HHS Public Access

Author manuscript

Methods Mol Biol. Author manuscript; available in PMC 2024 June 07.

Published in final edited form as:

Methods Mol Biol. 2024 ; 2724: 139–163. doi:10.1007/978-1-0716-3485-1_11.

Ribosome Profiling of Plants

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Abstract

Translation is a key step in control of gene expression, yet most analyses of global responses to a stimulus focus on transcription and the transcriptome. For RNA viruses in particular, which have no DNA-templated transcriptional control, control of viral and host translation is crucial. Here, we describe the method of ribosome profiling (ribo-seq) in plants, applied to virus infection. Ribo-seq is a deep sequencing technique that reveals the translatome by presenting a snapshot of the positions and relative amounts of translating ribosomes on all mRNAs in the cell. In contrast to RNA-seq, a crude cell extract is first digested with ribonuclease to degrade all mRNA not protected by a translating 80S ribosome. The resulting ribosome-protected fragments (RPFs) are deep sequenced. The number of reads mapping to a specific mRNA compared to the standard RNA-seq reads reveals the translational efficiency of that mRNA. Moreover, the precise positions of ribosome pause sites, previously unknown translatable open reading frames, and noncanonical translation events can be characterized quantitatively using ribo-seq. As this technique requires meticulous technique, here we present detailed step-by-step instructions for cell lysate preparation by flash freezing of samples, nuclease digestion of cell lysate, monosome collection by sucrose cushion ultracentrifugation, size-selective RNA extraction and rRNA depletion, library preparation for sequencing and finally quality control of sequenced data. These experimental methods apply to many plant systems, with minor nuclease digestion modifications depending on the plant tissue and species. This protocol should be valuable for studies of plant virus gene expression, and the global translational response to virus infection, or any other biotic or abiotic stress, by the host plant.

Keywords

RNA sequencing; Ribo-seq; translation; viral RNA; protein synthesis; translatome

1 Introduction

Translation, the key event in all living things in which the genetic code is decoded as proteins are synthesized, has been underappreciated as a regulatory stage of gene expression [1, 2]. Historically, gene expression monitoring has focused on the levels of mRNA (the transcriptome) determined by methods that continue to be more informative, such as the widely used high-throughput RNA sequencing (RNA-seq) [3]. However, monitoring RNA

abundance alone is not sufficient to determine protein expression level, because, as with transcription, the translation efficiencies can change due to an external stimulus [4] or during development [5], and changes in mRNA level do not always correlate with changes in protein level [6]. Proteomics addresses this question, but measuring protein levels combined with RNA-seq provides only an indirect measure of translation efficiency and is affected by differences in protein stability.

Ribosome profiling (ribo-seq) has emerged as a powerful method that directly measures the translational efficiency of mRNAs [7, 8]. Ribo-seq reveals the positions and relative amounts of ribosomes on every mRNA in the cell at single nucleotide resolution. Thus, it can reveal mRNAs that are up- or down-translated in response to a change in environment or developmental cue. It also detects sites of ribosome pausing that occur during critical translational control events. Ribo-seq is a modified RNA-Seq-based technique, in which, instead of sequencing and counting total mRNA, only the mRNA tracts that are occupied by translating ribosomes are sequenced. Depending on the research objectives, ribo-seq can provide a panorama of the whole translation process [9]. In this method, a translation-arrested cell lysate is digested with RNase to degrade mRNA not protected by a translating ribosome. Then, the resulting ribosome-protected fragments (**RPFs**) are deep sequenced. The number of RPFs that map to each position on an mRNA indicates the ribosome density and thus its translational efficiency (**TE**) and also reveals ribosome pause sites (Fig. 1).

1.1 Application of Ribosome Profiling in Plant–Virus Interactions

In addition to identifying unexpected coding regions and translational control mechanisms in host mRNAs, ribo-seq is invaluable for characterizing the numerous noncanonical translation events that are a hallmark of many positive-strand RNA viral genomes. For example, many viruses use recoding events such as stop codon read-through or ribosomal frameshifting [10]. Ribo-seq is the most accurate way of measuring the exact rate of read-through or frameshifting because the proportion of RPFs obtained from the ORFs before and after the recoding event is proportional to the number of ribosomes translating each ORF. This is more accurate than measuring ratios of viral or reporter proteins translated via the viral read-through/frameshift sequence, because the protein products may differ in stability. Thus, their levels would not be proportional to the efficiency of translation of the ORFs that generated them. Also, viruses encode many small proteins from small ORFs that may not appear significant [11]. Ribo-seq reveals all translated ORFs, including those that do not initiate with a canonical AUG start codon. Application of ribosome profiling to virus-infected human cells has revealed such highly quantitative details of coding capacity and gene expression [12, 13].

With regard to plant–pathogen interactions, Ribo-seq has revealed an unexpected translational upregulation of many genes in the plant immune system in response to bacterial infection [14]. This later led to the identification of a signal transduction pathway in which phosphorylation of translation factors released specific innate immune system genes from a translationally inhibited state, upon detection of a bacterial effector [15]. A Ribo-seq study of sugarcane mosaic virus-infected maize found that photo-synthetic and metabolic genes were both transcriptionally and translationally downregulated [16]. Given that many

plant viral RNAs, such as the cap-independent translation elements of tom-busvirids, and viral proteins, such as the genome-linked proteins (VPgs) of potyviruses and solemovirids, are known to bind key translation initiation factors [10, 17–19], these interactions might out-compete host mRNAs for the factors and alter their translation. Ribo-seq would be a powerful tool to answer these questions. Thus, we provide detailed methods here for plant virologists to employ Ribo-seq in the model plant Arabidopsis and the agronomic crop (and also a model organism) maize [20].

1.2 Bioinformatics

This chapter provides details only for preparing and sequencing RPFs at the laboratory bench. However, after that process, a substantial amount of sequence data analysis is necessary to confirm that the sequences represent true RPFs and to subsequently identify translational efficiencies, ribosome pause sites, and global control of translation. We provide a brief summary of data processing and analysis here. For more information on the processing and bionformatics of Ribo-seq data, we refer the readers to Kiniry et al. [21] and Xiao et al. [22]. Initial data treatment is the same for ribo-seq and RNA-seq data, starting by assessing the quality of raw data by tools such as FastQC [23], followed by processing via quality and adapter trimming by tools such as Cutadapt [24]. Bowtie2 [25] is used to align the processed reads to the organism's reference sequences for the rRNAs, tRNAs, and snoRNAs. The remaining reads are aligned to the reference transcriptome using Bowtie2 or to reference genome using splice-aware tools such as STAR [26]. There are two general approaches to counting reads: Consider only uniquely mapped reads or estimate the true location of multi-mapped reads. We recommend featureCounts [27] for counting only uniquely mapped reads and RSEM [28] for counting multi-mapped reads. Statistical analysis of counted reads can be performed with edgeR [29], DESeq2 [30], xtail [22], or riboVI [31]. The latter two are designed specifically for ribo-seq data. RiboVI is the most sensitive, as it identifies more genes as having statistically significant changes in translation efficiency (TE) with a lower false discovery rate than the other methods, but tends to give a lower change in TE for each gene. We advise working with an experienced statistician due to the complexity of the analyses. For RPF metagene analysis (discussed below), the authors recommend riboSeqR [32] if reference transcriptome mapping is used. If the RPFs are mapped to the reference genome, then RiboTaper [33] and RiboToolkit [34] are excellent tools for metagene analysis. Actively translating regions can be identified with RiboTaper [33]. Many of the above-listed programs and packages are used in a command-line environment and require some basic skills in scripting, R, and/or Python. Some web services perform all (RiboToolkit [34]) or many (RiboGalaxy [35]) of the above processes. Riboviz2 [36] and RiboVIEW [37] are command-line programs that perform many, but not all, of the steps above.

1.3 Hallmarks of High-Quality Ribo-seq Data

Ribo-seq data have specific hallmarks that are not present in RNA-seq data. However, some RNA secondary structures or ribonucleo-protein complexes not involved in translation may generate RPF-length fragments. Therefore, it is imperative to distinguish true RPFs from artifacts. The following features are hallmarks of high-quality Ribo-seq data with true RPFs:

(i) Narrow Length Distribution.—Depending on variety of organisms, ribosome-protected fragment length may vary. Still ribosomes protect predominantly one specific size of RNA for a given organism (e.g., 28 nt for Arabidopsis [8] and 30 nt for maize [20]). In high-quality Ribo-seq data, the length distribution is mostly enriched to a single RPF length (Fig. 2a) along with very few other RPF lengths, whereas in RNA-seq data there is a broad read length distribution (Fig. 2b). RPFs with good triplet periodicity (described below) show a narrow length distribution [8].

(ii) CDS Enrichment.—Almost all reads from ribo-seq data map to the coding sequence (CDS) because only the translating 80S ribosome protects mRNA from the RNase treatment used to obtain the RPFs. In contrast, RNA-seq data will always map to both CDS and UTRs (Fig. 2c–d).

(iii) Triplet Periodicity.—As mentioned above, ribosomes generate more RPFs at pause sites. Because ribosomes pause briefly at each codon, this pausing can be detected in the form of more reads mapping to every third base in a CDS (Fig. 2e). This triplet periodicity is absent in RNA-seq data because RNA-seq is obtained from random fragmentation of total RNA in the absence of ribosomes (Fig. 2f). Both the CDS enrichment and triplet periodicity in ribo-seq data and lack thereof in RNA-seq data can be observed in the same metagene analysis plot that maps positions of reads relative to codons in all mRNAs combined (Fig. 2e–h).

1.4 Translation Efficiency

Once the quality of RPFs has been confirmed, the translation efficiency (TE) can be calculated for each mRNA using software such as Xtail [22]. TE is the ratio of RPF abundance to mRNA abundance; as the more ribosomes are translating an mRNA, the more RPFs will be captured by ribo-seq. Thus, ribo-seq, when compared to the RNA-seq that is performed on the same sample, allows one to measure precisely how much change in expression of a given gene is due to transcription and how much is due to translation, the ultimate goal of ribosome profiling.

2 Materials

2.1 Enzymes, Chemical Reagents, and Kits

1. Nuclease-free water.
2. TURBO DNase with 10x buffer (Invitrogen).
3. RNase I (Invitrogen).
4. SUPERase-In (Invitrogen).
5. Proteinase K (Thermo Scientific).
6. T4PNK kit (Thermo Scientific).
7. ATP solution.
8. Acid–phenol: chloroform, pH 4.5 (with IAA 125:24:1, Invitrogen).

9. Chloroform–isoamyl alcohol (24:1, Sigma-Aldrich).
10. 15 mg/mL GlycoBlue Coprecipitant (Invitrogen).
11. 3 M sodium acetate pH 5.5.
12. 1 M Tris–HCl pH 7.5, autoclave, store at room temperature.
13. 2 M KCl, autoclave, store at room temperature.
14. 1 M $MgCl_2$, autoclave, store at room temperature.
15. 10% (v/v) Triton X-100, autoclave, store at room temperature.
16. 10% (v/v) IGEPAL CA-630, autoclave, store at room temperature.
17. 2 M sucrose, 0.45-μm filter-sterilize, store at 4 °C.
18. 50 mg/mL cycloheximide in ethanol, store at −20 °C.
19. 50 mg/mL chloramphenicol in ethanol, store at −20 °C.
20. 1 M dithiothreitol (DTT), do not autoclave, store at −20 °C in dark.
21. EDTA-free protease inhibitor (Thermo Scientific).
22. 10% (w/v) SDS, 0.22-μm filter-sterilize, do not autoclave, and store at room temperature.
23. 0.5 M EDTA pH 8.0, autoclave, store at room temperature.
24. 1 M Na_2CO_3, 0.22-μm filter-sterilize, store at room temperature.
25. 1 M $NaHCO_3$, 0.22-μm filter-sterilize, store at room temperature.
26. Ice-cold 100% isopropanol.
27. Ice-cold 100% ethanol.
28. Ice-cold 80% ethanol.
29. 15% TBE-Urea Gel.
30. 2x RNA loading Dye (NEB).
31. 10x TBE buffer.
32. SYBR Gold Nucleic Acid Gel Stain (Invitrogen).
33. Zymo RNA Clean & Concentrator −5 Kit (Zymo Res.).
34. Ribo-Zero rRNA depletion kit for plant leaf (Illumina).
35. NEXTflex Small RNA-Seq Kit v3 (Perkin Elmer).
36. Qubit dsDNA HS Assay Kit (Invitrogen).
37. Liquid nitrogen.

2.2 Buffers

1. Polysome extraction buffer (PEB):; 20 mM Tris–HCl pH 7.5, 140 mM KCl, 5 mM $MgCl_2$, 1% (v/v) Triton X-100, 0.5% (v/v) IGEPAL CA 630, 146 M sucrose, 100 μg/mL cycloheximide, 100 μg/mL chloramphenicol, 0.5 mM DTT, 0.5 μL/mL Turbo DNase, and EDTA-free protease inhibitor. Store the PEB without TURBO DNase, DTT, cycloheximide, and chloramphenicol at −20 °C. Add these components to the PEB right before use.
2. Proteinase K buffer: 10 mM Tris–HCl pH 7.5, 1% SDS, and 200 μg/mL Proteinase K.
3. Sucrose cushion, 1.022 M sucrose, 20 mM Tris–HCl pH 7.5, 140 mM KCl, 5 mM $MgCl_2$, 100 μg/mL cycloheximide, 100 μg/mL chloramphenicol, 0.5 mM DTT, 5 μL/mL SUPERase-in. Prepare fresh.
4. 2x Fragmentation buffer: 2 mM EDTA pH 8.0, 12 mM Na_2CO_3, 88 mM $NaHCO_3$. Prepare fresh. Verify pH ~ 9.0 using a pH strip.
5. Fragmentation stop solution: 0.3 M sodium acetate pH 5.5, 53.6 μg/mL GlycoBlue. Prepare fresh.
6. RNA gel extraction buffer: 0.3 M sodium acetate pH 5.5, 1 mM EDTA pH 8.0, 10 mM Tris–HCl pH 7.5, 0.25% (w/v) SDS. Store at room temperature.

2.3 Equipment and Consumables

1. Sorvall Mini-Ultracentrifuge (Discovery M150).
2. S150-AT fixed angle rotor (Thermo Scientific).
3. 1 mL Mini-Ultracentrifuge tubes (Thermo Scientific).
4. TissueLyser (Eppendorf).
5. Thermo-mixer (Thermo Scientific).
6. Blue light transilluminator.
7. 50-mL falcon tubes.
8. 4.8-mm stainless steel metal beads (MidSci) (sterilize in an oven at 200 °C overnight).
9. 2 mm zirconium oxide beads (Fisher Sci) (sterilize in an oven at 200 °C overnight).
10. 0.22-μm Spin-X cellulose acetate filter columns (Sigma-Aldrich).
11. Qubit Fluorometer (Invitrogen).
12. Qubit assay tubes (Invitrogen).
13. NanoDrop Spectrophotometer.
14. Low-retention 0.5-mL, 1.5-mL, and 2-mL microfuge tubes (Fisher Sci).

15. Low-retention barrier/filter pipette tips (for use in entire protocol for pipetting).
16. Square petri dish (VWR).
17. Razor blades (VWR) (sterilize in an oven at 200 °C overnight).
18. Syringe filters.
19. 18G syringe needle.
20. 0.22-μm syringe filter.
21. 0.45-μm syringe filter.
22. Magnetic tube rack for PCR tubes or/and 1.5-mL microfuge tubes (for use in magnetic bead RNA cleanup steps in rRNA depletion and library preparation).
23. Agilent Bioanalyzer.
24. Amber-colored goggles and blue transilluminator.

2.4 Size Markers

1. 28 nt RNA oligo (Sigma-Aldrich, HPLC purified): *5′* AUGUA CACGGAGUCGACCCGCAACGCGA-*3′*.
2. 34 nt RNA oligo (Sigma-Aldrich, HPLC purified): *5′* AUGUA CACGGAGUCGAGCUCAACCCGCAACGCGA-*3′*.
3. 10/60 nt single-stranded DNA ladder (IDT).

3 Methods

3.1 Lysate Preparation

1. Collect the plant leaves in liquid nitrogen-chilled 50-mL falcon tubes containing two 4.8-mm beads (*see* Notes 1 and 2).
2. Coarsely pulverize the leaves by vigorously shaking the tube and transfer them to liquid nitrogen-chilled 1.5-mL microfuge tubes containing 8–10 2-mm zirconium oxide beads.
3. Subsequently, pulverize the tissues into fine powder using a TissueLyser (1500 rpm for 30 s, twice), followed by adding 800 μL of PEB (*see* Note 3).
4. Incubate the crude lysate in ice for 20 min on a rocker followed by clarification using two rounds of centrifugation (21,100 × *g*, 15 min, 4 °C).
5. Subsequently, use NanoDrop to adjust the absorbance of the lysate with PEB to A_{254} (Lysate – PEB) ≈ A_{260} (Lysate – PEB) ≈ 6 (*see* Note 4).
6. Save a 200 μL aliquot of lysate to be used for total RNA extraction (Subheading 3.3). Dispense 400 μL aliquots of remaining lysate in 1.5-mL microfuge tubes for RPF purification (Subheading 3.2). If necessary, these can be flash-frozen and stored at −80 °C for future extraction (*see* Note 5).

3.2 RNase 1 Digestion, Monosome Collection, and RPF RNA Purification

1. Thaw 400 μL of the absorbance-adjusted lysate in ice.
2. Centrifuge (21,100 × *g*, 5 min, 4 °C) to remove any remaining debris and transfer the supernatant to new 2-mL microfuge tubes.
3. Add 200U RNAse1, mix by inverting the tube, and spin down.
4. Incubate at 28 °C for 60 min in a thermomixer at 400 rpm.
5. Immediately transfer the tubes to ice.
6. Add 5 μL SUPERase-In to terminate the reaction.
7. To the 400 μL RNase-treated lysate, add 350 μL ice-cold PEB to make the total volume ~ 750 μL, and carefully layer it on 350 μL ice-cold sucrose cushion in mini-ultracentrifuge tubes (*see* Note 6).
8. Ultracentrifuge at 57,000 rpm (131,500 × *g*) for 90 min at 4 ° C with slow acceleration and deceleration in a Sorvall mini-ultracentrifuge with 4 °C precooled S150-AT fixed angle rotor.
9. Carefully remove the supernatant by pipetting. The pellet consists of monosomes from which RPFs will be extracted below.
10. Use a 200-μL pipette to remove any residual supernatant.
11. Optional: Rinse the monosome pellet carefully with 500 μL nuclease-free water (*see* Note 7).
12. Add 600 μL Proteinase K buffer (prewarmed to 42 °C for 5 min) to the monosome pellet.
13. Incubate at room temperature for 5 min.
14. Resuspend the monosome pellet by pipetting and transfer to 2-mL microfuge tubes (*see* Note 8).
15. Incubate at 42 °C for 30 min followed by incubation at 65 °C for 5 min.
16. Immediately transfer the solution to 600 μL hot acid–phenol–chloroform that is pre-heated to 65 °C (*see* Note 9).
17. Mix by inverting the tubes.
18. Incubate the mix at 65 °C for 5 min (mix intermittently by inverting the tubes).
19. Spin down and incubate the tubes in ice for 5 min followed by centrifugation (21,100 × *g*, 2 min, 4 °C).
20. Transfer the ~600 μL aqueous phase to new 2-mL microfuge tubes.
21. Add 600 μL room temperature acid–phenol–chloroform to the aqueous phase and mix by inverting the tubes.
22. Incubate at room temperature for 5 min followed by centrifugation (21,100 × *g*, 2 min, 4 °C).

23. Transfer ~500 μL aqueous phase to new 2-mL microfuge tubes.
24. Add 500 μL chloroform–isoamyl alcohol and mix by inverting the tubes.
25. Incubate at room temperature for 1 min followed by centrifugation (21,100 × *g*, 2 min, 4 °C).
26. Transfer ~400 μL aqueous phase to new 1.5-mL tubes containing 2 μL GlycoBlue and 45 μL 3M sodium acetate pH 5.5.
27. Precipitate RPF RNA by the addition of ~450 μL ice-cold 100% isopropanol and overnight incubation at −80 °C.
28. Collect the RPF RNA pellet by centrifugation (21,100 × *g*, 45 min, 4 °C).
29. Wash the pellet by adding 1 mL ice-cold 80% ethanol (without disturbing the pellet), followed by centrifugation (21,100 × *g*, 10 min, 4 °C).
30. Wash the pellet for a total of two times.
31. Spin down and remove any residual ethanol using a 20-μL pipette tip.
32. Air-dry the pellet at room temperature for ~5 min.
33. Resuspend the pellet in 30 μL nuclease-free water followed by NanoDrop quantification. RNA can be stored at −80 °C at this stage.

3.3 Total RNA Extraction

1. Add 400 μL Proteinase K buffer (prewarmed at 42 °C for 5–10 min) to the 200 μL lysate aliquot that was set aside in Subheading 3.1, **step 6**.
2. Mix by inverting and spinning down.
3. Incubate at 42 °C for 30 min.
4. Immediately conduct RNA purification using the hot acid–phenol–chloroform method as follows (*see* Note 9).
5. Heat 600-μL acid–phenol–chloroform in 2-mL microfuge tubes to 65 °C.
6. Heat Proteinase K-treated lysate to 65 °C for 5 min and subsequently transfer to 600 μL hot acid–phenol–chloroform.
7. Mix by inverting the tubes.
8. Incubate the mix at 65 °C for 5 min (mix intermittently by inverting the tubes).
9. Spin down and incubate the tubes in ice for 5 min followed by centrifugation (21,100 × *g*, 2 min, 4 °C).
10. Transfer the ~600 μL aqueous phase to new 2-mL microfuge tubes.
11. Add 600 μL room temperature acid–phenol–chloroform to the aqueous phase and mix by inverting the tubes.
12. Incubate the mix at 65 °C for 5 min (mix intermittently by inverting the tubes).

13. Spin down and incubate the tubes in ice for 5 min followed by centrifugation (21,100 × *g*, 2 min, 4 °C).
14. Transfer the ~600 μL aqueous phase to new 2-mL microfuge tubes.
15. Add 600 μL room temperature acid–phenol–chloroform to the aqueous phase.
16. Mix by vortexing briefly.
17. Incubate at room temperature for 5 min followed by centrifugation (21,100 × *g*, 2 min, 4 °C).
18. Transfer ~500 μL aqueous phase to new 2-mL microfuge tubes.
19. Add 500 μL chloroform-isoamyl alcohol and mix by vortexing.
20. Incubate at room temperature for 1 min followed by centrifugation (21,100 × *g*, 2 min, 4 °C).
21. Transfer ~400 μL aqueous phase to new 1.5-mL tubes containing 2 μL GlycoBlue and 45 μL 3M sodium acetate pH 5.5.
22. Precipitate RNA by adding ~450 μL ice-cold 100% isopropanol and overnight incubation at −80 °C.
23. Collect the RNA pellet by centrifugation (21,100 × *g*, 45 min, 4 °C).
24. Wash the pellet by adding 1 mL ice-cold 80% ethanol (without disturbing the pellet), followed by centrifugation (21,100 × *g*, 10 min, 4 °C).
25. Wash the pellet for a total of two times.
26. Spin down and remove any residual ethanol using a 20-μL pipette tip.
27. Air-dry the pellet at room temperature for ~5 min.
28. Resuspend the pellet in 20 μL nuclease-free water, followed by NanoDrop quantification (*see* Note 10).
29. RNA can be stored at −80 °C at this stage.

3.4 DNase Treatment of RNase-Digested RNA and Total RNA

1. This step removes any gDNA contamination in the RNA preparations because the subsequent rRNA depletion step is sensitive to the presence of gDNA.
2. Prepare 50 μL reactions in PCR tubes with 10 μg RNA, 1 μL TURBO DNase, and 5 μL 10x TURBO DNase buffer.
3. Incubate at 37 °C for 30 min.
4. Add an additional 1 μL TURBO DNase and mix.
5. Incubate at 37 °C for 30 min.
6. Purify the DNase-treated RNA using the Zymo RNA Clean & Concentrator 5 Kit following the manufacturer's protocol (*see* Note 11).

7. Quantify DNase-treated RNA using NanoDrop and can store at −80 °C.
8. Total RNA integrity can be verified by electrophoresis of denatured RNA in a 15% TBE-Urea gel at 120 V for 5 min and 200 V for 50 min (*see* Note 12).
9. The quality of RPFs can be verified by electrophoresis of denatured RNA in a 15% TBE-Urea gel at 120 V for 5 min and 200 V for 75 min (*see* Note 13).

3.5 rRNA Depletion

1. Use half a reaction of Ribo-Zero (Illumina) per 5 ug of DNase-treated RNA (*see* Note 14).
2. Follow the manufacturer's protocol, except with one modification of omitting the 50 °C incubation step, only for DNase-treated ribo-seq samples.
3. Subsequently, purify the rRNA-depleted RNA using Zymo RNA Clean & Concentrator −5 kit according to the protocol described in Subheading 4.
4. For the final step, elute the RNA in 11 μL nuclease-free water.
5. Re-elute using the eluate (flow-through) to enhance recovery.
6. rRNA-depleted RNA can be stored at −80 °C at this stage.

3.6 Fragmentation of rRNA-Depleted Total RNA by Alkaline Hydrolysis

1. Add 10 μL of 2x fragmentation buffer to 10 μL rRNA-depleted total RNA and mix by pipetting.
2. Incubate the mix at 95 °C for 20 min followed immediately by adding 280 μL of fragmentation stop solution.
3. Precipitate RNA by adding ~750 μL ice-cold 100% ethanol and overnight incubation at −80 °C.
4. Collect the RNA pellet by centrifugation (21,100 × *g*, 45 min, 4 °C).
5. Wash the pellet by adding 1 mL ice-cold 80% ethanol (without disturbing the pellet), followed by centrifugation (21,100 × *g*, 10 min, 4 °C).
6. Wash the pellet twice.
7. Spin down and remove any residual ethanol using a 20-μL pipette tip.
8. Air-dry the pellet at room temperature for ~5 min.
9. Add 10 μL nuclease-free water to the pellet.
10. Let the pellet soften in ice and then resuspend it slowly by vortexing.
11. RNA can be stored at −80 °C at this stage.

3.7 Size Selection for RNA-Seq Samples

1. 10 μL fragmented RNA was mixed with 10 μL 2x RNA loading dye.

2. For size markers, 50 ng of IDT 10/60 nt ssDNA ladder was mixed with water and 2x RNA loading dye to 20 uL.
3. Denature RNA at 70 °C for 5 min followed immediately by incubation in ice for 2 min.
4. Clean the 15% TBE-Urea gel wells with the running buffer (1x TBE) by pipetting.
5. Immediately load the samples in the gel (*see* Note 15).
6. Run the gel at 120 V for 5 min and 200 V for 50 min.
7. Open the gel cassette and stain gel in the lid of a square petri dish with ice-cold SYBR gold staining solution (2 μL SYBR Gold in 20 mL 1x TBE buffer) for 1 min.
8. Discard the staining solution and immediately add 20 mL ice-cold 1x TBE.
9. Discard the solution as much as possible.
10. Place the lid of the petri dish (with the gel in it) on a blue light transilluminator.
11. Wear the amber-colored goggles and in the dark and excise the gel slice corresponding to the region between the top of the 30 nt and top of the 40 nt band of IDT 10/60 nt ssDNA ladder (*see* Note 16).
12. Transfer the gel slice to a pierced 0.5-mL tube placed in a 2-mL microfuge tube (*see* Note 17).
13. Centrifuge (21,100 × *g*, 2 min, 4 °C) so that the gel slice passes through 0.5-mL tube into the 2-mL tube.
14. Discard the pierced 0.5-mL tube.
15. To the crushed gel pieces in the 2-mL tubes, add 500 μL of RNA gel extraction buffer.
16. Seal the tube with parafilm.
17. Incubate overnight at 4 °C on a shaker or nutator.
18. Subsequently, vortex the tubes vigorously and spin down them.
19. Decant gel–RNA mix to a 0.22 μm Spin-X cellulose acetate filter columns.
20. Use 100 μL RNA gel extraction buffer to retrieve any residual gel–RNA mix from the original tube (*see* Note 18).
21. Centrifuge (16,000 × *g*, 2 min, 4 °C).
22. Add 50 μL RNA gel extraction buffer to the column membrane to retrieve any residual RNA.
23. Centrifuge (16,000 × *g*, 1 min, 4 °C).
24. Transfer the flow-through to new 1.5-mL microfuge tubes.

25. Add 2 μL GlycoBlue.
26. Precipitate the size-selected RNA by adding an equal volume (~600 μL) of ice-cold 100% isopropanol and overnight incubation at −80 °C.
27. Collect the RNA pellet by centrifugation (21,100 × *g*, 45 min, 4 °C).
28. Wash the pellet by adding 1 mL ice-cold 80% ethanol (without disturbing the pellet) followed by centrifugation (21,100 × *g*, 15 min, 4 °C).
29. Wash the pellet for a total of two times.
30. Spin down and remove any residual ethanol using a 20-μL pipette tip.
31. Air-dry the pellet at room temperature for ~5 min.
32. Add 3.25 μL nuclease-free water to the pellet.
33. Let the pellet soften in ice and then resuspend it slowly by pipetting.
34. RNA can be stored at −80 °C at this stage.

3.8 Size Selection for Ribo-seq Samples (See Note 19)

1. 10 μL rRNA-depleted ribo-seq RNA was mixed with 10 μL 2x RNA loading dye.
2. For size markers, 2 pmol each of 28 nt and 34 nt RNA oligos were mixed with water and 2x RNA loading dye to 20 ul.
3. Denature RNA at 70 °C for 5 min followed immediately by incubation in ice for 2 min.
4. Clean the 15% TBE–Urea gel wells with the running buffer (1x TBE) by pipetting.
5. Immediately load the samples in the gel (*see* Note 15).
6. Run the gel at 120 V for 5 min and 200 V for 75 min.
7. Open the gel cassette and stain it in the lid of a square petri dish with ice-cold SYBR gold staining solution (2 μL SYBR Gold in 20 mL 1x TBE buffer) for 1 min.
8. Discard the staining solution and immediately add 20 mL ice-cold 1x TBE.
9. Discard the solution as much as possible.
10. Place the lid of the petri dish (with the gel in it) on a blue light transilluminator.
11. Wear the amber-colored goggles and in the dark and excise the gel slice corresponding to the region between the bottom of the 28 nt and the bottom of the 34 nt RNA oligos (Fig. 4) (*see* Note 16).
12. Transfer the gel slice to a pierced 0.5-mL tube placed in a 2-mL microfuge tube (*see* Note 17).
13. Centrifuge (21,100 × *g*, 2 min, 4 °C) so that the gel slice passes through 0.5-mL tube into the 2-mL tube.

14. The subsequent steps are exactly the same as in Subheading 3.7 (size selection of RNA-seq samples).
15. Add 3.25 μL nuclease-free water to the pellet.
16. Let the pellet soften in ice and then resuspend it slowly by pipetting.
17. RNA can be stored at −80 °C at this stage.

3.9 Sequencing Library Preparation

1. Prior to sequencing library preparation, modify the ends of the RNA fragments according to the following steps. (*See* Note 20)
    a. Incubate 3.25 μL of size-selected RNA at 70 °C for 3 min. (b) Immediately transfer to ice.
    b. Add 0.5 μL of 10x T4 PNK buffer A (without ATP), 0.25 μL of SUPERase-In, and 0.5 μL of T4PNK enzyme.
    c. Incubate the reaction at 37 °C for 30 min.
    d. Add 0.5 μL of 10 mM ATP.
    e. Incubate the reaction at 37 °C for 1 h.
    f. Add 5.5 μL nuclease-free water to the reaction.
    g. Incubate at 75 °C for 10 min to terminate the reaction.
    h. Transfer the tubes to ice.
2. Prepare cDNA libraries with NEXTflex Small RNA-seq kit according to the manufacturer's protocol with the following specifications (*See* Note 21):
    a. Step A (3′ 4 N Adenylated Adapter Ligation): Dilute the 3′ 4 N adapter 1/three-fold (*see* Note 22) and conduct the incubation for ligation overnight.
    b. Step B (Excess 3′ Adapter Removal) and Step C (Excess Adapter Inactivation): follow the manufacturer's protocol.
    c. Step D (5′ 4 N Adapter Ligation): Dilute the 5' 4 N adapter 1/3-fold (*see* Note 22).
    d. Step E (Reverse Transcription-First Strand Synthesis): Follow the manufacturer's protocol.
    e. Step F (Bead Cleanup): Conduct bead cleanup for according to the "No size-selection" protocol (*see* Note 23).
    f. Step G (PCR Amplification): Conduct 11 cycles of PCR with barcoded primers (*see* Note 24).
    g. Step H2 (PAGE Size Selection & Cleanup): Follow the manufacturer's protocol.

3. Assess the quality of the libraries using Agilent High-Sensitivity DNA Assay Kit on a Bioanalyzer.
4. Quantify libraries using Qubit dsDNA HS Assay Kit.
5. Dilute and pool the libraries according to the sequencing platform and sequencing depth requirement.
6. Sequence the libraries on the Illumina NovaSeq 6000 platform to obtain at least 50-bp single-ended reads (*see* Note 25).

3.10 Ribo-seq and RNA-Seq Data Processing and Quality Assessment

1. Assess the quality of raw sequencing reads using FastQC [20].
2. Use Cutadapt [21] to remove the adapters from the raw sequencing reads using the following parameters: "-a TGGAATTCT CGGGTGCCAAGG—discard-untrimmed—minimum-length 23."
3. Use Cutadapt [21] with the parameters "-u 4 -u −4" to trim four nucleotides from the ends of ribo-seq and RNA-seq reads, which were added during library preparation.
4. Subsequently, retain the 27 to 32 nt ribo-seq reads and 25 to 40 nt RNA-seq reads and filter out the rest.
5. Use RiboToolkit [34] to assess ribo-seq-specific data characteristics, such as length distribution, triplet periodicity, frame enrichment, the distribution of reads over different mRNA features, etc.
6. Further data processing and analysis would largely depend on the experiment objectives and would include mapping the reads to ncRNA and using the ncRNA-unmapped reads to subsequently map to the organism's reference genome (*see* Note 26).

4 Notes

1. Preparing the lysate as soon as the plant tissue is collected is advisable. We recommend purifying RPF RNA from the lysate as early as possible. In case of storing at −80 °C, we recommend doing it after snap-freezing the lysate.
2. The amount of plant material collected would largely depend on the plant, the kind of tissues, and the age of the plant. Different amounts of tissues can be collected for different samples because, after lysate preparation, all the samples would be normalized to the same RNA/protein amounts using the absorbance at 254 and 260 nm.
3. The plant tissues should always be chilled in liquid nitrogen during tissue pulverization.
4. A_{254} and A_{260} refer to the absorbance measured at 254 and 260 nm wavelength, respectively, using NanoDrop. The absorbance of PEB is subtracted from the

absorbance of lysate to give A_{254} (Lysate – PEB) or A_{260} (Lysate – PEB). If this is less than 6, it means more tissue is required. If it is more, it can be lowered by dilution with PEB. The absorbance is adjusted to 6 because RNase 1 digestion is optimized according to these values.

5. The PEB is optimized to preserve ribosome occupancy on an mRNA and encourage RNase digestion by RNase 1. Therefore, the total RNA integrity is not guaranteed to be maintained in this buffer. To overcome this limitation, the total RNA extraction must be carried out immediately after the adjustment of lysate absorbance to ensure the collection of full-length mRNAs. An aliquot of lysate can be stored at −80 °C for the extraction of RPFs at a later stage.

6. Sucrose cushion is a dense sucrose pad that allows separation of tightly coupled active ribosomes without any mechanical stress. During ultracentrifugation with sucrose cushion, ribosome footprint complex will travel through the highly dense sucrose pad with high sedimentation rate to form a pellet, while other low molecular weight subunits (proteins/RNA) will remain in the supernatant. Though sucrose gradients can be used to separate ribosomes from other individual subunits according to their density, it may increase the chances of contamination with untranslated but protein-bound RNA molecules. Also, if the lysate volume is more than 400 μL, then add PEB accordingly to make the volume 750 μL before layering it on a sucrose cushion. Care must be taken when layering the lysate on a sucrose cushion to create a sharp junction between the two solutions with minimal to no mixing between them. Additionally, the time duration from sample layering to ultracentrifugation should be minimized. Furthermore, the pellet may be hard to see, and therefore, circle the area on the tube with a marker where the pellet is expected to form.

7. Rinsing the pellet with nuclease-free water is an optional step. Rinsing is done to wash away non-pelleted RNA and other contaminants. The risk of this step is that the pellet can be lost if it dissolves in the water or if it comes loose off the tube wall. Therefore, if rinsing is carried out, ice-cold nuclease-free water should be added on to the tube wall and removed by pipetting quickly but steadily on the tube wall opposite to the pellet side.

8. The pellet may not be completely dissolved at this stage. Therefore, care must be taken during transfer to make sure pellet pieces are not retained in the tube or in the pipette tip.

9. Acid–phenol–chloroform extraction must be carried in a fume-hood. Because phenol–chloroform is heated, the tubes usually open and splatter. Therefore, the necessary personal protective equipment must be worn, including safety glasses.

10. At this stage, total RNA can either be subjected to DNase treatment, followed by RNA integrity assessment by denaturing polyacrylamide gel electrophoresis, or stored at −80 °C. DNase treatment and gel electrophoresis of total RNA and RPFs can be conducted simultaneously.

11. A greater volume of ethanol is added to RPF RNA samples to ensure precipitation of the small ~28 nt RPFs.

12. Mix 0.5 μL DNase-treated RNA with 4.5 μL water and 5 μL 2x RNA loading dye. Similarly, for size markers, (i) mix 1 pmol each of 28 nt and 34 nt RNA oligos with water and RNA loading dye to 10 ul, and (ii) mix 50 ng 10/60 nt IDT ssDNA ladder with water and RNA loading dye to 10 ul. Denature the samples at 70 °C for 5 min and immediately transfer the samples to ice. Clean the wells of 15% TBE–Urea gel with the running buffer by pipetting and immediately load the denatured samples. Run the gel as indicated, open the gel cassette, and subsequently stain the gel in a square petri dish with ice-cold SYBR Gold staining solution (3 μL SYBR Gold in 30 μL 1x TBE buffer) for 2 min on a shaker. Cover the petri dish with aluminum foil to protect it from light. Discard the staining solution and add 30 mL ice-cold 1x TBE buffer. Immediately proceed to image the gel with a gel imager. For total RNA samples, the two larger rRNA bands should be present with minimal bands present between the RNA oligo size markers (Fig. 3a).

13. Mix 1 μL DNase-treated RNA with 4 μL water and 5 μL 2x RNA loading dye. Similarly, for size markers, (i) mix 2 pmol each of 28 nt and 34 nt RNA oligos with water and RNA loading dye to 10 ul and (ii) mix 50 ng 10/60 nt IDT ssDNA ladder with water and RNA loading dye to 10 ul. Denature the samples at 70 °C for 5 min and immediately transfer the samples to ice. Clean the 15% TBE-Urea gel wells with the running buffer (1x TBE) by pipetting and immediately load the denatured samples. Run the gel as indicated, open the gel cassette, and subsequently stain the gel in a square petri dish with ice-cold SYBR Gold staining solution (3 μL SYBR Gold in 30 μL 1x TBE buffer) for 2 min on a shaker. Cover the petri dish with aluminum foil to protect it from light. Discard the staining solution and add 30 mL ice-cold 1x TBE buffer. Immediately proceed to image the gel with a gel imager. One or a few sharp and distinct RPF bands should be observed between the RNA oligo size markers.

14. The sale of Illumina Ribo-Zero as a stand-alone kit has been discontinued by the time of writing this chapter. However, the components for rRNA depletion are included with the purchase of the Illumina TruSeq Library Preparation Kit. Alternatively, rRNA depletion kits from other vendors, such as the RiboMinus Plant Kit for RNA-seq from Thermo Fisher Scientific, may be used with appropriate modifications.

15. Load size markers on the gel after every two samples to ensure accurate and precise size selection.

16. Do not spend time visualizing the gel using a gel imager. As soon as the staining solution is added, the RNA starts diffusing out of the gel. Therefore, proceed to cut the gel immediately after staining. Firstly, cut the region of the samples horizontally and then vertically. Use a new baked blade (sterilized) for every cut. Then, using a new baked blade and a 10-μL tip, pick up the gel slice and transfer it to a pierced 0.5-mL tube in a 2-mL microfuge tube.

17. Using an 18G syringe needle, pierce a hole at the bottom of a 0.5-mL tube and then place this tube in a 2-mL microfuge tube. Upon centrifugation, the gel slice will be crushed into smaller pieces when it passes through the hole, getting then collected in the 2-mL tube.

18. The gel in the gel–RNA mix may block the pipette tip. Therefore, using a sterile blade, cut off the "tip" portion of the pipette tip, and use this for pipetting gel–RNA mix.

19. Optimal RNAse1 digestion conditions may vary for different plant tissue, and re-optimization may be needed. For example, 600 μL of lysate from maize seedling roots was digested with 1500 U RNase1 at 28 °C for 30 min [20]. To optimize the RNase1 digestion condition, titrate lysate volume, units of RNase 1, digestion temperature, and duration (as shown in Fig. 3) and analyze the sharpness of the band between 28 nt and 34 nt. Subsequently, run a small RNA bioanalyzer using Agilent bioanalyzer small RNA assay kit to assess the quality of the bands (Fig. 3b).

20. Alkaline hydrolysis and RNase 1 digestion result in RNA fragments with 5′ hydroxyl and 3′ phosphate ends. However, adapter ligation for library preparation requires 5′ phosphate and 3′ hydroxyl ends. Treat the RNA with T4PNK in the absence of ATP dephosphorylates the 3′ end. Subsequently incubate with ATP phosphorylates the 5′ end.

21. For steps A, B, etc., refer to the Steps mentioned in the manufacturer's protocol.

22. The one-third dilution of adapters was determined empirically based on the RNA yield after size selection. Users may need to determine the adapter dilution based on their RNA recovery.

23. Because the input RNA for library preparation was already size-selected, the bead cleanup after reverse transcription can be conducted with the modified, more straightforward protocol that does not need size selection. The modified protocol can be found on the manufacturer's website (perkinelmer-appliedgenomics.com/nextflex_small_rna_v3_no_size_selection_supplement-2/).

24. The optimal number of PCR cycles needs to be determined empirically. Because the reagents in the library preparation kit are very limited, we recommend the following approach to ensure optimal amplification for every library preparation. The bead cleanup after reverse transcription yields 18 μL o f cDNA. 9 μL of this cDNA can be amplified (Step G) in a 12.5 μL PCR with 11 cycles, thereby consuming only half the volume of PCR reagents and the cDNA. Assessing the PCR product can be done by PAGE. If the libraries are overamplified, the remaining 9 μL cDNA can be amplified with 9 or 10 cycles. In contrast, if the PCR product is not overamplified but after cleanup, the quantity is insufficient for sequencing, then the remaining 9 μL cDNA can be amplified with 12 or 13 cycles. This approach ensures the libraries are optimally amplified and yield sufficient DNA for sequencing.

25. The ribo-seq libraries yield fewer reads than RNA-seq libraries, for analysis after data processing and filtering steps. Therefore, ribo-seq libraries must be sequenced at least twice the depth of that of RNA-Seq libraries. We recommend using Illumina HiSeq 3000/4000 or NovaSeq 6000 to obtain high sequencing depth. For example, in ref [38], the libraries were sequenced on the Illumina NovaSeq platform with S1 flow cell (ribo-seq) and SP flow cell (RNA-seq) to obtain ~100 M and ~ 50 M raw reads, respectively, per sample. After data processing, ncRNA depletion, etc., the yield per sample was ~20 M reads, each for ribo-seq and RNA-seq data, which mapped to the Arabidopsis reference genome.

26. As of this writing, use Bowtie v.1.2 (parameters: "-v 2") [25] to map the processed data to ncRNA (rRNA, snoRNA, tRNA) sequences (TAIR10). Use STAR v.2.5 (parameters: "--outFilterMismatchNmax 2 --outFilterMultimapNmax 1") to map the ncRNA-unaligned reads to the Arabidopsis reference genome (TAIR10).

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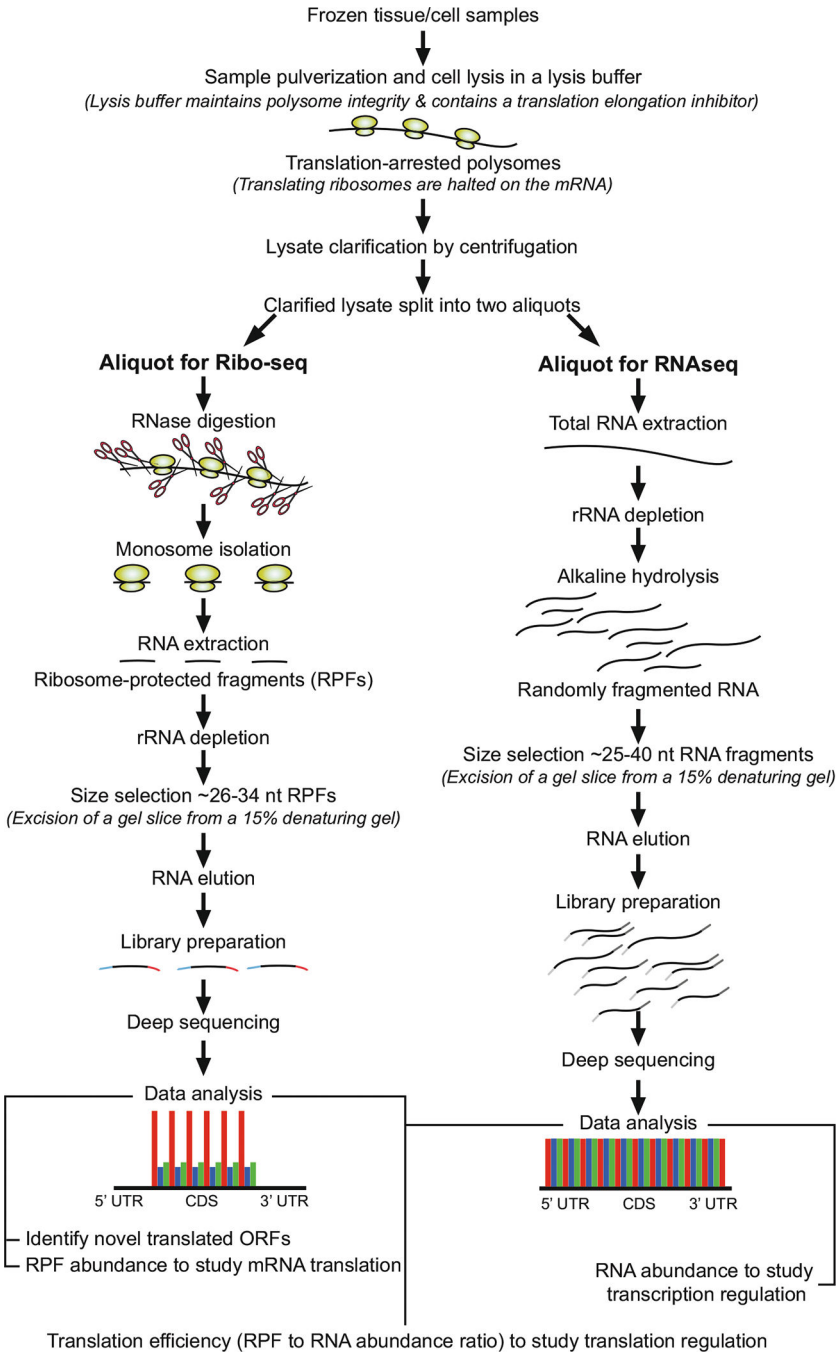

Fig. 1.

Outline of ribosome profiling and RNA sequencing methodology. (Modified slightly from Fig. 2a of reference 20)

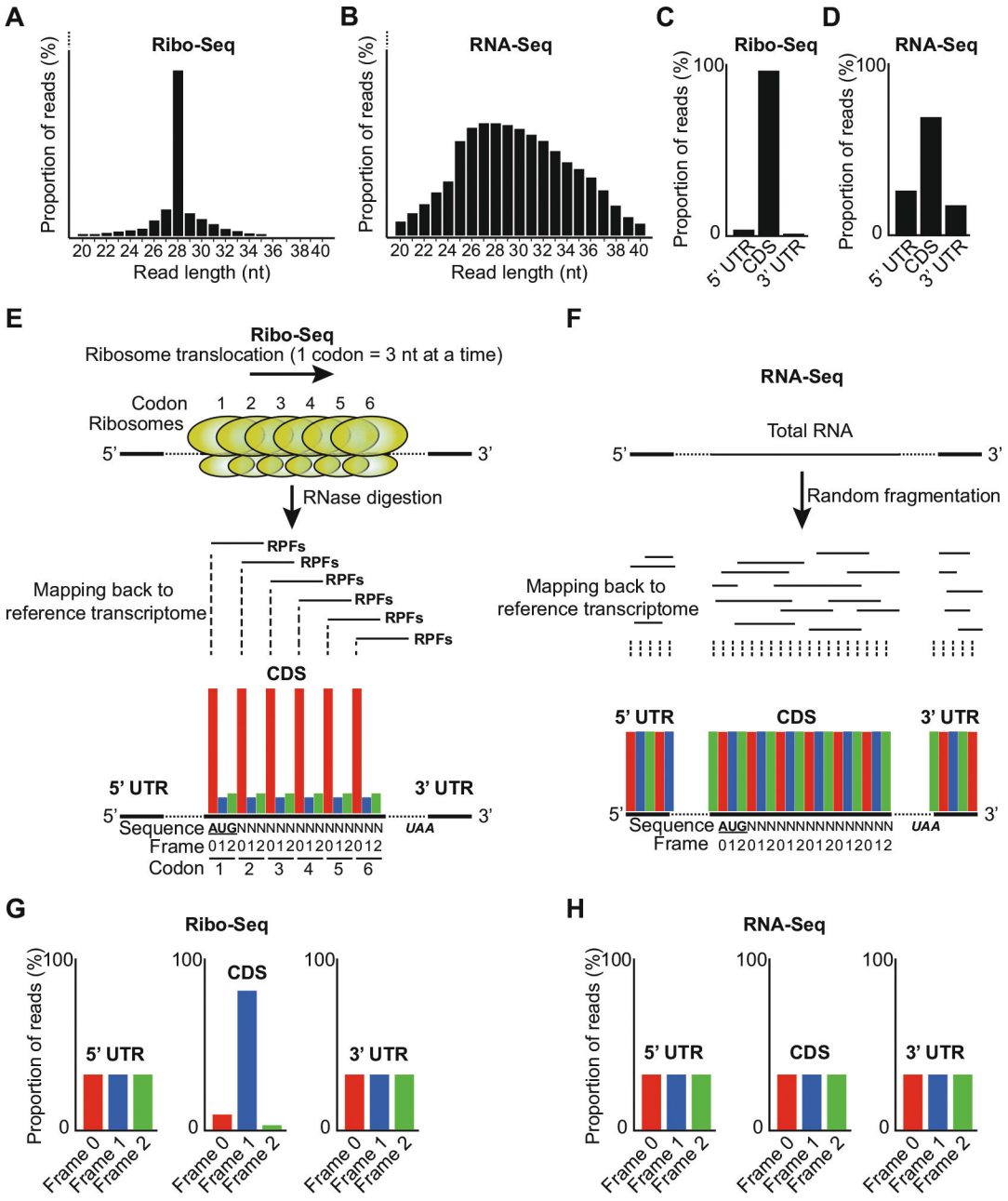

Fig. 2.

Illustration of specific hallmarks of Ribo-seq data. (**a**) Read length distribution of Ribo-seq data shows a unimodal distribution. (**b**) Read length distribution of RNA-seq data shows a relatively uniform and broad distribution. (**c**) Ribo-seq data map predominantly to the CDS of all the mRNAs, whereas only a few reads map to the UTRs. (**d**) A substantial number of reads from RNA-seq data map to the UTRs and the CDS. (**e**) Metagene analysis representing the triplet periodicity of ribo-seq data. Because ribosomes move 3 nt (one codon) at a time during translation elongation, most RPFs map to every third nt in the reference transcriptome. (**f**) Metagene analysis showing the lack of triplet periodicity of RNA-seq data because it is obtained from random fragmentation of ribosome-free total RNAs. (**g**)

Triplet periodicity of ribo-seq data is observed only for the reads that map to the CDS (true RPFs) and not for those that map to the UTRs. (**h**) No triplet periodicity is observed in RNA-seq data for reads that map either to the CDS or to the UTRs. The figure only includes illustrations of high-quality ribo-seq data and does not represent real experimental data. Nucleotide (nt), ribosome-protected fragments (RPFs), coding DNA sequence (CDS), untranslated regions (UTRs). Reproduced from Ph.D dissertation of Pulkit Kanodia (2021) with permission of the author [38]

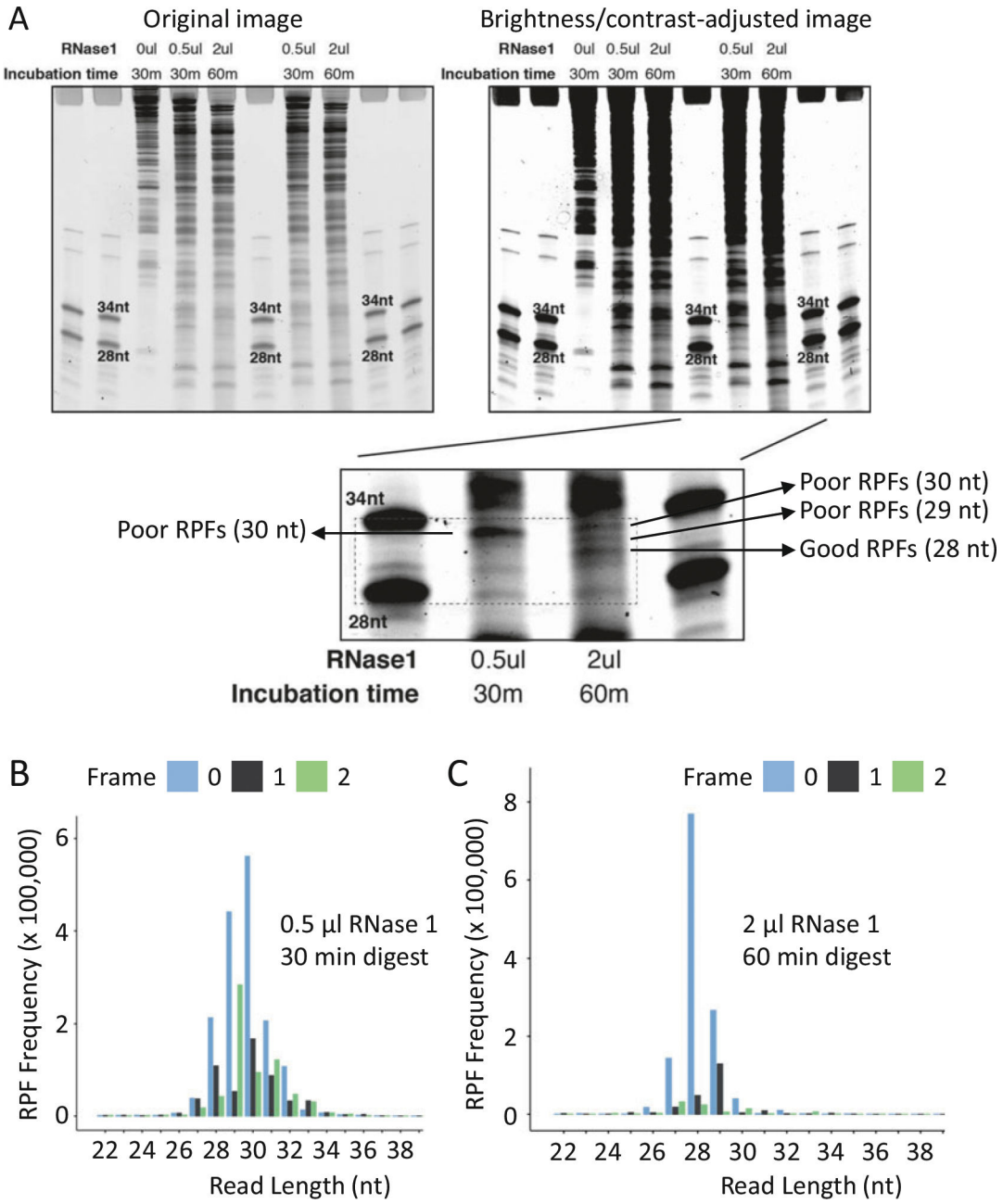

Fig. 3.

Optimization of RNase 1 digestion. (**a**) Denaturing 15% PAGE gel shows distinct sharp bands (inset) under different digestion conditions that may or may not give good RPFs (**b** and **c**). RiboToolkit analysis of ribo-seq data with two different RNase digestion conditions that show (b) suboptimal triplet periodicity obtained by 0.5 µL RNase1 with 30 min digestion for reads of all sizes and (c) excellent triplet periodicity (vast majority of reads map to one reading frame) of the most abundant RPF size class (28 nt) obtained by digestion with 2 µL RNase1 for 60 min

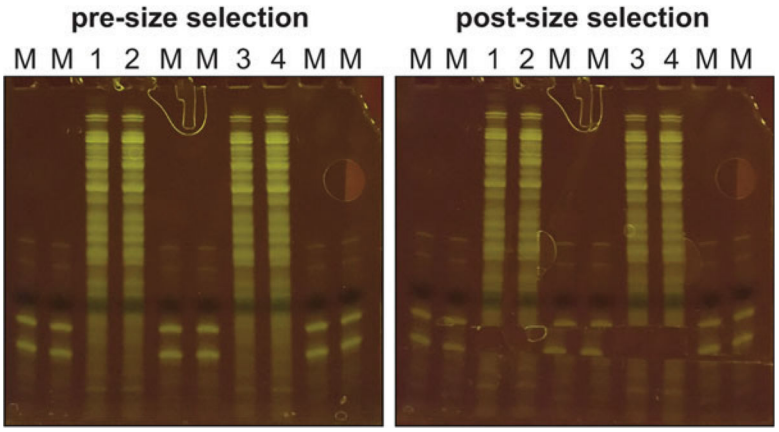

Fig. 4.

Image of gel used for RNA size selection. 6% polyacrylamide gel in TBE–urea buffer. Lanes 1, 2, 3, and 4 show RNA from RNase1-treated lysate. M indicates 28 and 34 nt RNA markers. RNA is stained with SYBR Gold, visualized on a blue light box through orange filter (Subheading 3.8). Note the blank regions on gel at right, from which RNA bands in the desired size range have been excised