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RIBO-seq in bacteria: a sample collection and library preparation protocol for NGS sequencing
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1 **TITLE:**
2 RIBO-seq in Bacteria: a Sample Collection and Library Preparation Protocol for NGS Sequencing
3

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22 **SUMMARY:**
23 Here we describe the stages of sample collection and preparation for RIBO-seq in bacteria.
24 Sequencing of the libraries prepared according to these guidelines results in sufficient data for
25 comprehensive bioinformatic analysis. The protocol we present is simple, uses standard
26 laboratory equipment and takes seven days from lysis to obtaining libraries.
27

28 **ABSTRACT:**
29 The ribosome profiling technique (RIBO-seq) is currently the most effective tool for studying the
30 process of protein synthesis *in vivo*. The advantage of this method, in comparison to other
31 approaches, is its ability to monitor translation by precisely mapping the position and number of
32 ribosomes on a mRNA transcript.
33

34 In this article, we describe the consecutive stages of sample collection and preparation for RIBO-
35 seq method in bacteria, highlighting the details relevant to the planning and execution of the
36 experiment.
37

38 Since the RIBO-seq relies on intact ribosomes and related mRNAs, the key step is rapid inhibition
39 of translation and adequate disintegration of cells. Thus, we suggest filtration and flash-freezing
40 in liquid nitrogen for cell harvesting with an optional pretreatment with chloramphenicol to
41 arrest translation in bacteria. For the disintegration, we propose grinding frozen cells with mortar
42 and pestle in the presence of aluminum oxide to mechanically disrupt the cell membrane. In this
43 protocol, sucrose cushion or sucrose gradient ultracentrifugation for monosome purification is
44 not required. Instead, mRNA separation using polyacrylamide gel electrophoresis (PAGE)

45   followed by ribosomal footprint excision (28-30 nt band) is applied and provides satisfactory
46   results. This largely simplifies the method as well as reduces the time and equipment
47   requirements for the procedure. For library preparation, we recommend using the commercially
48   available small RNA kit for Illumina sequencing from New England Biolabs, following
49   manufacturer's guidelines with some degree of optimization.
50
51   The resulting cDNA libraries present an appropriate quantity and quality required for next
52   generation sequencing (NGS). Sequencing of the libraries prepared according to the described
53   protocol results in 2 to 10 mln uniquely mapped reads per sample providing sufficient data for
54   comprehensive bioinformatic analysis. The protocol we present is quick and simple and can be
55   performed with standard laboratory equipment.
56
57   **INTRODUCTION:**
58   The ribosome profiling technique (RIBO-seq) was developed in the laboratory of Jonathan
59   Weissman at the University of California, San Francisco[1]. In comparison to other methods used
60   to study gene expression at the translational level, RIBO-seq focuses on each ribosome binding
61   to mRNA and provides information about its location and the relative number of ribosomes on a
62   transcript. It enables monitoring the process of protein synthesis *in vivo* and can provide single
63   codon resolution and accuracy allowing the measurement of ribosome density on both, the
64   individual mRNA and along the entire transcriptome in the cell. At the foundation of RIBO-seq
65   technique lies the fact that during translation the ribosome binds the mRNA molecule and thus
66   protects the buried fragment of the transcript from ribonuclease digestion. Upon addition of
67   ribonuclease, the unprotected mRNA is digested and the fragments enclosed by ribosomes -
68   typically of ~28-30 nt long - remain intact. These fragments, called ribosomal footprints (RF), can
69   then be isolated, sequenced and mapped onto the transcript they originated from resulting in
70   detection of the exact position of the ribosomes. In fact, the ribosome ability to protect mRNA
71   fragments has been used since the 1960s to study ribosomal binding and translation initiation
72   sites (TIS)[2-4]. However, with the advancement in deep sequencing technology, RIBO-seq has
73   become a gold standard for translation monitoring[5] which, through ribosome engagement, can
74   provide genome-wide information on protein synthesis[6]. Ribosome profiling filled the
75   technological gap that existed between quantifying the transcriptome and the proteome[6].
76
77   To conduct ribosome profiling we need to obtain cell lysate of the organism that had grown under
78   the investigated conditions. Disrupting these conditions during cell collection and lysis may
79   provide unreliable data. To prevent this, translation inhibitors, rapid harvesting and flash freezing
80   in liquid nitrogen are commonly used. Cells can be lysed by cryogenic grinding in a mechanical
81   homogenizer like mixer mill[7,8] or bead beater[9], and by trituration through a pipette[10] or with a
82   needle[11]. The lysis buffer can be added just before or shortly after pulverization of the cells. In
83   our protocol we use liquid nitrogen to precool mortar and pestle, as well as aluminum oxide as a
84   gentler approach to disruption of the bacterial cell wall, which prevents RNA shearing often
85   encountered when methods such as sonication are applied. After pulverization, we add ice-cold
86   lysis buffer into the cooled contents of the mortar. Selection of an appropriate lysis buffer is
87   important for obtaining the best resolution of ribosomal footprints. Since ionic strength affects
88   both RF size and reading frame precision, it is currently recommended to use lysis buffers with

89 low ionic strength and buffer capacity, even if it appears that buffer composition does not affect
90 ribosomal occupancy on mRNAs[11,12]. Important components of the lysis buffer are magnesium
91 ions, the presence of which prevents dissociation of the ribosomal subunits and inhibits
92 spontaneous conformational changes in the bacterial ribosomes[11,13]. Calcium ions also play a
93 significant role and are essential for the activity of micrococcal nuclease (MNase) used in the
94 bacterial ribosome profiling method[14]. Addition of guanosine 5'-[β,γ-imido]triphosphate (GMP-
95 PNP), a non-hydrolyzable analog of GTP, together with chloramphenicol inhibits translation
96 during lysis[15].
97
98 When the lysate is obtained, it is clarified by centrifugation and divided into two portions, each
99 for RIBO-seq and high-throughput total mRNA sequencing (RNA-seq) since they are performed
100 simultaneously (**Figure 1**). RNA-seq provides a point of reference which enables the comparison
101 of data from both RIBO-seq and RNA-seq during data analysis. The investigated translatome is
102 defined by normalization of ribosomal footprints to mRNA abundance[16]. Data from RNA-seq can
103 also help identify cloning or sequencing artifacts[17].
104
105 [Place **Figure 1** here]
106
107 Initial steps of the procedure of sample preparation for RIBO-seq and RNA-seq differ slightly
108 (**Figure 1**). For ribosomal profiling, the lysate needs to be digested by a specific endonuclease to
109 degrade the mRNA molecules not protected by the ribosomes. In standard protocols, the
110 obtained monosomes are recovered by sucrose cushion ultracentrifugation or sucrose gradient
111 ultracentrifugation[8,14]. In this article, we show that this step is not necessary to isolate RF
112 required for the RIBO-seq in bacteria, likewise for eukaryotic cells[18], and that size selection of the
113 appropriate length mRNA fragments from polyacrylamide gel is sufficient.
114
115 For RNA-seq, mRNA is obtained by depletion of rRNA from the total RNA - rRNA molecules
116 hybridize to the biotinylated oligonucleotide probes which bind to streptavidin-coated magnetic
117 beads. The rRNA-oligonucleotide-bead complexes are then removed from the sample with a
118 magnet resulting in a rRNA depleted sample[19,20]. The purified mRNA molecules are then
119 randomly fragmented by alkaline hydrolysis. The obtained fragments of mRNA as well as the
120 ribosomal footprints are converted into cDNA libraries and prepared for deep sequencing (**Figure
121 2**). This involves ends repair needed after alkaline hydrolysis (for mRNA) and enzymatic digestion
122 (for FT): dephosphorylation of 3' ends followed by phosphorylation of 5' ends. The next steps are
123 adaptors ligation and reverse transcription to create cDNA inserts framed by sequences required
124 for the next generation sequencing (NGS) using Illumina platform. The last phase of library
125 preparation is a PCR reaction in which the constructs are amplified and labelled with sample
126 specific barcodes to allow multiplexing and sequencing various samples on one channel. Before
127 sequencing, the quality and quantity of the libraries are assessed by high-sensitivity DNA on-chip
128 electrophoresis. cDNA libraries with appropriate parameters can then be pooled and sequenced.
129 Sequencing can be performed on different Illumina platforms, such as MiSeq, NextSeq or
130 HighSeq, depending on the number of libraries, required read length and sequencing depth. After
131 sequencing, the bioinformatic analysis is performed.
132

133   [Place **Figure 2** here]

134

135   The ribosome profiling is a universal method which can be easily modified and adjusted according
136   to the scientific question. Originally it was used in yeast[1], but shortly after it was applied to
137   bacterial cells[21] as well as eukaryotic model organisms including mouse[10], zebrafish[22], fruit fly[23]
138   and *Arabidopsis thaliana*[24]. It was also used for studying different ribosome types: cytoplasmic,
139   mitochondrial[25,26] and chloroplast[27,28]. In eukaryotes RIBO-seq is commonly adapted and refined
140   to investigate specific aspects of translation, including initiation[10,11,29-32], elongation[1,10,11,31,33],
141   ribosome stalling[33] and conformation change[33]. Most of the modifications involve the use of
142   different translation inhibitors. In bacteria however, analogous studies have been difficult to
143   conduct because of the paucity of inhibitors with the required mechanism of action[34]. The most
144   commonly used translation inhibitor in bacteria is chloramphenicol (CAM) which binds to the
145   peptidyl transferase center (PTC) and prevents correct positioning of the aminoacyl-tRNA in the
146   A-site. As a result, CAM prevents the formation of a peptide bond which leads to arresting the
147   elongating ribosomes[35]. Other examples of translation inhibitors in bacteria are tetracycline
148   (TET)[36], retapamulin (RET)[34] and Onc112[37] which have been used to investigate translation
149   initiation sites. TET, which prevents tRNA delivery to the ribosome by directly overlapping with
150   the anticodon stem-loop of tRNA at the A-site, was originally applied to verify the results
151   obtained from CAM treatment since they are both antibiotics inhibiting translation elongation[38].
152   TET was found to detect primary TIS, however was unable to reveal internal TIS[36]. RET binds in
153   the PTC of the bacterial ribosome, and prevents formation of the first peptide bond by interfering
154   with an elongator aminoacyl-tRNA in the A site. Applying RET results in arresting ribosomes at
155   both primary as well as internal TISs[34]. Onc112, a proline-rich antimicrobial peptide, binds in the
156   exit tunnel and blocks aminoacyl-tRNA binding in the ribosomal A site. As a result, Onc112
157   prevents initiation complexes from entering the elongation phase[37].

158

159   The main information ribosome profiling provides is ribosomes density and their position on the
160   mRNA. It was successfully applied to investigate differential gene expression at the level of
161   translation in various growth conditions[1,6], measure translational efficiency[1,38,39] and detect
162   translation regulation events such as ribosomal pausing[10]. RIBO-seq also allows for uncovering
163   the translation of annotated ncRNA, pseudogenes and unannotated small open reading frames
164   (ORF) leading to the identification of novel and/or very short protein coding genes[10,12,22,30,37]. In
165   such cases, RIBO-seq can fine-tune and improve genome annotation. With its high sensitivity for
166   identification of translated ORFs and its quantitative nature, ribosome profiling can also serve as
167   a proxy for the proteome determination or in aiding proteomics studies[31,34,39]. By mapping TIS,
168   ribosome profiling reveals N-terminally extended and truncated isoforms of known proteins[10,32].
169   RIBO-seq was also adapted to study co-translational folding of proteins[14,21,24]. The method
170   enables measuring of elongation rates[1,10,39] or decoding speeds of individual codons[6] and helps
171   in developing quantitative models of translation[17]. The ribosome profiling method is also capable
172   of providing mechanistic insights into ribosome pausing in bacteria[7,15,17], frameshifting[40], stop-
173   codon readthrough[21], termination/recycling defects[41,42] and ribosomal conformation changes[33]
174   in eukaryotes. RIBO-seq was also adapted to examine the impact of specific trans-acting factors
175   on translation such as miRNAs[6] and RNA-binding proteins in eukaryotes[16,43]. However, it is
176   important to acknowledge that the experimental design and the obtained resolution of RIBO-seq

177 determine the amount of information that can be extracted from the resulting sequencing data[12].
178

179 **PROTOCOL:**

180

181 **1.   Sample collection**

182

183 1.1.   Prepare a bacterial culture. We recommend a culture volume of 100 mL per sample.

184

185 1.2.   Prepare equipment and reagents for sample collection: two scoopulas per sample, sterile
186 0.45 μm mixed cellulose esters membrane (MCE) filters, 50 mL sterile tubes, liquid nitrogen, 50
187 mg/mL chloramphenicol in 70% (vol/vol) ethanol (optional). Puncture the lid of 50 mL tubes to
188 allow liquid nitrogen evaporation and prevent the explosion of closed tubes. Decontaminate
189 scoopulas with laboratory detergent and 70% ethanol.

190

191 1.3.   Prewarm the filtering equipment and one of the scoopulas to growth temperature of the
192 bacterial culture.

193

194 NOTE: We recommend all-glass filtration apparatus with funnel, fritted base, ground joint flask
195 and 47 mm Ø spring clamp.

196

197 1.4.   Before sample harvesting, add chloramphenicol into the bacterial culture to the final
198 concentration of 100 μg/mL and incubate 1 minute (optional).

199

200 1.5.   Pour liquid nitrogen into 50 mL sterile tube and place the second scoopula inside the tube
201 to cool.

202

203 CAUTION! Liquid nitrogen can cause closed containers to explode due to pressure change upon
204 evaporation. To prevent this, it is necessary to create a few holes in the lid of 50 mL tubes to
205 allow liquid nitrogen evaporation.

206

207 1.6.   Collect cells by filtration. Stop filtering when medium has passed through the membrane,
208 but do not allow the filter to dry completely.

209

210 1.7.   Collect bacterial pellets by rapidly scraping the cells off of the filter disc using a
211 prewarmed scoopula. Immediately place the entire scoopula with the harvested cells in 50 mL
212 tube filled with liquid nitrogen. The harvested pellet should be completely covered in liquid
213 nitrogen.

214

215 1.8.   Let the pellet freeze thoroughly and dislodge frozen cells using a previously cooled
216 scoopula. Close the punctured lid and transfer the tube to -80°C. STOP point

217

218 NOTE: Disinfect scoopulas after each sample harvesting.

219

220 **2.   Cell lysis**

221

222 2.1 Prepare 0.1 M GMP-PNP in 10 mM Tris pH 8, DNase I and 1.5 mL reaction tubes for lysates
223 and keep them on ice. Cool the centrifuge to 4 °C.

224

225 2.2. Prepare lysis buffer (20 mM TRIS pH 7.6, 10 mM MgAcet, 150 mM KAcet, 0.4% TRITON X-
226 100, 6 mM β-mercaptoethanol, 5 mM $CaCl_2$ and optional 1 mM chloramphenicol). Aliquot 500 µL
227 of lysis buffer per sample into 1.5 mL reaction tubes and place them on ice.

228

229 2.3 Decontaminate mortar and pestle with laboratory detergent and 70% ethanol, and dry them.
230 Cool mortar and pestle by pouring liquid nitrogen into the mortar.

231

232 2.4 Transfer frozen pellet into pre-chilled mortar and grind it to a powder. With a spatula, add
233 approximately 1 volume of aluminum oxide and continue grinding. Keep mortar, pestle and cells
234 cool by pouring liquid nitrogen when needed, do not let the contents of the mortar to thaw.

235

236 2.5 Just before using lysis buffer, add GMP-PNP and DNase I into the lysis buffer aliquot to the
237 final concentration of 2 mM and 100 U/mL respectively. Transfer the solution into the mortar
238 with cells and aluminum oxide and continue grinding. Let the lysate thaw slowly while grinding
239 and transfer the mixture into the pre-cooled 1.5 mL reaction tube and immediately place on ice.

240

241 2.6 Centrifuge lysates at 20 000 x g for 5 minutes at 4 °C. Transfer supernatants into new, pre-
242 cooled 1,5 mL reaction tubes and keep them on ice.

243

244 2.7 Measure the RNA concentration in each sample with NanoDrop. Use 1:10 dilutions of
245 samples and 1:10 dilution of lysis buffer in nuclease-free water as a blank.

246

247 NOTE: Be aware that chloramphenicol shows significant absorption at 260 nm.

248

249 2.8 Divide each lysate into two portions: one for RIBO-seq (0.5 – 1 mg of RNA) and the second for
250 RNA-seq (the rest).

251

252 2.9 Clean the samples for RNA-seq using a commercially available RNA clean-up kit according to
253 the manufacturer's protocol.

254

255 NOTE: We recommend using Zymo RNA Clean & Concentrate -25 kit (see **Table of Materials**). We
256 also recommend adding 4.5 volumes of ethanol (compared to sample volume) into the mix of
257 sample and Binding Buffer for ribosomal footprints and fragmented mRNA, in order to increase
258 the purification efficiency of short RNA fragments.

259

260 2.10 Store the samples intended for RNA-seq at -80 °C. The procedure for RNA-seq will continue
261 from step 5.

262

263 **3. MNase digestion of samples for RIBO-seq**

264

265 3.1 To 1 mg of RNA add 3.8 μL of 187.5 U/μL MNase in 10 mM Tris pH 8 and top it up with lysis
266 buffer to the total volume of 500 μL.

268 3.2 Incubate at 25 °C, 300 RPM, for 45 minutes in thermomixer.

270 3.3 Clean the samples with a commercially available RNA clean up kit (as in step 2.9).

272 3.4 Store the samples for RIBO-seq at -80 °C (STOP point) or proceed to size selection.

274 **4. Polyacrylamide gel electrophoresis (PAGE) and size selection of samples for RIBO-seq**

276 4.1 Prepare 15% polyacrylamide-TBE gel with 8 M urea and place it in a tank with TBE buffer. Pre-
277 run for minimum 10 minutes at a constant voltage of 200 V.

279 4.2 Mix samples with TBE-Urea Sample Buffer, denature at 95 °C for 1 minute and place them
280 immediately on ice.

282 4.3 Wash out the urea by injecting TBE buffer into gel wells using a syringe. Load the samples
283 leaving one well space between them to separate each sample and prevent cross-contamination.
284 Use 29 nt oligonucleotide and a mix of 26 nt and 32 nt oligonucleotides as markers. Run
285 electrophoresis at a constant voltage of 180 V.

287 4.4 Prepare sterile buffer for overnight incubation (5 mM EDTA, 10 mM NaOAc pH 5).

289 4.5 After electrophoresis, stain the gel in a SYBR Gold-bath for about 2-3 minutes and rinse the
290 gel with nuclease-free water.

292 4.6 Excise fragments of gel between 26 and 32 nt with a sterile needle or a razor blade and place
293 the gel fragments in separate 1.5 mL tubes for each sample. Change the needle or the razor blade
294 between the samples.

296 4.7 Add 200 μL of the overnight incubation buffer to each reaction tube.

298 4.8 Incubate the samples at 10 °C, 1000 RPM overnight in a thermomixer.

300 4.9 Next day clean the samples as in step 2.9. Elute the footprints in 80 μL of nuclease-free water.

302 4.10 Store the samples at -80 °C. STOP point. The procedure for RIBO-seq will continue from step
303 7.

305 **5. Purification of bacterial mRNA by rRNA depletion from samples for RNA-seq**

307 1.9.   5.1 Remove rRNA from samples with a bacterial mRNA purification kit (e.g., Invitrogen
308   Microbexpress). Follow the manufacturer's protocol.

309

310   5.2 Clean the samples as in step 2.9. Elute the footprints in 50 μL of nuclease-free water.

311

312   5.3 Store the samples for RNA-seq at -80 °C (STOP point) or continue to alkaline fragmentation.

313

314   **6. Alkaline fragmentation of samples for RNA-seq**

315

316   6.1 Prepare alkaline hydrolysis buffer (mix 220 μL of 0.1 M $NaHCO_3$, 30 μL of 0.1 M Na_2CO_3 and 1
317   μL of 0.5 M EDTA). Mix 1 volume (80 μL) of alkaline buffer with 1 volume (80 μL) of the sample
318   and incubate at 95 °C for 25 minutes.

319

320   6.2 Add 5 μL of 3 M NaOAc pH 5.5 to stop the reaction.

321

322   6.3 Clean the samples as in step 2.9 and elute the samples in 80 μL of nuclease-free water.

323

324   6.4 Possible STOP point: store RNA-seq samples at -80 °C. However, we recommend to go directly
325   to step 7 in order to avoid unnecessary freezing and thawing.

326

327   **7. Dephosphorylation and phosphorylation of samples for both RNA- and RIBO-seq**

328

329   7.1 Add 10 μL of 10x reaction buffer PNK and 5 μL T4 PNK to each sample. Incubate at 37 °C for
330   1.5 hour in order to dephosphorylate the 3' ends.

331

332   7.2 Add 3 μL of 1 mM ATP and incubate at 37 °C for 1 hour in order to phosphorylate the 5' ends.

333

334   7.3 Clean the samples as in step 2.9, elute in 30 μL nuclease-free water.

335

336   7.4 Store the samples at -80 °C. STOP point.

337

338   **8. Library preparation using NEBNext Multiplex Small RNA Library Prep Set for Illumina**

339

340   8.1 Prepare 100-1000 ng of input RNA (obtained mRNA fragments and ribosomal footprints) in 6
341   μL of nuclease-free water and add 1 μL of 3' SR Adaptor. Incubate according to the
342   manufacturer's protocol.

343

344   8.2 Add 10 μL of 3' Ligation Reaction Buffer (2X) and 3 μL of 3' Ligation Enzyme Mix and incubate
345   at 37 °C for 2.5 hour, instead of 1 hour at 25 °C as the manufacturer's protocol specifies.

346

347   8.3 Hybridize the Reverse Transcription Primer according to the manufacturer's protocol with a
348   modified program: 5 minutes at 75 °C, 30 minutes at 37 °C and hold at 4 °C.

349

350   8.4 Ligate the 5´ SR Adaptor. Follow the manufacturer's protocol with one change: perform the
351   incubation at 37 °C for 2.5 hour, instead of 1 hour at 25 °C.

352

353 8.5 Perform Reverse Transcription according to the manufacturer's protocol.
354
355 8.6 Possible STOP point: incubate the samples containing synthesized cDNAs at 70 °C for 15
356 minutes to inactivate the reverse transcriptase and store them at -80 °C. However, we
357 recommend to proceed directly to the next steps to avoid unnecessary freezing and thawing of
358 the samples.
359
360 8.7 Store half of each cDNA library at -80 °C as a back-up.
361
362 8.8 Perform PCR amplification according to the manufacturer's protocol. Use half of the reaction
363 mix. Store the obtained libraries at -80 °C. STOP point.
364
365 **9. Size selection of cDNA libraries using PAGE**
366
367 9.1 Prepare 6% polyacrylamide-TBE gel and place it in a tank with TBE buffer. Pre-run for
368 minimum 10 minutes at a constant voltage of 200 V.
369
370 9.2 Mix the samples with Gel Loading Dye, Blue (6X) and load them on the gel. Use Quick-Load
371 pBR322 DNA-MspI Digest as a ladder. At the beginning run electrophoresis at a constant voltage
372 of 120 V to allow DNA to enter the gel and then change the voltage to 180 V.
373
374 9.3 When electrophoresis is finished, stain the gel in a SYBR Gold-bath for about 2-3 minutes and
375 rinse the gel with nuclease-free water.
376
377 9.4 Excise gel fragments containing the libraries. For RNA-seq samples excise between 135-180
378 nt and for RIBO-seq between 135-170 nt. Use sterile needle or razor blade and place the excised
379 gel fragments in separate 1.5 mL reaction tubes. Remember to change the needle or razor blade
380 between the samples.
381
382 NOTE: The adapters and barcode from NEBNext Multiplex Small RNA Library Prep Set for Illumina
383 have 119 nt in total which determines the lower excision cut-off.
384
385 9.5 Add 100 μL of nuclease-free water to each excised gel fragment.
386
387 9.6 Incubate the gel fragments at 10 °C, 450 RPM overnight in a thermomixer.
388
389 9.7 Clean and concentrate the cDNA libraries with a commercial DNA clean-up kit according to
390 the manufacturer's protocol. NOTE: We recommend using Zymo DNA Clean & Concentrate -5 kit
391 (see Table of Materials).
392
393 9.8 Store purified cDNA libraries at -80 °C. STOP point.
394
395 **REPRESENTATIVE RESULTS:**
396 The exemplary results presented here were obtained in a study examining translation regulation

397 in sporulating WT *Bacillus subtilis* cells. Overnight cultures were diluted to OD_{600} equal to 0.1 in
398 100 mL of rich medium and incubated at 37 °C with vigorous shaking until OD_{600} reached 0.5-0.6.
399 The rich medium was then replaced with minimal medium to induce sporulation process and the
400 incubation was continued for up to four hours. Cells were harvested every hour beginning with
401 T0 - sporulation induction - resulting in five time points. One minute before harvesting, the
402 cultures were treated with chloramphenicol as described in the protocol above. Cells were
403 collected by filtration in a prewarmed glass filtration system using 0.45 μm mixed cellulose esters
404 membrane (MCE) filters and flash-frozen in liquid nitrogen.

406 The amount of ribonucleic acid obtained in the lysate depends on growth conditions, culture
407 volume and density. Following our protocol, 100 mL of bacterial culture (*Bacillus subtilis*) yields
408 1-4 mg of RNA on average. Examples of lysates obtained from the experiment described above
409 are shown in **Table 1**.

411 [Place **Table 1** here]

413 When the amount of RNA is lower than 1 mg, 0.25 mg of RNA is used for RIBO-seq instead of 1-
414 0.5 mg and the amount of MNase is then adjusted respectively. The representative RNA amount
415 and concentration after nuclease digestion is shown in **Table 2**. The average amount of RNA after
416 digestion and cleaning is 50 μg from 0.5 mg of RNA input.

418 [Place **Table 2** here]

420 After MNase digestion, size selection is performed with the aid of PAGE. An example of a 15%
421 TBE-urea polyacrylamide gel with the digested samples is shown in **Figure 3**. Fragments of gel
422 between 26 and 32 nt were excised with a sterile razor blade and incubated overnight in the
423 overnight incubation buffer. The next day, ribosomal footprints were cleaned with the
424 commercial RNA clean-up kit.

426 [Place **Figure 3** here]

428 Parallel to nuclease digestion, the lysates for RNA-seq were cleaned with the commercial RNA
429 clean-up kit and the obtained concentrations of RNA were measured with NanoDrop. The
430 representative results are presented in **Table 3**. After cleaning, the amount of ribonucleic acid
431 decreased to 40-500 μg.

433 [Place **Table 3** here]

435 The obtained RNA concentrations of the samples for RNA-seq were used to define RNA input for
436 rRNA depletion. 8 μg of RNA from each RNA-seq sample were used with MicrobExpress bacterial
437 mRNA purification kit (see **Table of Materials**) according to the manufacturer's protocol and were
438 cleaned with the commercial RNA clean-up kit afterwards. The representative results of rRNA
439 depletion are shown in **Table 4**.
440

441   [Place **Table 4** here]

442

443   The obtained mRNA was next fragmented by alkaline hydrolysis and cleaned with a commercial
444   kit as described in the protocol. Next, the fragmented mRNA and ribosomal footprints were
445   dephosphorylated at 3' ends and phosphorylated at 5' ends according to the above protocol. The
446   samples were then used to construct cDNA libraries for Illumina sequencing, as described in the
447   protocol. **Figure 4** shows an example of a 6% polyacrylamide gel with the obtained libraries. Gel
448   fragments in the range of 135-180 nt for RNA-seq and 135-170 nt for RIBO-seq were excised with
449   a sterile razor blade and incubated overnight in nuclease-free water. Libraries were cleaned with
450   the commercial DNA clean-up kit and quality and quantity was assessed by high-sensitivity DNA
451   on-chip electrophoresis using Agilent 2100 Bioanalyzer.

452

453   [Place **Figure 4** here]

454

455   The examples of virtual gel images and electropherograms obtained from Agilent 2100
456   Bioanalyzer are shown in **Figure 5**. Bands and peaks representing the RIBO-seq libraries are more
457   narrow and better defined compared to these representing RNA-seq libraries. According to the
458   on-chip electrophoresis, the libraries are approximately 150 bp long, which is the expected library
459   size. The adapters and barcode used in library preparation are 119 nt long and the inserts are
460   either 29-30 nt long (for RIBO-seq) or range between 25 and 50 nt (for RNA-seq). The additional
461   peaks closer to 200 bp obtained from RIBO-seq libraries may indicate PCR artefacts which can be
462   discarded during bioinformatic analysis when the data obtained from sequencing is trimmed and
463   rRNA/tRNA is filtered. The average quantity of DNA is 32 ng providing sufficient quantity of
464   material required for the next generation sequencing.

465

466   [Place **Figure 5**]

467

468   After quality control by on-chip electrophoresis, libraries were pooled for single-end 50-bp deep
469   sequencing on Illumina's NextSeq500 platform (10 libraries per channel, with a minimum of 350
470   mln reads per channel). Sequencing yielded 30-57 million reads per sample for RNA-seq libraries
471   and 30-50 million reads per sample for RIBO-seq libraries. Trimming the adaptors and poor
472   quality sequences resulted in 24-47 mln reads per sample for RNA-seq samples and 25-50 mln
473   reads per sample for RIBO-seq samples. Mapping, performed with STAR[44], yielded 2.4-9.6 million
474   of uniquely mapped reads per sample for RNA-seq samples (which constitute 8-16.8% of the total
475   number of reads) and 2.3-10.4 million uniquely mapped reads (7.7-22.1%) for RIBO-seq samples.
476   The obtained data was quality control checked with FastQC[45]. Examples of the quality plots
477   produced by FastQC for the trimmed sequences are shown in **Figure 6**. The sequences of the
478   length of interest, which is <32 nt for RIBO-seq and <50 nt for RNA-seq, had proper quality. The
479   examples of plots of GC content and distributions of sequence lengths over all trimmed
480   sequences are shown in **Figure 7**. The additional peak at 72% in GC distribution plot of RIBO-seq
481   library may potentially indicate rRNA contamination which can be removed during bioinformatic
482   analysis by rRNA filtration[46,47,48]. The sequence lengths distribution is very narrow for RIBO-seq
483   libraries, with a peak at 26-27 nt whereas the length distribution for RNA-seq libraries is broader
484   and ranges between 15 and 50 nt.

485

486   [Place **Figure 6** and **Figure 7** here]

487

488   To verify whether the RIBO-seq data provides information of true ribosomal footprints rather
489   than random mRNA fragments of the selected length, we compared sequencing data from RIBO-
490   seq and RNA-seq using RiboToolkit, a web server for RIBO-seq data analysis[49]. Ribo-seq data
491   exhibits triplet periodicity and a tall, narrow peak corresponding to the initiating ribosomes and
492   characteristic of the RF as shown in **Figure 8A**, which is not observed in the RNA-seq data (**Figure
493   8B**). Moreover, the metagene plot showing the profiles of average coverage of RF and mRNA
494   fragments on the coding sequences (CDSs) revealed a higher proportion of reads mapped to the
495   CDSs in the RIBO-seq dataset (**Figure 8C**), as expected.

496

497   [Place Figure **8** here]

498

499   To compare whether the RIBO-seq protocol presented here yields results similar to those
500   obtained by standard method of RF recovery (sucrose gradient ultracentrifugation), we paralleled
501   the sequencing results from libraries prepared following the two methods. The experiments
502   investigating translation regulation during sporulation in WT *B. subtilis* were conducted according
503   to the presented RIBO-seq protocol, as well as following the standard protocol which includes
504   monosomes recovery by sucrose gradient ultracentrifugation. The results of ribosome coverage
505   profiles obtained from the two experiments are shown in **Figure 9**. The visualisation of mapped
506   RFs is very similar between the two experiments and yielded similar results upon bioinformatic
507   analysis.

508

509   [Place **Figure 9** here]

510

511   **FIGURE AND TABLE LEGENDS:**
512   **Figure 1. Schematics of mRNA sample preparation for RIBO-seq and RNA-seq.** For RIBO-seq
513   library preparation, RNA is digested with MNase (A), followed by size selection of RF of ~28-30 nt
514   length (B); for RNA-seq RNA is isolated (C) depleted of rRNA (D) and the resulting mRNA is
515   randomly fragmented into fragments of varying length (E).

516

517   **Figure 2. Library preparation.** Library preparation includes ends repair, adapters ligation, reverse
518   transcription and amplification with barcoding.

519

520   **Table 1. RNA concentration and amount in representative samples of B. subtilis lysates.** The
521   table includes samples from wild type (WT) *B. subtilis* which were harvested before sporulation
522   induction (0) and one hour (1), two (2), three (3) or four (4) hours post sporulation induction.
523   Lysis was performed with 550 µL of lysis buffer containing chloramphenicol. It can be noticed
524   that as the sporulation proceeds the amount of obtained RNA decreases.

525

526   **Table 2. The amount and concentration of RNA after nuclease digestion of the representative**
527   **samples for RIBO-seq.** The table includes samples from wild type (WT) *B. subtilis* which were
528   harvested before sporulation induction (0) and one hour (1), two (2), three (3) or four (4) hours

529   post sporulation induction; the letter "R" refers to samples for RIBO-seq. Because RNA
530   concentration of WT4-R sample was low, 0.25 mg of RNA was digested, while 0.5 mg of the RNA
531   for the remaining samples was used for ribosomal footprint generation. The amount of MNase
532   for digestion was 178.125 U for 0.25 mg of RNA in WT4-R, and 356.25 U for 0.5 mg of RNA in
533   other samples. After digestion the samples were cleaned with the commercial RNA clean-up kit
534   and ribonucleic acid concentration was measured with NanoDrop.
535
536   **Figure 3. 15% TBE-urea polyacrylamide gel with representative samples after MNase digestion.**
537   The figure includes samples from wild type (WT) *B. subtilis* which were harvested before
538   sporulation induction (0) and one hour (1), two (2), three (3) or four (4) hours post sporulation
539   induction; the letter "R" refers to sample for RIBO-seq. 15 µL of denatured samples was loaded
540   into the gel in order: WT2-R, WT3-R, WT4-R, WT0-R, WT1-R and the remaining volume was stored
541   at -80 °C as a back-up. 29 nt oligonucleotide and mix of 26 nt and 32 nt oligonucleotides were
542   used as markers. Electrophoresis was performed as described above and the gel was stained in a
543   SYBR Gold-bath.
544
545   **Table 3. RNA amount and concentration of samples for RNA-seq after RNA purification.** The
546   table includes samples from wild type (WT) *B. subtilis* which were harvested before sporulation
547   induction (0) and one hour (1), two (2), three (3) or four (4) hours post sporulation induction; the
548   letter "m" refers to samples for RNA-seq (mRNA).
549
550   **Table 4. The representative RNA amount and concentration after rRNA depletion.** The table
551   includes samples from wild type (WT) *B. subtilis* which were harvested before sporulation
552   induction (0) and one hour (1), two (2), three (3) or four (4) hours post sporulation induction; the
553   letter "m" refers to samples for RNA-seq. 8 µg of RNA from samples for RNA-seq were used for
554   rRNA depletion and cleaned with the commercial RNA clean-up kit afterwards.
555
556   **Figure 4. 6% polyacrylamide gel of cDNA libraries before and after size selection.** Quick-Load
557   pBR322 DNA-MspI Digest was used as a ladder. 25 µL of samples were loaded into the gel in the
558   following order: three RIBO-seq libraries and six RNA-seq libraries. The remaining volumes of
559   samples were stored at -80 °C as a back-up. The figure includes samples from wild type (WT) *B.*
560   *subtilis* which were harvested one hour (1), two (2), three (3) or four (4) hours post sporulation
561   induction; the letter "R" refers to samples for RIBO-seq and the letter "m" refers to samples for
562   RNA-seq.
563
564   **Figure 5. The examples of electropherograms and virtual gel images from high-sensitivity DNA**
565   **on-chip electrophoresis.** The figure includes samples from wild type (WT) *B. subtilis* which were
566   harvested before sporulation induction (0) and one hour (1), two (2), three (3) or four (4) hours
567   post sporulation induction; the letter "R" refers to RIBO-seq libraries and the letter "m" refers to
568   RNA-seq libraries.
569
570   **Figure 6. The examples of box-and-whisker plots of quality scores across all bases for RIBO-seq**
571   **and RNA-seq libraries after trimming.** The figure includes samples from wild type (WT) *B. subtilis*
572   which were harvested before sporulation induction (0) and four (4) hours post sporulation

573   induction; the letter "R" refers to RIBO-seq samples and the letter "m" refers to RNA-seq samples.
574

575   **Figure 7. The examples of plots of GC content and sequence length distribution of all sequences**
576   **for RIBO-seq and RNA-seq libraries after trimming.** The figure shows samples from wild type
577   (WT) *B. subtilis* which were harvested four (4) hours post sporulation induction; the letter "R"
578   refers to sample for RIBO-seq and the letter "m" refers to sample for RNA-seq.
579

580   **Figure 8. Metagene periodicity plots and average coverage of RF and mRNA fragments on CDSs.**
581   Metagene profiles were obtained with RiboToolkit, an integrated web server[49]. CDS length was
582   normalized to a value of 1 and each CDS was divided into 100 equal bins. The figure shows
583   samples from wild type (WT) *B. subtilis* which were harvested before sporulation induction (1);
584   the letter "R" refers to sample for RIBO-seq and the letter "m" refers to sample for RNA-seq.
585

586   **Figure 9. The visualisation of obtained RF mapped onto the genome.** The figure includes RF
587   obtained from wild type *B. subtilis* harvested four hours post sporulation induction and prepared
588   according to the presented protocol (WT4-R) or the standard protocol (WT4-R-s).
589

590   **DISCUSSION:**
591   The key technical challenge of ribosome profiling is the need to rapidly inhibit translation in order
592   to capture a snapshot of ribosomes on mRNAs at a particular physiological state of interest. To
593   accomplish this, translation inhibitors, rapid harvesting and flash freezing in liquid nitrogen are
594   commonly used. Applying antibiotics is optional since they can cause artifacts. Chloramphenicol
595   is the commonly used drug to arrest elongating ribosomes in bacterial RIBO-seq. However, it does
596   not prevent initiation, resulting in accumulation of ribosomes about six codons downstream of
597   the translation initiation site[14]. Moreover, its ability to inhibit the peptidyltransferase reaction
598   can be dependent on the nature of the ribosomal P- and A-site substrates. Presumably, CAM
599   arrests ribosomes more effectively with Gly, Ala, Ser, Cys, or Thr as penultimate amino acid of
600   the nascent peptide[17,38,50]. This is worth taking under consideration during experimental design
601   and bioinformatic analysis, especially when studying ribosomal pausing[17]. CAM has also been
602   shown to influence translational accuracy by promoting stop codon readthrough and
603   frameshifting[51]. Still, CAM inhibition is relatively fast and sufficient for typical RIBO-seq research[14]
604   and does not induce misincorporation[51]. Some of the artifacts caused by antibiotics can be
605   avoided by increasing the drug amount[9]. CAM concentration applied in our protocol is the typical
606   amount used in RIBO-seq experiments[7,14,21], however, the concentration may vary for different
607   microorganisms and should be optimized before sample harvesting. Due to the possibility of
608   occurrence of artifacts caused by applying translation inhibitors, it is recommended to avoid the
609   inhibitor treatment if not necessary for the research question and if the rapid harvesting is
610   possible[14]. Fast cells collection is crucial, especially if it is performed without the inhibitor
611   treatment, since prolonged harvesting can result in loss of polysomes and/or change in the
612   translatome as a response to the changing environmental conditions[14]. Thus, we recommend
613   using filtration as a harvesting method as it can be performed under the same or similar
614   conditions as the culture growth, including temperature and/or shaking. During filtration cells
615   collected at the filter surface are constantly flushed with growth medium, preventing them from
616   sensing shifts in cell densities, oxygenation and level of amino acids and other nutrients[14].

617 Filtration is gentler and may be faster than centrifugation, if the density of the culture is
618 moderate, otherwise a large number of cells can clog the filter pores and slow down the process.
619 If there is probability for prolonged filtration we recommend chloramphenicol pre-treatment of
620 the culture, which results in translation arrest and allows to extend harvesting time. If filtration
621 becomes difficult to complete due to filter clogging, we suggest to stop the filtration, remove the
622 excess culture and flash freeze fraction of the culture collected on the filter. We observed that
623 50 mL of dense bacterial culture is sufficient to perform RIBO-seq. The subsequent flash freezing
624 of cells in liquid nitrogen is the most universal and effective approach to immediately stop
625 translation[8,21,52].
626
627 Ribonuclease digestion is a critical step required to obtain good quality RF. Selecting the right
628 enzyme is important since the ribosomal subunits are composed of RNA molecules that can be
629 digested, disrupting the integrity of the ribosome, causing rRNA contamination and destroying
630 RF[44]. Originally, RNase I was used for digestion of mRNA and RF generation in eukaryotes[1]. Since
631 this enzyme seemed inactive in bacteria, it was replaced with micrococcal nuclease (MNase) from
632 *Staphylococcus aureus*[21]. It was later showed that RNAse I can in fact perform digestion of
633 bacterial samples, however MNase remains commonly used for this purpose[53]. The disadvantage
634 of MNase lies in the sequence bias of its catalytic activity, which is 30-fold increased proximal to
635 A or T, resulting in 80% of mRNA fragments starting with A or T at the 5' end[14]. However, this has
636 been overcome by the observation that most of the variation in length occurs at the 5'-end of RF,
637 and assigning ribosome density to the 3'-end yields precise and accurate information about the
638 ribosome position[7]. The MNase activity is affected by the enzyme concentration, pH and
639 digestion time and should be determined for each new MNase batch. An increased nuclease
640 activity results in increased rRNA contamination, while decreased activity causes less accurate
641 cleavage of ribosomal footprints. This may reduce the overall yield of RF in the size-selective gel
642 purification procedure and provide less accurate information on ribosome positions on mRNA[14].
643 Thus, the required amount of MNase should be determined carefully. For our experiments we
644 apply 712.5 U of micrococcal nuclease per 1 mg of RNA while the concentration reported in the
645 literature varies between 50 U[38], 450 U[34], 1000 U[14] and 1500 U[15,21,36] per 1 mg of RNA. As
646 mentioned above, MNase requires presence of calcium ions for its activity. In standard protocols
647 EGTA, a chelating agent for divalent ions which has a higher affinity for calcium compared to
648 magnesium[54], is used for stopping the digestion by binding calcium and inactivating MNase.
649 However, in our protocol the digestion reaction is stopped by using RNA cleaning kit directly after
650 incubation with the nuclease.
651
652 In standard protocols, the next step is ribosomes recovery by sucrose cushion ultracentrifugation
653 or sucrose gradient ultracentrifugation[8,14]. A sucrose gradient centrifugation enables the
654 selective purification of monosomes from subunits and polysomes, however it requires specific
655 equipment such as a gradient mixer and an ultracentrifuge. Also, the throughput of this method
656 is limited to 500-700 μL of lysate. On the other hand, sucrose cushion ultracentrifugation pellets
657 most ribosomal particles, enables processing of over dozen milliliters of lysate and is less
658 demanding in terms of the instrumentation needed. However, both approaches are lengthy as
659 they take minimum 4 to 6.5 hours[14] and require an ultracentrifuge. In our protocol this step is
660 omitted as we proceed directly to size selection in 15% TBE-urea polyacrylamide gel. The

661 electrophoresis is performed under denaturing conditions in order to unfold RNA secondary
662 structures and prevent intramolecular interactions. We use 26 nt, 29 nt and 32 nt
663 oligonucleotides as markers for excising a narrow range of ribosomal footprints. We assume that
664 the concentration of actual RF significantly exceeds potential contamination with false RF
665 considered as random, unprotected by the ribosome mRNA or rRNA fragments of length in that
666 range.. The results obtained using our protocol indeed showed enriched fraction of RF in the
667 selected size range. The quality check with Agilent 2100 Bioanalyzer shows RIBO-seq libraries as
668 single, well define bands and peaks, in contrast to RNA-seq libraries which are visualized as
669 significantly wider smears and peaks (Figure 5). Also, obtained Ribo-seq data exhibits triplet
670 periodicity (Figure 8A), which is not observed in the RNA-seq data (Figure 8B). Moreover, the
671 visualizations of the obtained RF mapped onto the genome show very similar expression patterns
672 regardless of the footprint isolation method which was further confirmed in our bioinformatical
673 analyses (Figure 9). Still, we are aware that false RF could map to the genome which may disturb
674 the obtained signal. However, the fact that false RF are randomly generated implies that they
675 should not accumulate to one place on the genome and hence, should not result in artifacts and
676 false result. Also, it has to be noted that there is no consensus in bacterial studies regarding the
677 size of RFs that should be isolated[53,55] and that selecting only a subfraction of length range may
678 lead to misinterpretations of the results[17]. It is suggested that a wide range of lengths
679 characteristic for bacterial footprints is a consequence of the prokaryotic ribosome behaviour
680 rather than MNase digestion[55]. For example, complementary interaction between the 3' end of
681 16S rRNA in small ribosomal subunit and a Shine–Dalgarno motif in mRNA can result in longer
682 RF[56]. Considering a wide range of lengths of bacterial footprints, Mohammad *et al*. recommend
683 to perform size selection between 15 and 40 nt[17,55]. This should lead to isolation of potentially an
684 entire range of RFs and ensure obtaining a comprehensive representation of ribosomes in various
685 stages of the translational cycle. Because of the ongoing discussion, the excision range should be
686 considered carefully and it is important to remember that the incorrect choice may exclude some
687 subfractions of RF and lead to bias in the subsequent analysis.

688

689 Due to the size selection performed directly after nuclease digestion and with omitting the
690 monosome isolation by ultracentrifugation, our protocol is quick, relatively easy and can be
691 conducted with standard laboratory equipment. Using dedicated kits for cleaning RNA and DNA,
692 purification of mRNA and library preparation ensures standardization and repeatability. Appling
693 more volume of ethanol during RNA cleaning increases the purification efficiency of <200 nt RNA
694 fragments[57]. Modifications to the library preparation - extending the incubation time and
695 lowering the temperature during adaptors ligation - are applied to decrease ligation efficiency
696 for methylated RNA as rRNA and to reduce rRNA contamination[58]. Our protocol has been used
697 to investigate translation regulation in various growth conditions (publications in process) but
698 can be applied to study other aspects of translation like TIS detecting or to improve genome
699 annotation and assist proteomic studies. Additional modification to the protocol, dependent on
700 the research question, can be easily included, e.g. crosslinking the ribosome with the protein of
701 interest or affinity purification of the ribosomes of interest resulting in the enriched fractions of
702 ribosomes. With our protocol the RIBO-seq procedure is more accessible for scientists and thus
703 may stimulate new discoveries and accelerate developments in the field.

704

DISCLOSURES:
The authors have nothing to disclose.

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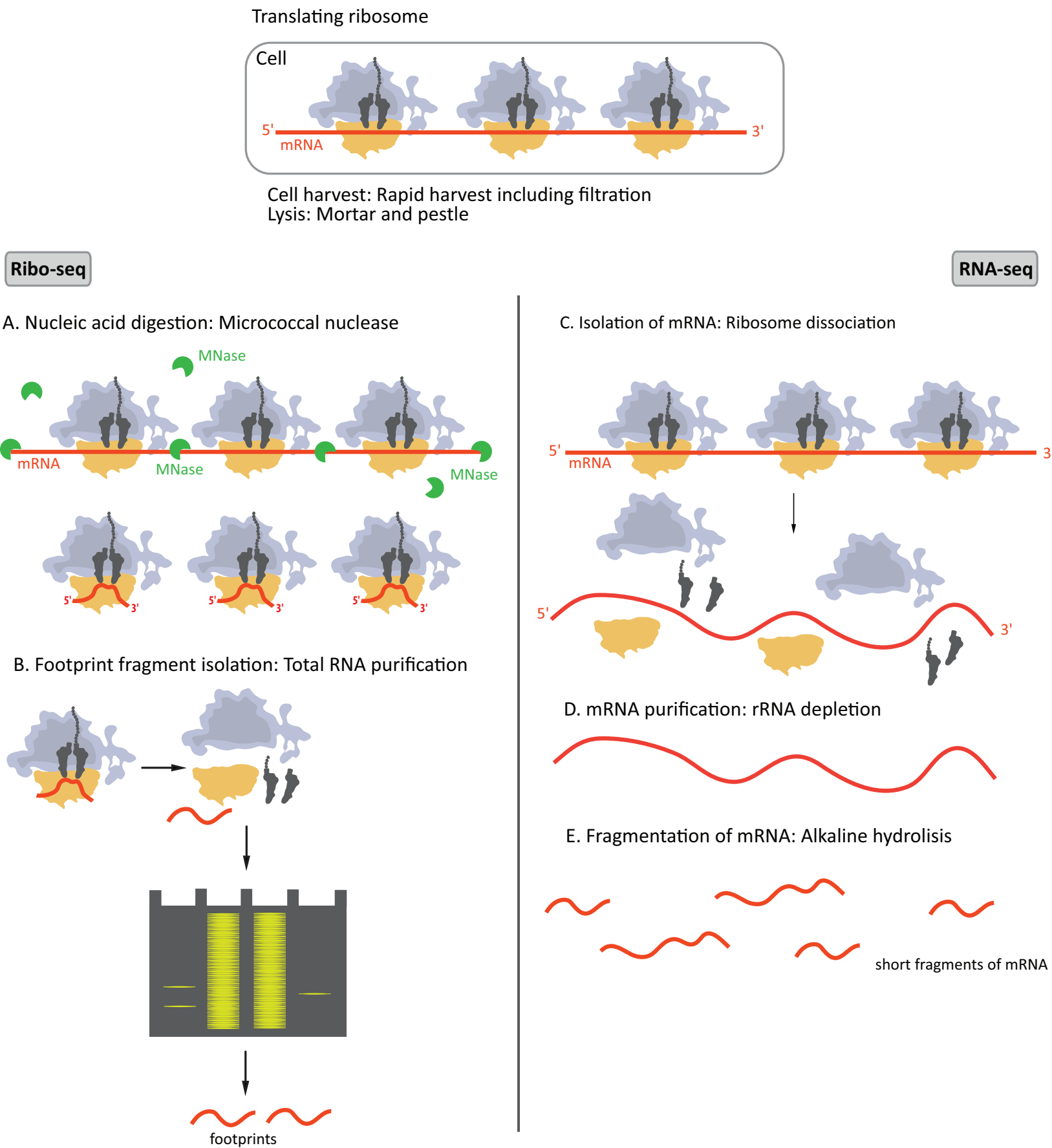838 *Chemistry*. **295**, 8999–9011 (2020).

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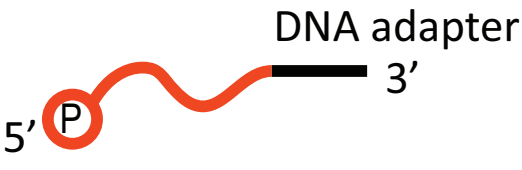
Figure 1

Translating ribosome

Cell

5'

mRNA

3'

Cell harvest: Rapid harvest including filtration
Lysis: Mortar and pestle

Ribo-seq

RNA-seq

A. Nucleic acid digestion: Micrococcal nuclease

MNase

MNase

mRNA

MNase

MNase

5' 3'   5' 3'   5' 3'

B. Footprint fragment isolation: Total RNA purification

footprints

C. Isolation of mRNA: Ribosome dissociation

5'

mRNA

3'

5'

3'

D. mRNA purification: rRNA depletion

E. Fragmentation of mRNA: Alkaline hydrolisis

short fragments of mRNA

Figure 2

Footprint isolation

RF or fragmented mRNA

5' P 3'

MNase

**3'Dephosphorylation/
5'Phosphorylation**

T4 PNK

OH 3'

5' P

3'Adapter ligation

DNA adapter

3'

5' P

**Hybridization
of RT primer**

3'

RT primer

5' P

5'Adapter ligation

DNA adapter   DNA adapter

5'   3'

RT primer

**Reverse
transcription**

5'   3'

3'   5'

cDNA

PCR

barcode   footprint

5'   3'

3'   5'
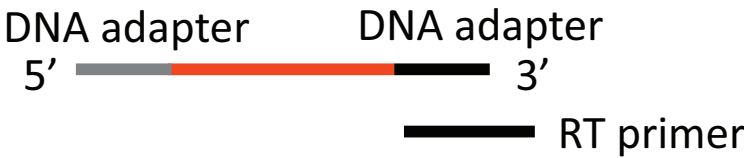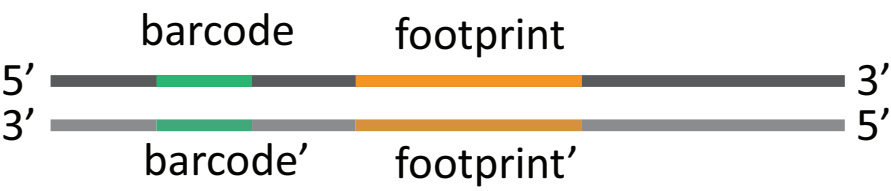
barcode'   footprint'

**Sequencing of
barcode /
footprint fragment**

barcode'   footprint'

3'   5'

Barcoding primer

L1

Chip

Figure 3

Figure 4

before excision   after excision

Figure 5

Figure 6

Figure 7

A — GC distribution over all sequences (WT4-R)
B — GC distribution over all sequences (WT4-m)
C — Distribution of squence lengths over all sequences (WT4-R)
D — Distribution of squence lengths over all sequences (WT4-m)

Figure 8

Figure 9

A. WT4-R-s Coverage

B. WT4-R Coverage

gene

cotX

Table 1

	RNA concentration [ng/µL]	RNA amount [mg]	A260/A280	A260/A230
WT0	5845	3.21	1.57	0.83
WT1	6275	3.45	1.58	0.79
WT2	3525	1.94	1.38	0.56
WT3	3717	2.04	1.37	0.61
WT4	1653	0.91	1.11	0.43

	RNA concentration [ng/µL]	RNA amount [mg]	A260/A280	A260/A230
WT0				

Table 2

	RNA concentration [ng/µL]	RNA amount [µg]	A260/A280	A260/A230
WT0-R	1015	50.75	2.12	1.84
WT1-R	1157.4	57.87	2.10	2.19
WT2-R	847.2	42.36	2.10	2.00
WT3-R	983.6	49.18	2.09	2.16
WT4-R	206	10.30	2.13	1.00

	RNA concentration [ng/µL]	RNA amount [µg]	A260/A280	A260/A230
WT0-R	1015	50.75	2.12	1.84
WT1-R	1157.4	57.87	2.10	2.19

Table 3

	RNA concentration [ng/µL]	RNA amount [µg]	A260/A280	A260/A230
WT0-m	10274.1	513.70	2.14	2.35
WT1-m	10118.3	505.92	2.12	2.33
WT2-m	5599.7	279.98	2.15	2.29
WT3-m	6562.2	328.11	2.08	2.31
WT4-m	757	37.85	2.01	1.95

	RNA concentration [ng/µL]	RNA amount [µg]	A260/A280	A260/A230
WT0-m	10274.1	513.70	2.14	2.35
WT1-m	10118.3	505.92	2.12	2.33
WT2-m	5599.7	279.98	2.15	2.29
WT3-m	6562.2	328.11	2.08	2.31
WT4-m	757	37.85	2.01	1.95

Table 4

	RNA concentration [ng/µL]	A260/A280	A260/A230	RNA amount [µg]
WT0-m	62.7	1.99	1.75	3.1
WT1-m	59.3	2.00	2.16	2.9
WT2-m	93.7	1.97	1.69	4.7
WT3-m	68.3	1.99	2.33	3.4
WT4-m	85.0	1.97	2.00	4.2

	RNA concentration [ng/µL]	A260/A280	A260/A230	RNA amount [µg]
WT0-m	62.7	1.99	1.75	3.1

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Table of Materials

JoVE_Materials.xls

Reviewers' comments:

Reviewer #1:

Figure 8 is useful and to some extend is reassuring, however, it is unclear how the metagene profiles were obtained, it looks like ORFs were truncated as even in RNA-seq data the region upstream of stop is not covered. Also I did not understand what is shown in the panel C, what are the CDS bins?

Metagene profiles were obtained with RiboToolkit, an integrated web server. Also, we have now improved our bioinformatic pipeline – we changed the default offset settings to the data specific ones. The resulting plot of normalized read coverage over the CDSs is now an adequate representation of the obtained data (Fig. 8A and 8B).

The lengths of all coding sequences in the reference genome of *Bacillus subtilis* were normalized to a value of 1 and next, each CDS was divided into 100 equal fragments – bins (Fig. 8C).

Although the authors added the statement "However, it should be noted that the excision range should be considered individually depending on the research question and that the incorrect choice may lead to bias in the subsequent analysis" on lines 579-581, I think this is insufficient. Based on authors response "Indeed, for more precise experiments, like studying ribosomal pausing, conformation changes or frameshifting, the wider excision range should be considered." I suspect that the authors underapreicate the importance of wide range selection of read lengths. It seems to me that the authors think that length variation is a minor issue affecting pause sites or frameshifting. First, I am not sure how such variation would be relevant to pausing and frameshifting, secomd strong pause and especiallt frameshifting are rare events. However, read length variation is widespread as it is associated with SD like sequences which are very frequent. Selecting only long reads would lead to increased density at purine rich sequences, while slecting only short reads would result in deppletion of coverage at such sequences.

We further elaborated on the topic of size selection and a variety in footprint lengths (lines 605-617). We highlighted the recommendation to isolate footprints between 15 and 40 nt as suggested in the literature, and commented on the hazards of excluding certain RF subfractions.