

Article

Abridged Ribosome Profiling for Accurate Bacterial Translation Measurements

Marc Follmer , Korbinian Pürckhauer  and Klaus Neuhaus * 

Core Facility Microbiome, ZIEL Institute for Food & Health, Technical University of Munich, 85354 Freising, Germany; marc.follmer@tum.de (M.F.); korbinian.puerckhauer@tum.de (K.P.)

* Correspondence: neuhaus@tum.de

Abstract

Ribosome profiling, or Ribo-Seq, is a powerful tool for studying translation. It maps the positions of translating ribosomes on mRNAs, providing insights into actively expressed genes. Unlike mass spectrometry, Ribo-Seq is not affected by the same biases that limit mass spectrometry, such as protein size, concentration, trypsin digestibility, or hydrophobicity. Thus, the translome has previously been used to discover unannotated genes, including small and overlapping ones that were missed by mass spectrometry or gene prediction models. However, a major limitation of classical ribosome profiling is its complexity, involving multiple steps such as sucrose density gradient centrifugation and gel electrophoresis. These make the method costly, time-consuming, and limit its throughput. Here, we compared the classical method using gradient centrifugation and size exclusion by gel electrophoresis with shortened versions to evaluate experimental performance and achieved reductions. Our results show that the sucrose density gradient centrifugation is essential for obtaining accurate Ribo-Seq data, whereas gel electrophoresis for size selection can be omitted (although this requires increased sequencing depth). Thus, future experiments can be conducted with reduced sample input and hands-on time while still achieving a reliable quantification of translation.

Keywords: ribosome profiling; Ribo-Seq; microbial translomics; translation quantification; shortened workflow; bacterial gene expression

1. Introduction

In the past, global gene expression was measured using the mRNA or protein content of cells. A more fine-grained analysis is possible by measuring translational events. Early attempts involved affinity purification of translating ribosomes. For instance, Heiman et al. [1] labeled a ribosomal protein with GFP and used this as a “handle” to pull down ribosomes along with the entrapped mRNA. Around this time, Ingolia et al. [2] published the currently most widely used method for ribosome profiling (Ribo-Seq). Here, all RNA not protected within the ribosome is digested using an RNase treatment. The resulting monosomes are collected via gradient centrifugation. Subsequently, the footprints (i.e., the ribosome-protected mRNA fragments) are sequenced. This workflow has now been widely used to monitor and quantify translation across all domains of life [2–4]. By capturing the exact location of ribosomes at the moment of cell harvest, which indicates translation, Ribo-Seq is a superior method for identifying protein synthesis compared to mRNA abundance alone [2]. In addition, the achieved “translome” is a good proxy for the proteome acquired by mass spectrometry (MS). Unlike MS, Ribo-Seq is not affected



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by protein size, concentration, or other biases (trypsin digestibility, hydrophobicity) [5,6]. Therefore, it gives a more detailed picture of translation than MS; however, subsequent factors such as post-translational modifications and protein stability cannot be examined [6]. Unfortunately, achieving single-codon resolution in bacteria is challenging, even though it would greatly facilitate the detection of novel genes. Codon-level resolution is currently only possible in the summed signal [7,8]. Nevertheless, Ribo-Seq can successfully be used to detect novel, often small and weakly expressed genes that remained hidden from MS or escaped gene prediction models (e.g., [9–11] and references in [12]). The potential was further exploited by stalling ribosomes at start codons, greatly facilitating discovery [13,14]. To aid data analysis, especially in annotating novel genes, several tools for Ribo-Seq data have been developed [15–17]. Vice versa, predicted small genes have been verified using Ribo-Seq in a human microbiome [18]. In addition, the combination of ribosome profiling with RNA sequencing can be used to detect genes that are translationally regulated by measuring the translation efficiency [19]. Moreover, some supposedly non-coding RNAs were found to be coding using Ribo-Seq [20]. However, despite great opportunities, several limitations remain, such as the lack of true codon periodicity when just using MNase, which has been attempted to be circumvented by using a mixture of several RNases, the need for a relatively large quantity of sample, and a cumbersome workflow, including steps of digestion, gradient centrifugation, and gel electrophoresis [8,10,21]. After preparing the footprints, the short mRNA fragments are transformed into a strand-specific sequencing library and are sequenced using short-read next-generation sequencing [2]. All steps combined are time-, budget-, and sample-intensive, with a major limitation for many labs being the need for an ultracentrifuge to perform gradient centrifugation. Here, we test for the necessity of the most cumbersome steps, namely, sucrose density centrifugation and size selection by gel electrophoresis.

2. Materials and Methods

2.1. Bacterial Cultivation

Escherichia coli LF82 $\Delta tolC$ was cultivated at 37 °C and 150 rpm overnight in Schaedler broth or M9 minimal broth supplemented with MEM vitamins. Final cultures were inoculated 1:100 with the overnight culture in 300 mL of the corresponding broth. Subsequently, cultures were incubated at 37 °C and 150 rpm until an OD₆₀₀ of 0.8 was reached.

2.2. Harvesting and Lysis of Bacteria

Bacterial cultures were centrifuged (5 min, 12,000× g, 4 °C) and the pellet was dissolved in 200 µL of polysome lysis buffer for each 50 mL of culture (20 mM Tris-HCl, pH 8; 10 mM MgCl₂, 100 mM NH₄Cl, 5 mM CaCl₂, 0.1% NP-40, and 0.4% Triton X-100), according to Woolstenhulme et al. [4]. Of note, neither DNase I nor chloramphenicol was applied. Chloramphenicol is known to cause artifacts, which are prevented by simply conducting snap-freezing [7]. Thus, the buffer was supplemented with 3.2 µL of Superscript-In (20 U/µL) for every 50 mL of culture. Bacterial pellets were flash-frozen in liquid nitrogen. Cell pellets were kept frozen and ground in liquid nitrogen with a mortar and pestle. After thawing, cell debris was removed by centrifugation, three times for 5 min at 12,000× g and 4 °C, and after each step, the clear supernatant was transferred into the same tube. Finally, the tube was centrifuged three times for 5 min at 20,000× g and 4 °C, and as before, the supernatant was transferred into a fresh tube to remove any leftover cell debris.

2.3. RNase Digestion and Sucrose Density Centrifugation

To obtain footprints, 1 mg of total RNA (measured using a NanoDrop, Thermo Fisher Scientific, Waltham, MA, USA) was digested for 1 h at 25 °C, while shaking at 900 rpm, using

750 U MNase (Thermo Fisher Scientific, Waltham, MA, USA), 5 U XRN-1 (New England Biolabs, Ipswich, MA, USA), 50 U RNase R (LGC Biosearch Technologies, Teddington, UK) and 12 U Exonuclease T (New England Biolabs, Ipswich, MA, USA). An RNase mixture was used, since MNase alone has bias; for instance, it prefers AT-rich regions and causes mainly observed variations at the 5'-end [4]. Therefore, to aid digestion of fragments derived from initial cuts by MNase, we also added XRN-1, a 5'→3' exonuclease, although this enzyme itself cannot process 5'-triphosphate ends (i.e., primary bacterial transcripts [22]). Furthermore, we added the 3'→5' exonucleases RNase R and Exonuclease T, which preferentially act on structured and unstructured RNA, respectively [23,24]. Thus, RNase R should limit structured ncRNA carryover. The digestion was supplemented with CaCl₂ for a concentration of 1 mM, 55 µL of NEB buffer 4, and molecular biology grade water up to 500 µL of total volume. Quenching was conducted with 6 µL of 500 mM EGTA and 5 µL of Suprase-In (20 U/µL). The sample was transferred onto a sucrose gradient. Here, nine 1.5 mL layers of 50% to 10% sucrose (bottom to top) in polysome gradient buffer (20 mM Tris-HCl, pH 8; 10 mM MgCl₂, 100 mM NH₄Cl, 2 mM DTT) were used according to Woolstenhulme et al. [4]. Each layer was supplemented with 0.1875 µL of SYBR Gold (10,000× concentrate). The gradient with sample was centrifuged for 3 h at 28,000 × rpm (corresponds to 107,000× *g* at *r*_{av}) and 4 °C, using an SW28.1 rotor (L7 ultracentrifuge, Beckman Coulter, Brea, CA, USA). The monosome fraction was visualized with UV light. The centrifugation tube was pierced at the bottom, and drops with the monosome fraction were collected into a fresh reaction tube.

2.4. RNA Extraction

RNA was extracted from 200 µL of lysate (for RNA-Seq) or the monosome fraction, which had been aliquoted into 200 µL portions (for Ribo-Seq), by adding 1 mL of TRIzol per 200 µL of liquid. After 5 min at room temperature (RT), 200 µL of chloroform was added. The mixture was briefly vortexed and incubated for an additional 5 min at RT. After centrifugation for 15 min at 12,000× *g* and 4 °C, the aqueous phase was transferred to fresh tubes, and 500 µL of isopropanol and 1 µL of 20 mg/mL glycogen were added. After incubation for 30 min at RT, the sample was centrifuged for 10 min at 12,000× *g* and 4 °C. The pellet was washed twice with 70% or 80% ethanol, respectively, for total RNA and ribosomal footprints. Finally, RNA was dissolved in molecular-grade water. RNA integrity for RNA sequencing was verified using a 1.5% agarose gel (Figure A1) and the Agilent RNA 6000 Nano Kit for the Agilent 2100 BioAnalyzer (Agilent Technologies, Santa Clara, CA, USA).

2.5. Size Selection of Ribosomal Footprints

The 15% urea–polyacrylamide (PAA; Table A1) gels were pre-run at 200 V for 20 min for equilibration. Prior to loading, up to 55 µg of RNA per gel was incubated at 80 °C for 2 min with an equal volume of 2× Novex TBE-urea sample buffer. The denatured RNA was loaded into the wells. Next to the RNA samples, a size ruler consisting of random ssRNA oligo of 20 and 40 nt was loaded. The RNA-size ruler was mixed with buffer and heated as described for the sample. The gels were run for 70 min at 200 V. For RNA staining, the gels were incubated in 50 mL TBE with 15 µL of SYBR Gold for 15 min under moderate shaking. Footprints were selected between the RNA size ruler, i.e., between 20 nt (N20) and 40 nt (N40; Figure A2a). The cut gel section was centrifuged for 2 min at 15,700× *g* at RT in self-made gel-breaker tubes. Gel-breaker tubes were made from a 0.5 mL tube in which we punched two separate holes in the bottom with a diameter of 0.9 mm each. This tube was placed into a 2-mL tube, allowing the gel fragments to be collected while spinning. The crushed gel was supplemented with 400 µL of gel extraction buffer (300 mM

sodium acetate, 1 mM EDTA) and 1 μ L of Suprase-In. For footprint elution, samples were incubated overnight at 23 °C under shaking at 800 rpm. The final mixture was transferred onto a 0.22 μ m spin filter (Corning, Corning, NY, USA) and centrifuged at 9300 $\times$ g for 2 min. Centrifugation was repeated after loosening the gel in the filter to maximize eluate volume. The eluate was incubated with 1 mL of 100% ethanol, 1 μ L of 20 μ g/ μ L glycogen, and 40 μ L of 3 M sodium acetate at -75 °C for several hours. Subsequently, samples were centrifuged for 20 min at 12,000 $\times$ g and 4 °C, and pellets were washed at least twice with 80% ethanol. RNA was resuspended in RNase-free water. RNA fragments were not further assessed.

2.6. DNA Digestion for RNA Sequencing

For DNA digestion, 20 μ g of total RNA was incubated with 2 μ L of Turbo DNase, 10 μ L of 10 $\times$ Turbo DNase buffer, and 2.5 μ L of Suprase-In in a total volume of 100 μ L for 1 h at 37 °C. The DNase is inactivated by adding EDTA to a final concentration of 15 mM and incubating for 10 min at 65 °C. For precipitation, 1380 μ L of precooled 100% ethanol, 55.2 μ L of 3 M sodium acetate, and 1 μ L of 20 μ g/ μ L glycogen were added. The mixture was incubated at -20 °C overnight or at -75 °C for several hours. The mixture was centrifuged for 20 min at 12,000 $\times$ g and 4 °C, and the pellet was washed twice with precooled 70% ethanol. Finally, the RNA was resuspended in water for molecular biology.

The success of DNA digestion was verified using a 25 μ L PCR targeting *rrsH* of *E. coli* (sequence taken from GCF_000005845.2). For the PCR, 2.5 μ L of Taq buffer, 0.5 μ L of dNTP mix, 0.5 μ L of *rrsH*-F (AAT GTT GGG TTA AGT CCC GC), 0.5 μ L of *rrsH*-R (GGA GGT GAT CCA ACC GCA GG) primer, and 0.125 μ L of Taq polymerase (NEB) were used. One microliter of the digested sample was used as a template. As a positive control, genomic DNA was used as a template. Cycling included an initial step of 95 °C for 5 min, followed by 30 cycles of 95 °C for 30 s, 52 °C for 30 s, and 68 °C for 45 s, and a final step of 68 °C for 5 min. Absence of amplicons was tested on a 1.5% agarose gel with 5 μ L of the PCR (Figure A1).

2.7. Depletion of rRNA and Preparation for Sequencing

rRNA was depleted using riboPOOL™ Kits for *E. coli* (siTools Biotech GmbH, Planegg/Martinsried, Germany) following the instructions of the manufacturer, which was shown to be a good choice [25]. Both the Ribo-Seq and RNA-Seq samples were depleted using the riboPOOL™ RNA-Seq kit in the first experiment (designated as Protocols 1_{Exp1}, 2_{Exp1}, 3_{Exp1}, and 4_{Exp1}, and RNA-Seq_{Exp1}). In the second experiment (designated Protocols 1_{Exp2}, 2_{Exp2}, and RNA-Seq_{Exp2}), the riboPOOL™ Ribo-Seq and riboPOOL™ RNA-Seq kit were used according to the sample type. Up to 5 μ g of RNA was used for the first experiment and up to 3 μ g for the second, following the manufacturer's updated instructions. In any case, RNA was precipitated using ethanol as described in the manual at -75 °C for several hours.

The depleted total RNA sequencing samples (i.e., no footprints) were fragmented using a BioRuptor Plus sonication device (Diagenode, Seraing, Belgium) in "high" mode two times for 30 s at 4 °C, with a 30 s pause in between.

To reset the phosphorylation status of all fragments in Ribo-Seq and RNA-Seq, dephosphorylation was conducted by adding 10 U of Antarctic phosphatase, 2.7 μ L of Antarctic phosphatase buffer, and 0.5 μ L of Suprase-In to each 25 μ L of sample. The samples were incubated for 30 min at 37 °C and cleaned using the miRNeasy mini Kit (Qiagen, Venlo, Netherlands) following the manufacturer's instructions. Here, the RWT buffer was added to each sample for a total volume of 700 μ L. To increase yield, the eluate was put on the column a second time. Next, RNA fragments were phosphorylated. Each 30 μ L of the

sample was supplemented with 20 U of T4 Polynucleotide kinase (T4PNK), 3.5 µL of T4 DNA Ligase buffer, and 0.5 µL of Suprase-In. Reactions were incubated for 1 h at 37 °C. The mono-phosphorylated RNA was cleaned up as above.

2.8. Library Preparation

Libraries for sequencing were prepared according to the TruSeq® Small RNA Library Prep Reference Guide (Document #15004197 v02) with a few changes. After library amplification, 10% PAA gels were used for gel electrophoresis, which were run for 70 min at 145 V (Table A2, Figure A2b). For this, 50 µL of the sample was mixed with 25 µL of 6× DNA loading dye. As a size standard, the ladders included in the kit, i.e., CRL and HRL, were used. For staining, the gel was incubated for 15 min in 50 mL TBE supplied with 15 µL of SYBR Gold (10,000× concentrate) at moderate shaking. Steps 7–11 were skipped as suggested in the manual, when concentrating the final libraries, as conducted here. The final library was eluted by incubating the crushed gel overnight in 300 µL of ultrapure water at 22 °C and shaking at 700 rpm. The sample was pipetted on a 0.22 µm spin filter, and the library was obtained by centrifuging for 1 min at 6000× g and RT. The library was precipitated as given in the manual at −75 °C for several hours. The obtained pellets were washed and briefly air-dried. The library was resuspended in 10 mM Tris-HCl, pH 8.5. Final libraries were sent to Novogene (Planegg/Martinsried, Germany) for partial lane sequencing on a NovaSeq (Illumina, San Diego, CA, USA) with PE150 reads.

2.9. Analysis of Sequencing Data

Only forward read files are used for analysis (as they contain fewer sequencing errors than the reverse reads) and for determining strand specificity (achieved by directional ligation of different adapters to the 5' and 3' ends of the RNA fragments). Adapters were trimmed with Cutadapt v5.0, and the output read length was set between 20 and 40 nt [26]. The resulting reads were mapped onto the genome of *E. coli* LF82 (GCF_021398935.1) using Bowtie2 v2.5.4 with the following parameters: -R 3, -D 20, -N 0, -L 19 [27]. These parameters are close to the settings in the preset “very-sensitive option”, but importantly, -L is set to 19 to slightly increase the overall sensitivity for these short reads. Multimapping reads were handled by default as stated [28]. Samtools v1.21 was used to convert sam-files to bam-files and also to sort them. Unmapped reads were discarded. Using Bedtools v2.31.1 (“bedtools intersect”), reads mapping to known tRNA and rRNA regions were excluded, and remaining reads were written into a new bam-file using samtools. These remaining reads were analyzed further. To calculate sense mapping reads to features for RPM calculation, featureCounts v2.1.1 was used with the following prompts: -a genomic.gtf -o “CDS.txt”/“transcript.txt” -R BAM -s 1 -t “CDS”/“transcript” -T 20 [29]. For coding sequences, the strings “CDS.txt” and “CDS” were used, while for ncRNA, the strings “transcript.txt” and “transcript” were used. The GTF-file from GCF_021398935.1 (annotation date 05/30/2025) was used as genomic.gtf. “Effective reads” are defined as reads mapping to the genome but not to rRNA and tRNA, and were determined using samtools. RPM values were then calculated by multiplying “reads per gene” by 1,000,000 and dividing the result by “total number of effective reads”. Read lengths were determined by running “bedtools bamtoBED -i \$input | awk '{print \$3-\$2}' | sort | uniq -c | sort -gk2,2.” Graphs were made, and correlations were calculated using Excel 2019. Graphs were subsequently edited using Affinity Designer 2 v2.6.5.

3. Results

Ribosome profiling was evaluated using the complete workflow and different shortcut approaches (Figure 1). Protocol 1 is the established “classical” method, which is used

as a reference. Here, sucrose density gradient centrifugation and gel electrophoresis are conducted. Protocol 2 has the centrifugation step, but omits the gel. Conversely, in Protocol 3, centrifugation is omitted, but gel electrophoresis is used. Finally, Protocol 4 does not involve centrifugation or gel electrophoresis. All workflows were conducted using a single bacterial sample, including RNA-Seq. Further, Protocols 1 and 2 were processed together until after the centrifugation. The experiment with bacteria grown in Schaedler medium (i.e., rich medium) was designated Protocols 1_{Exp1}, 2_{Exp1}, 3_{Exp1}, and 4_{Exp1}, and for RNA sequencing, RNA-Seq_{Exp1}. Protocols 1 and 2 (together with RNA-Seq) were also evaluated in M9 minimal medium, and the output was designated Protocols 1_{Exp2}, 2_{Exp2}, and RNA-Seq_{Exp2}.

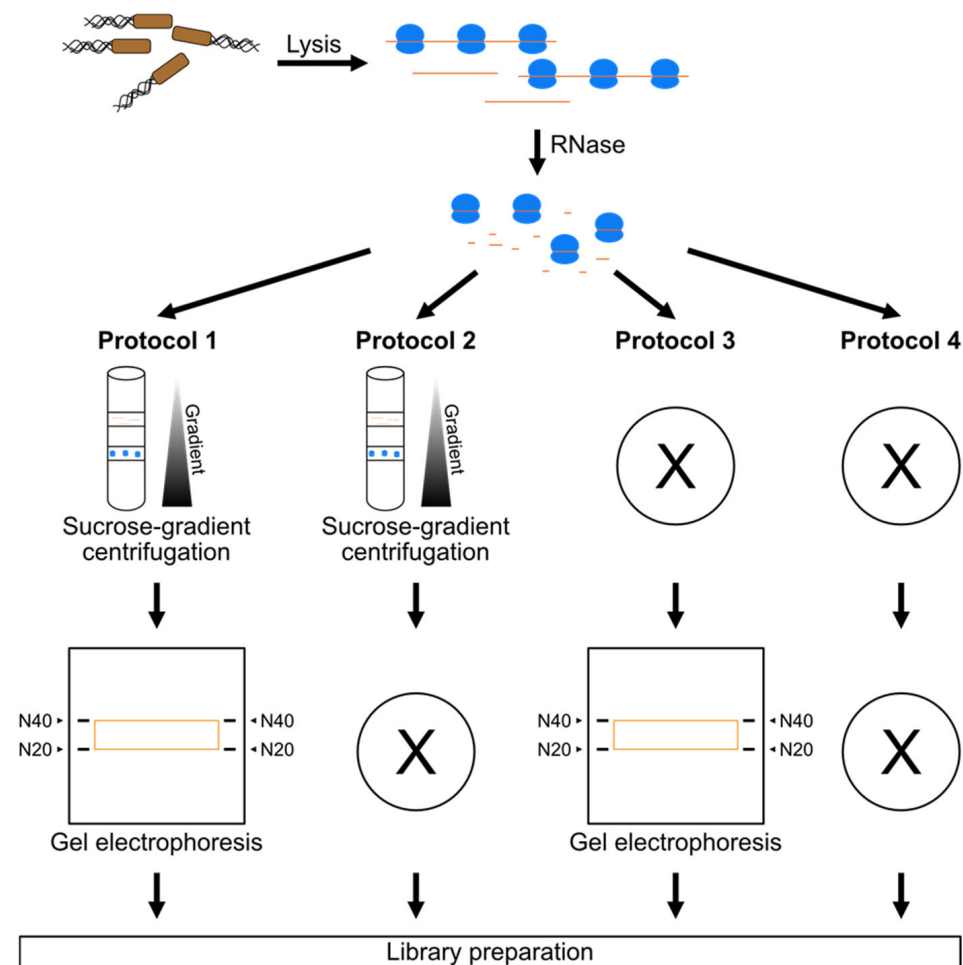


Figure 1. Schematic overview of four ribosome profiling strategies tested. A circled X indicates omissions in all cases. **Protocol 1** includes sucrose density gradient centrifugation and gel electrophoresis. **Protocol 2** includes sucrose density gradient centrifugation but omits gel electrophoresis. **Protocol 3** omits sucrose density centrifugation but includes gel electrophoresis. **Protocol 4** omits both. N20/N40 are random ssRNA oligonucleotides of 20 and 40 nt.

First, the distribution of sequenced reads was compared between the different approaches. Next, we compared normalized translational values, expressed as reads per million (RPM), for all genes using linear regression. Finally, we analyzed the fraction of effective reads mapping to coding sequences and the prevalence of non-coding RNAs (ncRNAs) to assess whether the reads represent true ribosome footprints or indicate excessive carryover of non-footprint RNA, thereby reflecting the quality of the signal.

3.1. Evaluating Raw-Read Distribution for Their Usability in Downstream Analysis

To assess the efficiency of each approach, we compared the distribution of raw reads across five distinct read categories: (1) effective reads, (2) rRNA/tRNA reads, (3) reads shorter than 20 nt, (4) reads longer than 40 nt, and (5) reads not mapping to the bacterial genome. Effective reads are the most relevant category. They are defined as reads mapping outside rRNA and tRNA and should correspond to mRNA. For Protocols 1_{Exp1} and 1_{Exp2}, 29% and 19% of the reads were effective, respectively. Protocols 2_{Exp1} and 2_{Exp2} yielded roughly half the number of effective reads, 15% and 10%, respectively. Therefore, gel electrophoresis allows for approximately double the relative abundance of effective reads. In contrast, reads from Protocols 3_{Exp1} and 4_{Exp1} predominantly map to rRNA and tRNA, resulting in only 6–7% of effective reads. Thus, omitting the density-gradient centrifugation decreases the number of effective reads substantially (Figure 2).

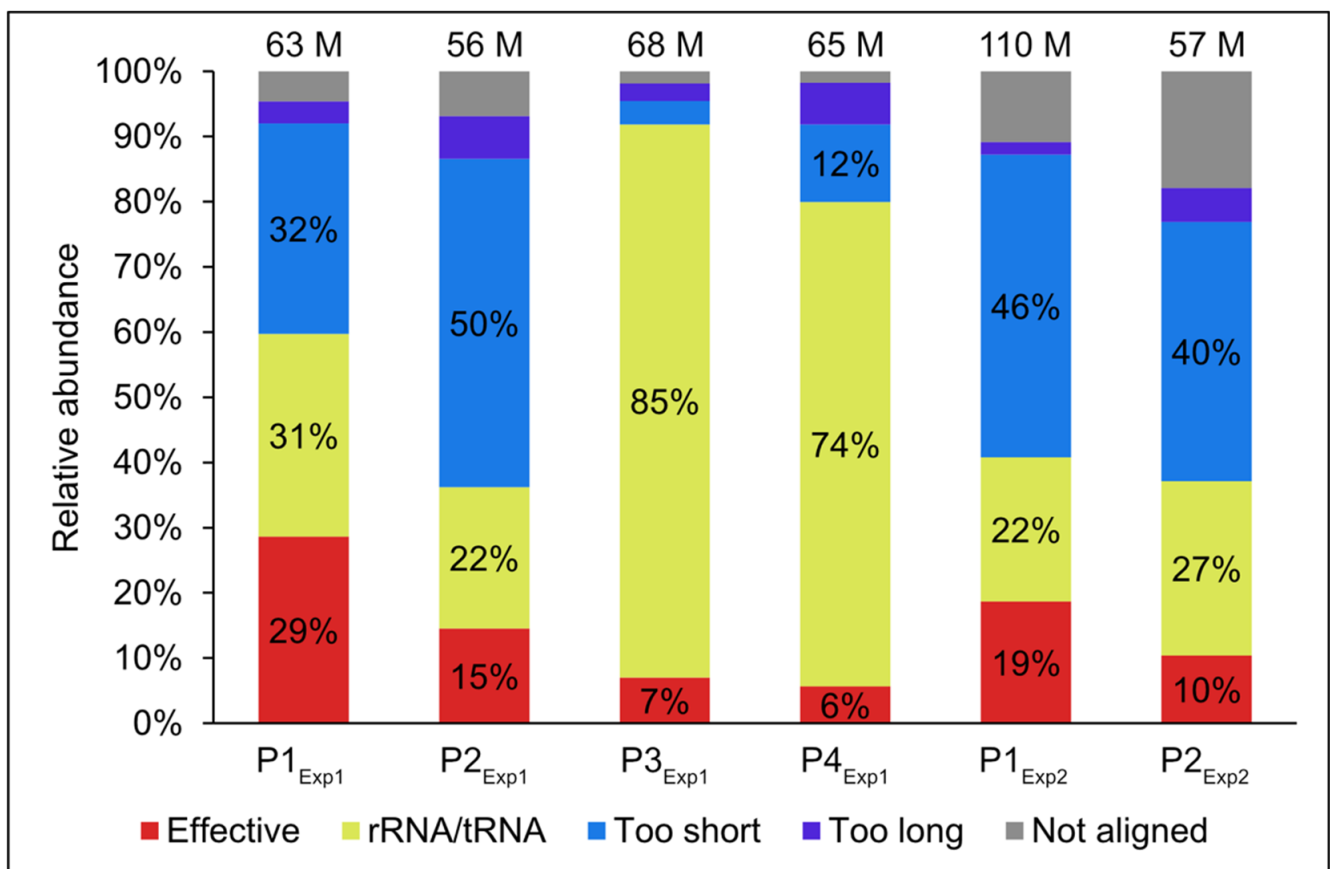


Figure 2. Overview of relative counts for read categories as indicated concerning the workflows tested. The total number of reads is indicated above each column. Reads are categorized as (1) effective reads, (2) reads mapping to rRNA or tRNA, (3) reads that are too short, (4) reads that are too long, and (5) reads that could not be aligned. Protocols 1 and 2 were tested twice, but in different media. Of note, Protocol 2 yields half the effective reads of Protocol 1, while Protocols 3 and 4 result in even fewer. Thus, Protocol 1 has nearly twice as many effective reads compared to Protocol 2 and is much better than Protocols 3 and 4. Actual read numbers for this bar plot are given in Table A3.

3.2. Comparison of Gene Expression Values Between Different Approaches

To assess whether one of the shortened workflows is able to accurately quantify translation, we evaluated global gene coverage by calculating RPM values and assessed the linear correlation of these values across the different approaches with an intercept at 0.0. When comparing coding sequences with RPM values ≥ 1.0 (Figure 3), it becomes evident that

Protocols 1 and 2 yield highly similar results and can be considered interchangeable. The R^2 values between Protocols 1 and 2 reach or exceed 0.97 when comparing log-transformed RPM values. Protocols 3 and 4 show moderate correlation in RPM values to the “gold standard” Protocol 1.

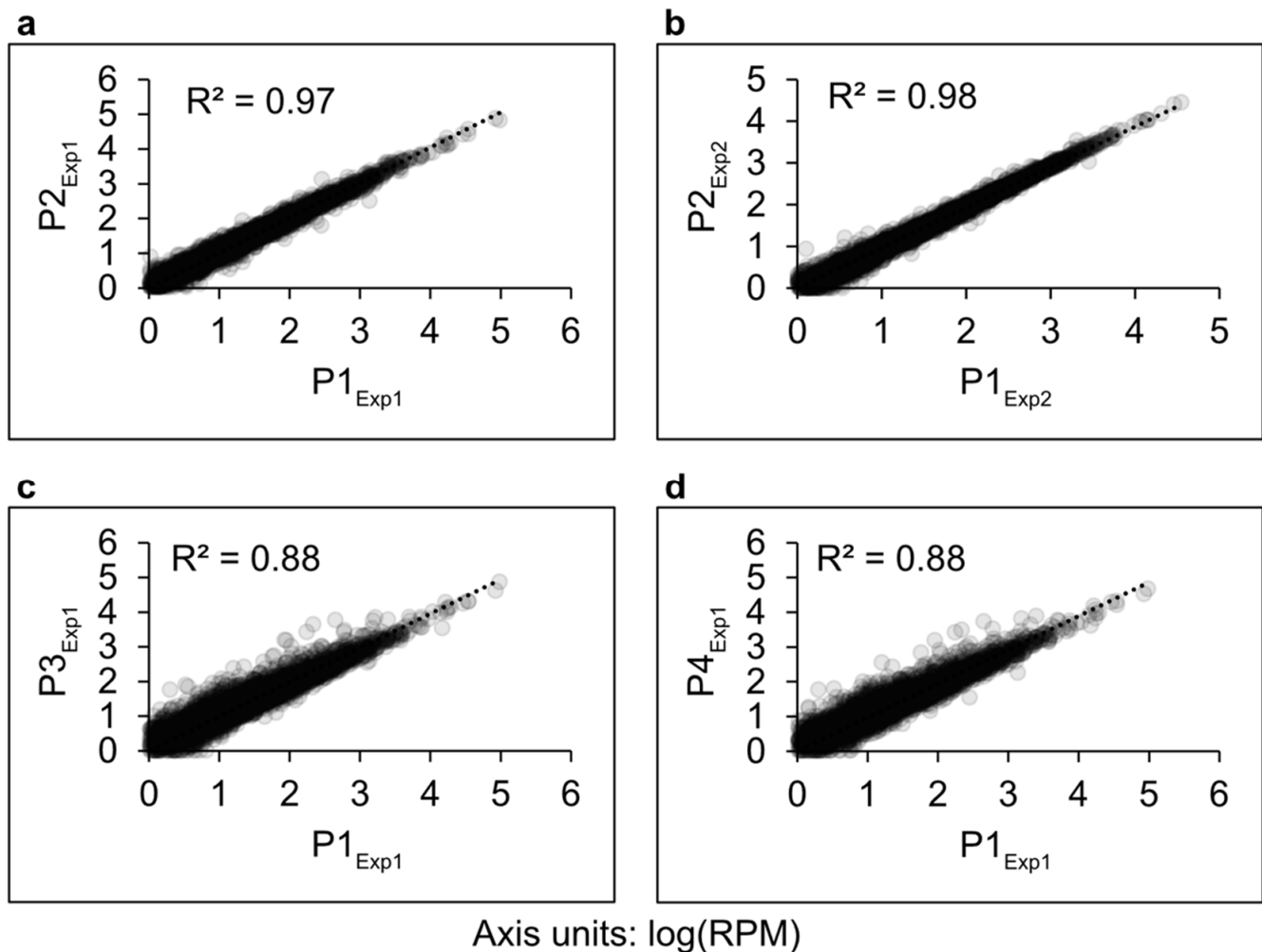


Figure 3. Protocols 1 and 2 correlate very well across two growth conditions. XY-diagrams of log-transformed RPM values above 1.0. Correlations are compared between workflows as indicated. The intercept is set to 0.0. (a,b) Protocol 1 compared to Protocol 2 in two experiments. (c,d) Protocol 1 compared to Protocols 3 and 4 for the first experiment, respectively. The drop in correlation indicates that Protocols 3 and 4 are inferior compared to Protocol 1.

3.3. Carryover of Non-Coding RNA Reveals Further Limitations in Shortened Ribosome-Profiling Procedures

To verify that the generated data represent genuine ribosomal footprints of mRNA rather than contaminating untranslated RNAs, we compared the number of reads mapping to coding regions to the number of effective reads in the sample. In rich medium (Experiment 1), more than 90% of effective reads map to coding regions for Protocol 1 (94%) and Protocol 2 (91%). Protocols 3 (76%) and 4 (66%) display much lower values; however, the values are still higher than RNA-Seq (59%). In minimal medium (Experiment 2), a decreased ratio is observed. Here, for Protocols 1 and 2, the values drop to 78% and 66%, respectively, while RNA-Seq stays at a value of 59%. Even though the *E. coli* genome is well annotated, we reasoned that some ncRNAs exist, which could constitute a contamination. Nevertheless, ncRNA genes are the only regions that we assume with high certainty are untranslated. Thus, we compared RPM values of known ncRNA genes between workflows,

i.e., *ffs*, *rnpB*, *ssrS*, *rprA*, and *RtT* sRNA (Figures 4 and A3; Table A4). Here, Protocols 1 and 2 displayed the lowest RPM values for ncRNA consistently, while either Protocol 3 or 4 had the highest values. Solely, *rnpB* showed some elevated levels for the tested Protocols 1 and 2 when compared to RNA-Seq.

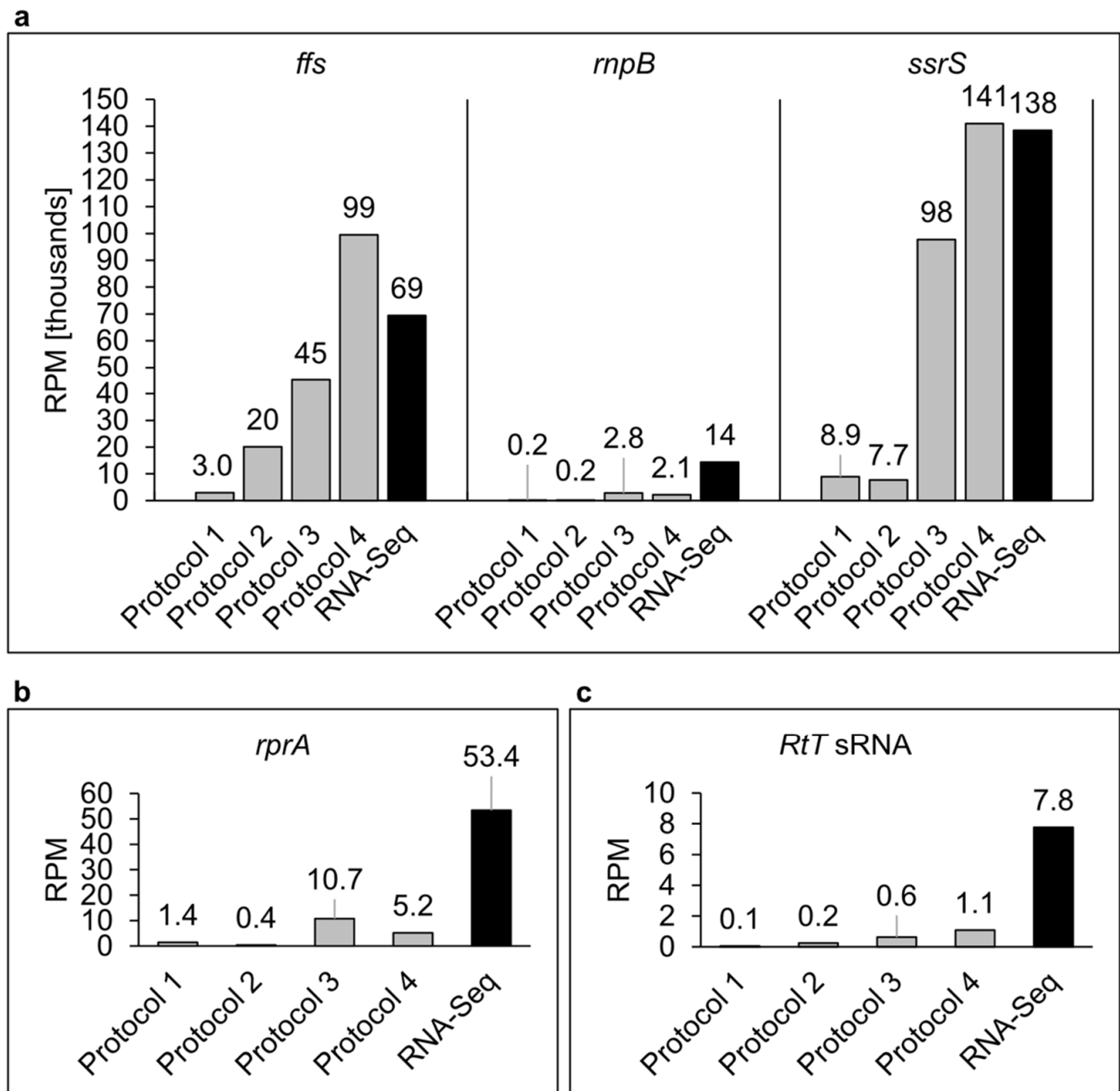


Figure 4. Protocols 1 and 2 effectively limit contamination of ncRNA in the first experiment, while Protocols 3 and 4 do not. (a) *ffs*, *rnpB*, and *ssrS*; (b) *rprA*; and (c) *RtT* sRNA.

3.4. Influence of Gel Electrophoresis on Read Lengths

Previous analysis of ribosome profiling data suggested that reads corresponding to ribosome-protected fragments yield a peak between 24 and 27 nt. However, a wider distribution can be seen when isolating a broader fragment size range in size selection. In addition, fragment sizes vary depending on the concrete experimental design [7,30]. We expected to see a peak around the mentioned size in our data. Here, Protocol 1 serves as the reference as before. The RNA-Seq sample was fragmented using sonification, which

should yield a broader pattern without specific peaks as seen for the ribosome footprints. Comparing the shortened workflows to the classical method (Protocol 1) and to RNA-Seq data, we examined the read-length distributions across the different workflows. We found that read lengths are similar between Protocols 1 and 2, whereas Protocols 3 and 4—as well as the RNA-Seq data—show broader size distributions (Figure 5; Tables A5 and A6). This is in line with our previous observations that Protocols 1 and 2 produce similar results, while workflows without sucrose density gradient centrifugation seem to have issues.

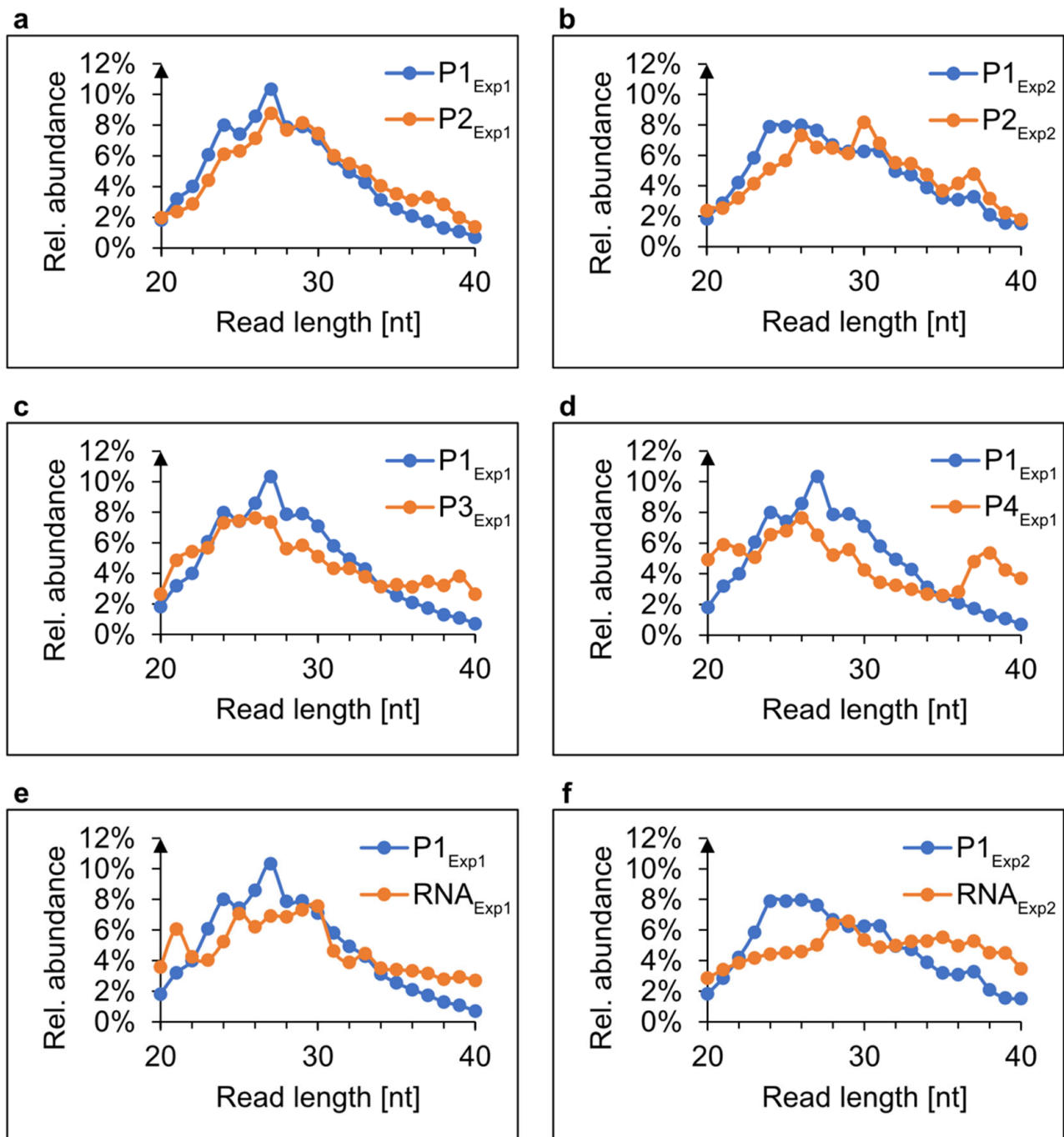


Figure 5. Comparing read lengths for different workflows. (a,b) Comparison between Protocols 1 and 2. (c) Comparison between Protocols 1 and 3. (d) Comparison between Protocols 1 and 4. (e,f) Comparison between Protocol 1 and RNA-Seq for the two experiments. Protocols 1 and 2 display similar read distribution patterns when comparing the length of reads. For actual numbers of mean, median, and peak read lengths, and relative abundancies, see Tables A5 and A6.

4. Discussion

“Classical” ribosome profiling is labor- and budget-intensive. Here, we tested whether omitting the sucrose density centrifugation or gel electrophoresis, or both, has a significant influence on the outcome. Omitting sucrose density gradient centrifugation would be particularly desirable, as not every lab has access to an ultracentrifuge. Unfortunately, gradient omission leads to a significant decrease in effective read numbers (approx. fourfold fewer). However, even when performing centrifugation, only about half the number of effective reads are obtained when no UREA-PAA gel is used, compared to the classical method. This could limit the ability to analyze very small or weakly translated genes. If a study indeed targets novel, weakly translated genes, the omission of gradient centrifugation might be compensated for by deeper sequencing [20,30]. Nevertheless, RPM values of the protein-coding genes varied substantially between workflows with and without gradient centrifugation. For example, Protocol 2 correlated strongly with Protocol 1, but Protocols 3 and 4 showed poorer correlation with the latter. Nevertheless, Protocols 3 and 4 correlated well with each other, suggesting that sucrose density gradient centrifugation is the main factor driving the decreased correlation.

More importantly, differentiating between translated and untranslated regions of the genome is crucial for the discovery of novel genes. Ideally, reads corresponding to untranslated regions, such as ncRNA, should not be present in ribosome-profiling data. However, in workflows that omitted density gradient centrifugation (Protocols 3 and 4), markedly elevated levels of ncRNA were found along with much lower numbers of effective reads corresponding to coding regions. Concerning *rnpB*, such structured ncRNAs sometimes may appear at elevated levels in Ribo-Seq data, since their structure protects them partially from nuclease digestion [31,32]. Here, elevated levels of *rnpB* occurred in the second experiment using minimal medium. In parallel, we observed a decrease in effective reads mapping to coding regions in comparison to the first experiment. Indeed, for the first experiment, we grew bacteria in Schaedler broth (a rich medium), while for the second experiment, as said, M9 minimal medium was used. It has been described before that in nutrient-limited environments, bacteria grow more slowly, and fewer numbers of (active) ribosomes are present. This lowers the number of translating ribosomes that can be harvested from a gradient, which, in turn, leads to a less complex library [33]. Consequently, a higher fraction of reads originates from non-footprint RNA, causing increased noise observed in the second experiment using minimal medium [6]. Nonetheless, RPM values of ncRNA and the distribution of read lengths correlate well across both experiments. Thus, Protocols 1 and 2 continuously show similar and the best performance. Therefore, denaturing gels could be omitted (i.e., following Protocol 2) without compromising final data quality, whereas the initial gradient centrifugation remains essential for high-quality results. It should be noted, however, that in the second experiment in minimal medium, the gels made a notable difference in the fraction of reads mapping to CDS, but gel omission did not change the correlation of translational events. In our hands, Protocol 1 required up to 18 times more RNA than Protocol 2 and two additional days of processing (Table A7). The reduction in effective reads observed in Protocol 2 compared to Protocol 1 can be compensated for by approximately doubling the sequencing depth. However, this would double the sequencing costs, which may not be affordable for all laboratories. In minimal medium, the cost-increase will be higher compared to experiments in rich medium because of lower effective read numbers. Therefore, Protocol 1 requires deeper, more expensive sequencing in this case, and this cost increase doubles for Protocol 2. On top, in nutrient-limited environments, the UREA/PAA gels notably increased mapping to CDS. Taken together, the lower number of effective reads across both experiments and the pronounced decrease in CDS-mapping reads in minimal medium for Protocol 2 must be

carefully weighed against the reduced sample input and time, and therefore labor cost, when choosing between workflows. Taken together, Protocol 2 produces a highly accurate, though less deep, snapshot of the transcriptome. The high correlation in gene expression values validates its use for differential expression analysis, especially when sample input is limited, provided the reduction in sequencing depth is accounted for.

A limitation of our study is the absence of biological replicates for all experiments. However, our focus here is not on biological reproducibility but on the comparability of the different workflows. Accordingly, we tested the protocols using split samples under two different conditions. We chose *E. coli* because it remains the most widely used model organism. Previous work from our laboratory has demonstrated the applicability of Protocol-1-like workflows to a high-GC, Gram-negative bacterium, namely *Pseudomonas* [34]. Of course, we are not the first to be interested in shortening ribosome profiling. Others have published procedures with fewer steps, including substituting gradient centrifugation with size exclusion spin-columns, but the comparative outcome in terms of overall performance remains vague. Latif et al. [35] did not use split samples as conducted here, making direct comparisons difficult. Furthermore, Kopik et al. [36] only show footprints of a single gene when comparing workflows with and without gradient centrifugation, but additional comparisons are missing. Certainly, approaches without gradient centrifugation do produce data, but their reliability appears limited, especially when detecting novel genes. In any case, library preparation from footprints after gradient centrifugation can be shortened by using magnetic beads for all necessary steps in library preparation [37]. However, even in the shortcut described in [37], the procedure starts from footprints recovered after sucrose gradient centrifugation and gel electrophoresis. Certainly, the proposed library preparation requires less time and considerably lowers the initial number of footprints (and hence, bacteria) needed. Similarly, the currently used “TruSeq Small RNA Library Preparation Kit” can be replaced by bead-based kits, which will reduce the required hands-on time and sample input. In closing, although omitting the centrifugation step would have been most desirable, sucrose gradient centrifugation for obtaining monosomes with footprints remains the gold standard, especially in gene-finding approaches. Omitting the subsequent gel electrophoresis can still provide an accurate quantification of the transcriptome, although the loss in effective reads must be compensated for by deeper sequencing. Subsequently, other library preparation kits may be used to shorten time and effort.

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Conflicts of Interest: The authors declare no conflicts of interest.

Appendix A

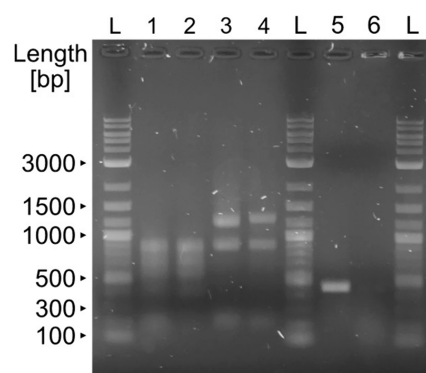


Figure A1. Agarose gel analysis to verify RNA integrity. The gel demonstrates that the RNA-Seq sample remains intact before and after DNA digestion. Successful DNA removal is further confirmed by a negative PCR result after 30 cycles. Lanes are as follows: L: 1 kb Plus DNA Ladder (NEB, USA), 1: P3_{Exp1} and P4_{Exp1}, 2: P1_{Exp1} and P2_{Exp1}, 3: RNA-Seq_{Exp1} before DNA digestion, 4: RNA-Seq_{Exp1} after DNA digestion, 5: PCR of *E. coli* LF82 $\Delta tolC$ DNA (positive control), and 6: PCR of digested RNA-Seq sample.

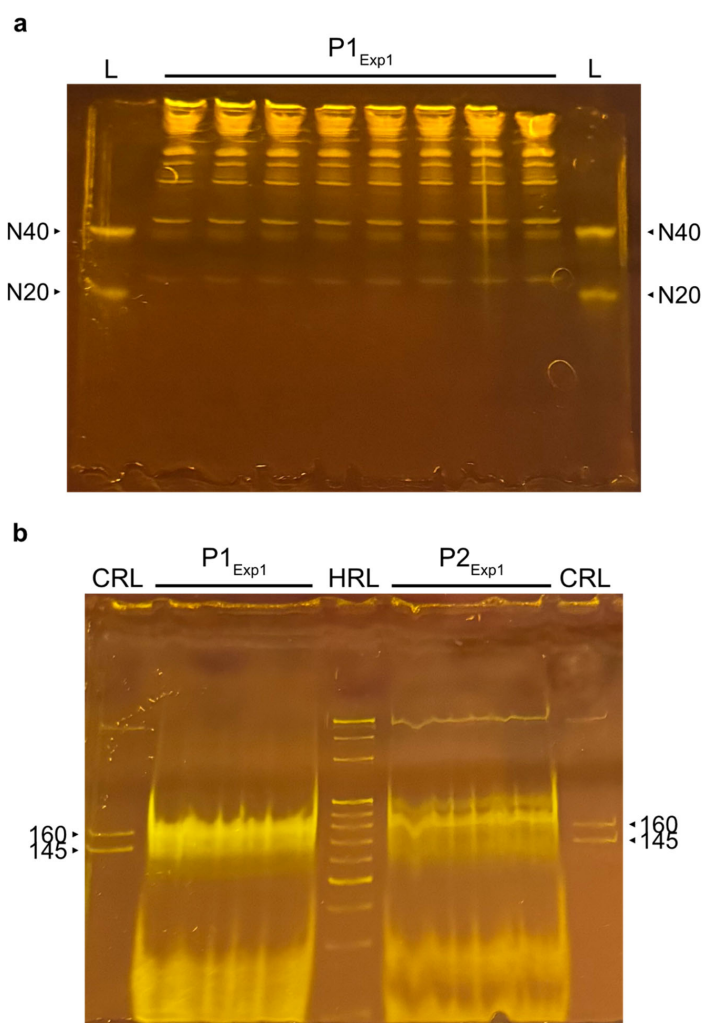


Figure A2. Gel electrophoresis for size selection. (a) 15% UREA/PAA gel for Protocol 1 in the first experiment. The area between the two ladder lines (L) and between N20/N40 (random ssRNA oligo of 20 and 40 nt) was excised. (b) 10% PAA gel for size selection during library preparation. The area between 145 and 160 bp was excised. CRL: Custom RNA Ladder; HRL: High Resolution Ladder.

Table A1. Recipe for one 15% UREA/PAA gel with the dimensions 8.3 cm × 7.3 cm × 0.1 cm (W × L × H) prepared in a Mini-PROTEAN[®] tetra cell (Bio-Rad Laboratories, USA). ROTIPHORESE solutions were obtained from Carl Roth, Germany.

Reagent Solution	Volume [mL]
ROTIPHORESE Sequencing gel diluent	1.95
ROTIPHORESE Sequencing gel concentrate	4.8
ROTIPHORESE Sequencing gel buffer concentrate	0.75
10% APS	0.0375
TEMED	0.00375

Table A2. Recipe for one 10% PAA gel with the dimensions 8.3 cm × 7.3 cm × 0.1 cm (W × L × H) prepared in a Mini-PROTEAN[®] tetra cell (Bio-Rad Laboratories, USA). DEPC-H₂O and ROTIPHORESE solutions were both obtained from Carl Roth, Karlsruhe, Germany.

Reagent Solution	Volume [mL]
DEPC-H ₂ O	4.2
ROTIPHORESE 30 NF (29:1)	2.475
10× TBE	0.75
10% APS	0.075
TEMED	0.0045

Table A3. Read numbers corresponding to Figure 2. Total reads are shown for every workflow and divided into five categories: raw reads (sum of all reads), reads shorter than 20 nt (too short), reads longer than 40 nt (too long), correctly sized reads that are not aligned, reads mapping to rRNA/tRNA, and effective reads.

Workflow	Raw	Too Short	Too Long	Not Aligned	rRNA/tRNA	Effective
P1 _{Exp1}	63,043,337	20,380,286	2,117,443	2,906,484	19,600,273	18,038,851
P2 _{Exp1}	56,398,517	28,392,436	3,711,784	3,860,817	12,238,108	8,195,372
P3 _{Exp1}	67,661,477	2,416,862	1,818,311	1,263,775	57,417,613	4,744,916
P4 _{Exp1}	65,479,688	7,773,788	4,184,376	1,146,711	48,689,030	3,685,783
RNA-Seq _{Exp1}	65,961,328	25,058,570	5,804,620	9,968,212	21,011,035	4,118,891
P1 _{Exp2}	110,235,104	51,156,993	2,156,125	11,940,433	24,415,302	20,566,251
P2 _{Exp2}	57,084,268	22,684,357	2,976,269	10,215,310	15,264,158	5,944,174
RNA-Seq _{Exp2}	21,744,825	7,928,534	1,435,295	2,571,209	8,758,941	1,050,846

Table A4. Percentages of effective reads mapping to either the ncRNAs or to all coding sequences are shown.

Gene	P1 _{Exp} [%]	P2 _{Exp1} [%]	P3 _{Exp1} [%]	P4 _{Exp1} [%]	RNA-Seq _{Exp1} [%]	P1 _{Exp2} [%]	P2 _{Exp2} [%]	RNA-Seq _{Exp2} [%]
<i>ffs</i>	0.2971	2.0093	4.5172	9.9484	6.9387	0.7720	2.2465	11.7452
<i>RtT</i> sRNA	0.0000	0.0000	0.0001	0.0001	0.0008	0.0000	0.0000	0.0009
<i>rprA</i>	0.0001	0.0000	0.0011	0.0005	0.0053	0.0035	0.0023	0.0312
<i>ssrS</i>	0.8918	0.7706	9.7773	14.0963	13.8442	1.5947	1.0650	5.3584

Table A4. Cont.

Gene	P1 _{Exp} [%]	P2 _{Exp1} [%]	P3 _{Exp1} [%]	P4 _{Exp1} [%]	RNA-Seq _{Exp1} [%]	P1 _{Exp2} [%]	P2 _{Exp2} [%]	RNA-Seq _{Exp2} [%]
<i>rnpB</i>	0.0225	0.0193	0.2845	0.2136	1.4374	2.0413	4.5918	0.7420
CDS	94.1	91.2	76.4	66.1	58.8	78.1	66.4	58.6

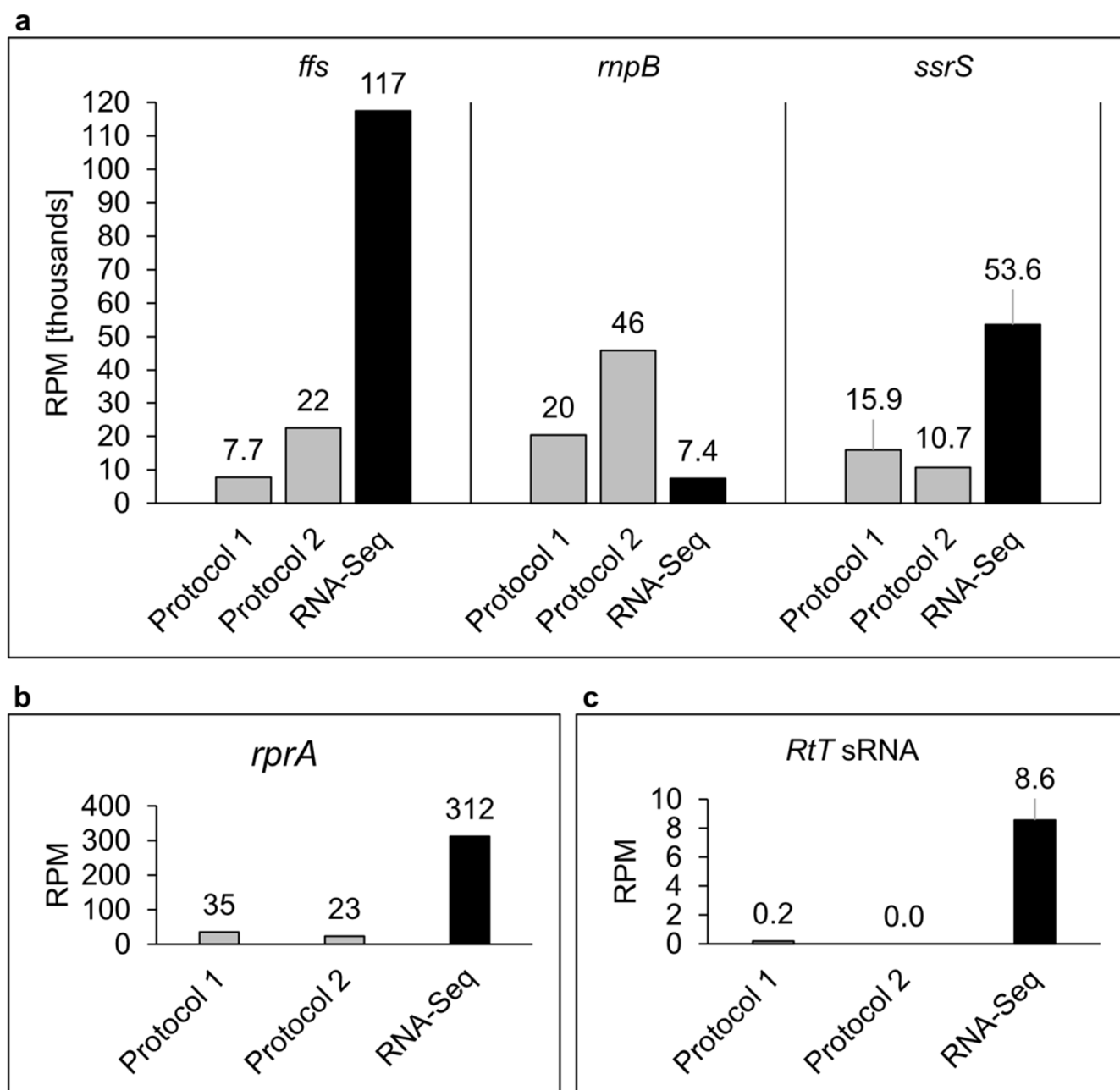


Figure A3. Carryover of different ncRNAs was compared across the different Ribo-Seq workflows and standard RNA sequencing in the second experiment in M9 broth. (a) *ffs*, *rnpB*, and *ssrS*; (b) *rprA*; and (c) *RfT sRNA*. Of note, the RNA-Seq sample contained just over 1 Mio of effective reads.

Table A5. Quantitative data for the read length distribution. Mean, median, and peak read lengths are shown for all workflows.

Workflow	Mean	Median	Peak
P1 _{Exp1}	28.1	28	27
P2 _{Exp1}	29.2	29	27
P3 _{Exp1}	28.8	28	26
P4 _{Exp1}	28.9	28	26
RNA-Seq _{Exp1}	28.9	28	30
P1 _{Exp2}	28.7	28	26
P2 _{Exp2}	29.7	30	30
RNA-Seq _{Exp2}	30.4	30	29

Table A6. Relative abundance of all read lengths across all samples.

Read Length [nt]	P1 _{Exp1}	P2 _{Exp1}	P3 _{Exp1}	P4 _{Exp1}	RNA-Seq _{Exp1}	P1 _{Exp2}	P2 _{Exp2}	RNA-Seq _{Exp2}
19	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
20	1.82%	1.97%	2.65%	4.93%	3.58%	1.84%	2.36%	2.86%
21	3.20%	2.38%	4.87%	5.91%	6.05%	2.87%	2.54%	3.41%
22	4.01%	2.88%	5.42%	5.56%	4.25%	4.21%	3.20%	3.86%
23	6.08%	4.42%	5.68%	5.08%	4.05%	5.85%	4.15%	4.18%
24	8.00%	6.12%	7.30%	6.56%	5.24%	7.89%	5.11%	4.42%
25	7.43%	6.33%	7.43%	6.81%	7.07%	7.89%	5.67%	4.52%
26	8.59%	7.13%	7.62%	7.65%	6.22%	7.98%	7.30%	4.60%
27	10.34%	8.76%	7.37%	6.52%	6.92%	7.63%	6.54%	5.04%
28	7.88%	7.68%	5.61%	5.23%	6.85%	6.68%	6.49%	6.38%
29	7.91%	8.15%	5.85%	5.58%	7.32%	6.26%	6.13%	6.59%
30	7.11%	7.46%	5.11%	4.25%	7.57%	6.26%	8.17%	5.35%
31	5.81%	6.01%	4.32%	3.44%	4.63%	6.27%	6.79%	4.88%
32	4.94%	5.49%	4.33%	3.26%	3.88%	4.96%	5.51%	4.99%
33	4.29%	5.02%	3.78%	2.99%	4.46%	4.73%	5.47%	5.26%
34	3.13%	4.05%	3.14%	2.69%	3.52%	3.89%	4.74%	5.28%
35	2.55%	3.54%	3.26%	2.60%	3.41%	3.21%	3.69%	5.53%
36	2.10%	3.12%	3.11%	2.83%	3.33%	3.09%	4.16%	4.99%
37	1.73%	3.30%	3.48%	4.80%	3.16%	3.28%	4.78%	5.28%
38	1.30%	2.84%	3.20%	5.36%	2.79%	2.11%	3.16%	4.52%
39	1.08%	1.97%	3.82%	4.25%	2.94%	1.56%	2.22%	4.50%
40	0.70%	1.38%	2.64%	3.71%	2.70%	1.52%	1.78%	3.47%
41	0.00%	0.00%	0.01%	0.00%	0.04%	0.01%	0.00%	0.06%
42	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.02%
43	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%

Table A6. Cont.

Read Length [nt]	P1 _{Exp1}	P2 _{Exp1}	P3 _{Exp1}	P4 _{Exp1}	RNA-Seq _{Exp1}	P1 _{Exp2}	P2 _{Exp2}	RNA-Seq _{Exp2}
44	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
45	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
46	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%

Table A7. Specification of the RNA amounts used. For Protocols 1 and 2, the first RNA measurement can only be performed after extraction from the monosomes. For Protocols 3 and 4, the first RNA measurement is possible immediately after RNase digestion.

Workflow	Amount of RNA [μg]	Protocol Step
Protocol 1	55	After gradient, input for UREA/PAA gel
Protocol 2	5/3	After gradient, input for RiboPools™
Protocol 3	55	After RNase, input for UREA/PAA gel
Protocol 4	5	After RNase, input for RiboPools™

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