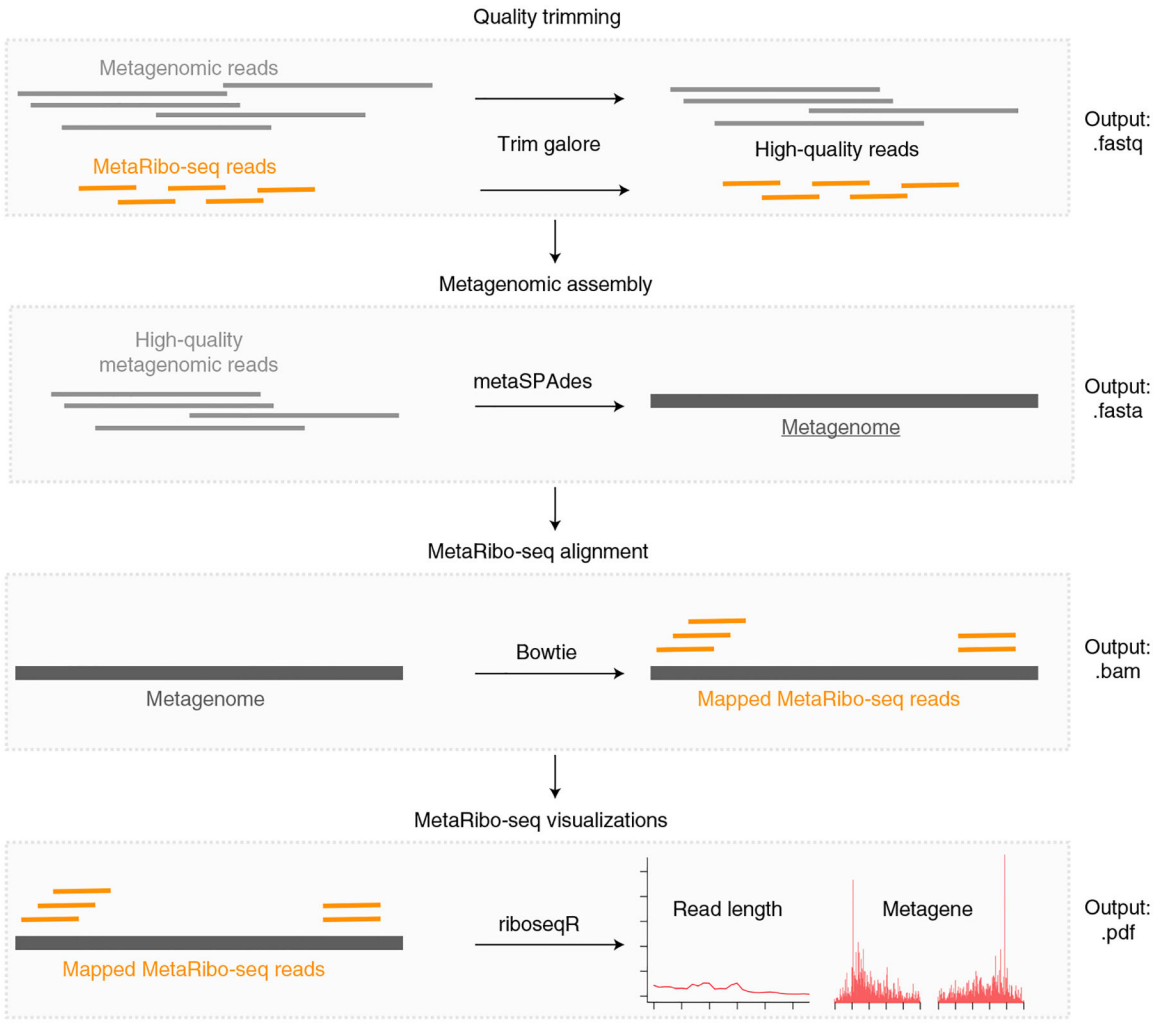

Fig. 2 |. MetaRibo-Seq bioinformatic workflow.

After sequencing, this computational workflow assesses MetaRibo-Seq library quality and serves as a starting point for downstream analyses. After quality trimming all relevant reads, this pipeline uses metagenomic reads for a given sample to create a de novo reference and then aligns MetaRibo-Seq reads corresponding to the sample to the assembly. These alignment files are used to create visualizations, including aligned footprint length distributions, footprint length specific triplet periodicity, and metagene plots for various footprint sizes.

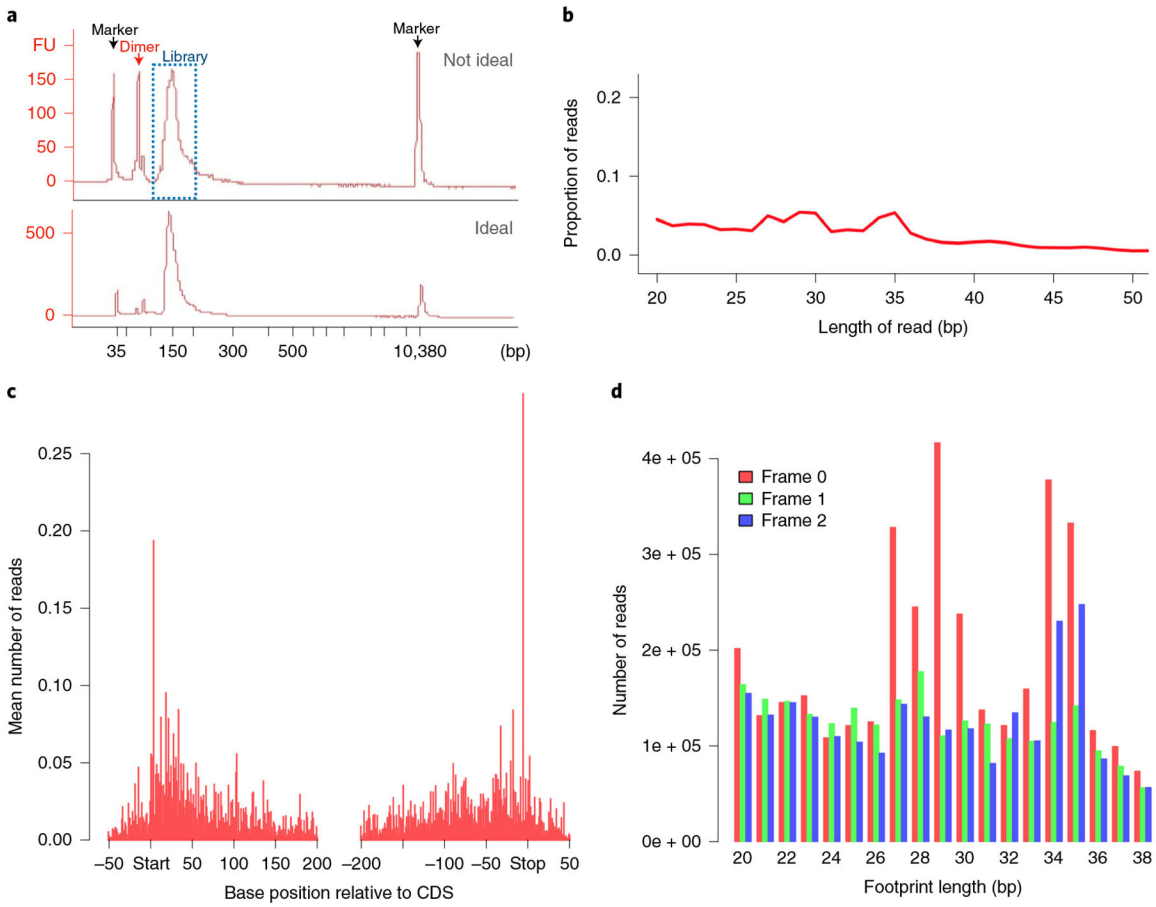

Fig. 3 |. MetaRibo-Seq quality.

Assessing the quality of MetaRibo-Seq libraries experimentally and computationally. **a**, Bioanalyzer trace of a low-quality (top, with primer dimers) and high-quality (bottom, with minimal primer dimers) MetaRibo-Seq library. The bioanalyzer trace is labeled to indicate which peaks correspond to markers used by the bioanalyzer, primer dimer and library. **b**, Aligned read length distribution histogram of the MetaRibo-Seq library. **c**, Metagene plot of the MetaRibo-Seq library. **d**, Footprint length-specific triplet periodicity histogram of the MetaRibo-Seq library.

Table 1 |

Troubleshooting table

Step	Problem	Possible reason	Solution
13	Difficult to resuspend pellet	Much variability exists in fecal samples. Some precipitations can be more crude than others	Use a pipette tip to gently break apart the pellet first, then gently vortex for a few minutes
14	Low yield (<80 μg total)	Low biomass of microbes in fecal sample. In this case, the RNA extractions will also be low yield. Alternatively, it could be an issue of RNA degradation during sample storage. For example, the RNAlater may not have been mixed well with the sample	Increase the number of extractions per sample. Also, it is acceptable to proceed with 40 μg, cutting the MNase treatment concentration in half as a last resort. Although the libraries will work, you will likely have just enough input for each downstream step
34	Not enough RNA for library preparation	RNA was degraded during rRNA depletion, from too many freeze-thaw cycles, or during the T4 PNK reaction	Backtrack and quantify how much RNA remained after rRNA depletion Try to avoid some pausing points to minimize freeze-thaws, and try to complete the entire protocol in 2 d Ensure that RNase inhibitors are being added diligently
38	Substantial amount of primer dimers	Too much primer was added	We recommend adding 1.5 instead of 2.5 μL of primers as the manufacturer recommends. If need be, this can be reduced to 1 μL Although less ideal, a few rounds of Ampure beads (1.3× ratio of beads to sample) can reduce the dimers in the library