Chapter 1

Monitoring Bacterial Translation Rates Genome-Wide

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Abstract

Modern DNA sequencing technologies have allowed for the sequencing of tens of thousands of bacterial genomes. While this explosion of information has brought about new insights into the diversity of the prokaryotic world, much less is known of the identity of proteins encoded within these genomes, as well as their rates of production. The advent of ribosome profiling, or the deep sequencing of ribosome-protected footprints, has recently enabled the systematic evaluation of every protein-coding region in a given experimental condition, the rates of protein production for each gene, and the variability in translation rates across each message. Here, I provide an update to the bacterial ribosome profiling approach, with a particular emphasis on a simplified strategy to reduce cloning time.

Key words Ribosome profiling, Ribo-seq, Bacterial ribosome profiling, Bacterial translation

1 Introduction

Capturing translation rates can reveal which genes are being made, how much, and when they are needed [1, 2]. Recent advances in sequencing technologies have now made it possible to systematically assess translation rates under any experimental condition. Ribosome profiling, or the deep sequencing of ribosome-protected mRNA footprints, allows for the genome-wide monitoring of ongoing protein synthesis and complements other global approaches (such as RNA-seq and mass spectrometry-based proteomics) that enable exploration and discovery of diverse facets of gene regulation [3]. Indeed, ribosome profiling of various bacterial species has been used (i) to define unannotated open reading frames, especially those that encode for small polypeptides [4, 5]; (ii) to find translation initiation sites [6–9]; (iii) to investigate specific aspects of translation, including initiation [10] and frameshifting [11, 12]; (iv) to measure translation efficiencies and examine causes of their variation [13]; (v) to estimate stoichiometries of protein complexes [14]; (vi) to ascertain sites of ribosome pausing and their causes [15–17]; (vii) to determine codon usage patterns

Vyacheslav M. Labunskyy (ed.), *Ribosome Profiling: Methods and Protocols*, Methods in Molecular Biology, vol. 2252, https://doi.org/10.1007/978-1-0716-1150-0_1,

[18]; (viii) to characterize the mechanism of antibiotic action [19, 20]; and (ix) to identify stress-induced regulons [21–23].

While ribosome profiling was originally developed for the budding yeast [24], fundamental differences in bacterial translation demanded a substantial overhaul of the eukaryotic protocol [25, 26]. Firstly, bacteria can sense the immediate loss of nutrients by rapidly shutting down translation initiation, causing ribosomes to run off, which, in turn, leads to a localized paucity of ribosome occupancy at the 5′ end of messages. To minimize this effect, a rapid filtration strategy was developed for cell harvesting in lieu of traditional methods based on centrifugation. Secondly, the nuclease used to generate ribosome-protected footprints for eukaryotes, RNase I, had long been known to be inhibited by the 30S subunit of bacterial ribosomes [27]. This necessitated a new digestion strategy. While micrococcal nuclease was chosen for its ability to be quenched, use of other ribonucleases might prove beneficial [28]. Additionally, recent protocols have provided advancements that should be taken into consideration [29, 30]. However, it is worth noting that the cloning of mRNA footprints into a sequenceable library is time-consuming, taking up to 6 days of continuous work. Much of this time is spent on purifying each enzymatic step through lengthy gel extractions. Here, I describe a protocol that eliminates two gel purification steps, by substituting them with SPRI (solid phase reverse immobilization)-based purifications.

2 Materials

2.1 Cell Harvesting by Rapid Filtration

1. Growth media: LB broth (Lennox), LB broth (Miller), or MOPS minimal medium containing 0.2% glucose.
2. 15 mL culture tube.
3. 1 L baffled flask.
4. 90 mm filtration apparatus.
5. Vacuum line.
6. Nitrocellulose filter discs, 90 mm diameter, 0.2 μm pore size.
7. Scoopula spatula.
8. Liquid nitrogen.
9. 50 mL conical and 18 gauge needle.

2.2 Cell Pulverization

1. Styrofoam box.
2. Liquid nitrogen.
3. Mixer mill, 10 mL grinding jar, and 12 mm grinding ball (*see* **Note 1**).
4. 1 M tris(hydroxymethyl)aminomethane (Tris), pH 8 stock solution in nuclease-free water.

5. 1 M NH_4Cl stock solution in nuclease-free water.
6. 1 M $MgCl_2$ stock solution in nuclease-free water.
7. 10% Triton X-100 stock solution in nuclease-free water.
8. 10% Nonidet P-40 stock solution in nuclease-free water.
9. 100 mM chloramphenicol stock solution in 100% ethanol (store at −20 °C).
10. DNase I, RNase-free (*see* **Note 2**).
11. Lysis buffer: 20 mM Tris, pH 8, 100 mM NH_4Cl, 10 mM $MgCl_2$, 0.4% Triton X-100, 0.1% Nonidet P-40, 1 mM chloramphenicol, and 100 unit/mL DNase I, RNase-free in nuclease-free water.
12. 50 mL conical and 18 gauge needle.
13. Rounded-edge spatula.

2.3 mRNA Enrichment and Fragmentation

1. 1 M ethylenediaminetetraacetic acid (EDTA), pH 8 stock solution in nuclease-free water.
2. 3 M sodium acetate (NaOAc), pH 5.5 stock solution in nuclease-free water.
3. Resuspension buffer: 10 mM EDTA and 60 mM NaOAc in nuclease-free water.
4. MICROBExpress bacterial mRNA enrichment kit (*see* **Note 3**).
5. 10 mM Tris, pH 7 in nuclease-free water (prepare from a 1 M stock solution).
6. MEGAclear clean-up kit (*see* **Note 3**).
7. 2× alkaline hydrolysis buffer: 1 μL of 0.5 M EDTA, pH 8, 30 μL of 0.1 M Na_2CO_3, and 220 μL of 0.1 M $NaHCO_3$ (prepare fresh each time).
8. GlycoBlue coprecipitant (15 mg/mL stock) or equivalent carrier.
9. Isopropanol.
10. Microcentrifuge.
11. 80% ethanol in nuclease-free water (prepare from a 200 proof stock solution).

2.4 Extract Preparation, MNase Footprinting, and Monosome Isolation

1. Tabletop centrifuge compatible for spinning 50 mL conicals.
2. 1.5 mL tubes.
3. Microcentrifuge.
4. RNA/DNA spectrophotometer or Nanodrop.
5. Micrococcal nuclease (MNase) (*see* **Note 4**). Prepare a stock solution containing 250 units of MNase/μL in 10 mM Tris, pH 8 (store aliquots at −80 °C, avoid freeze-thaw cycles).

6. Superase•In RNase inhibitor (*see* **Note 5**).
7. 100 mM $CaCl_2$ stock solution in nuclease-free water.
8. 0.5 M ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*,'*N*'--tetraacetic acid (EGTA), pH 8 stock solution in nuclease-free water.
9. SW 41 Ti swinging buckets, rotor, and ultracentrifuge.
10. Sucrose.
11. 1 M Tris, pH 8 stock solution in nuclease-free water.
12. 1 M NH_4Cl stock solution in nuclease-free water.
13. 1 M $MgCl_2$ stock solution in nuclease-free water.
14. 100 mM chloramphenicol stock solution in 100% ethanol (store at −20 °C).
15. 10% sucrose solution: 10% sucrose (w/v), 20 mM Tris, pH 8, 100 mM NH_4Cl, 10 mM $MgCl_2$, and 1 mM chloramphenicol in nuclease-free water.
16. 50% sucrose solution: 50% sucrose (w/v), 20 mM Tris, pH 8, 100 mM NH_4Cl, 10 mM $MgCl_2$, and 1 mM chloramphenicol in nuclease-free water.
17. SETON open-top polyclear tubes (*see* **Note 6**).
18. BIOCOMP Gradient Master and Piston Gradient Fractionator (*see* **Note 6**).
19. 2 mL screw cap tube.
20. Liquid nitrogen.

2.5 RNA Extraction

1. 20% sodium dodecyl sulfate (SDS) in nuclease-free water.
2. Acid phenol, pH 4.5.
3. Microcentrifuge.
4. 1.5 mL tubes.
5. Chloroform.
6. 3 M sodium acetate (NaOAc), pH 5.5.
7. Isopropanol.
8. 80% ethanol in nuclease-free water (prepare from a 200 proof stock solution).
9. 10 mM Tris, pH 7 in nuclease-free water (prepare from a 1 M stock solution).

2.6 Footprint Size Selection

1. RNA/DNA spectrophotometer or Nanodrop.
2. 10 mM Tris, pH 7 in nuclease-free water (prepare from a 1 M stock solution).
3. Agencourt AMPure XP beads (*see* **Note 7**).
4. Magnetic stand compatible for 1.5 mL tubes.

5. 1.5 mL tubes.
6. Isopropanol.
7. 80% ethanol in nuclease-free water (prepare from a 200 proof stock solution).

2.7 Dephosphorylation

1. NEB T4 polynucleotide kinase (PNK) (*see* **Note 7**).
2. 10× T4 PNK buffer (supplied with enzyme).
3. Superase•In RNase inhibitor.
4. 10 mM Tris, pH 7 in nuclease-free water (prepare from a 1 M stock solution).
5. 3 M sodium acetate (NaOAc), pH 5.5.
6. GlycoBlue coprecipitant (15 mg/mL stock).
7. Isopropanol.
8. Microcentrifuge.
9. 80% ethanol in nuclease-free water (prepare from a 200 proof stock solution).

2.8 Linker Ligation

1. NEB T4 RNA ligase 2, truncated K227Q (*see* **Note 7**).
2. 50% (w/v) PEG 8000 (supplied with enzyme).
3. 10× T4 RNA ligase reaction buffer (supplied with enzyme).
4. Superase•In RNase inhibitor.
5. 20 μM solution of oCJC88 oligonucleotide /5rApp/GATCGGAAGAGCACACGT/3ddC/ (*see* **Note 8**) dissolved in 10 mM Tris, pH 7.
6. Agencourt AMPure XP beads.
7. Isopropanol.
8. Magnetic stand compatible for 1.5 mL tubes.
9. 10 mM Tris, pH 7 in nuclease-free water (prepare from a 1 M stock solution).

2.9 Reverse Transcription

1. 10 mM dNTP stock solution.
2. 20 μM solution of oCJC160 oligonucleotide /5Phos/AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT/iSp18/GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC (*see* **Note 8**) dissolved in 10 mM Tris, pH 7.
3. SuperScript III reverse transcriptase (*see* **Note 7**).
4. 5× First strand buffer (FSB) (supplied with enzyme).
5. 0.1 M DTT (supplied with enzyme).
6. Superase•In RNase inhibitor.
7. 1 N NaOH.
8. 2× Novex TBE-urea sample buffer (*see* **Note 7**).

9. 10% Novex TBE-urea gel, 12 wells (*see* **Note 7**).
10. 1× Tris-borate-EDTA (TBE) running buffer. Contains 0.089 M Tris-borate and 0.002 M EDTA buffered to pH 8.3.
11. SYBR gold nucleic acid gel stain (*see* **Note 7**).
12. 18 gauge needle, 0.5 mL tubes, and 1.5 mL tubes.
13. Microcentrifuge.
14. 10 mM Tris, pH 8.
15. Costar Spin-X centrifuge filters (*see* **Note 7**).
16. 5 M NaCl.
17. 0.5 M EDTA, pH 8.
18. GlycoBlue coprecipitant (15 mg/mL stock).
19. Isopropanol.
20. 80% ethanol in nuclease-free water (prepare from a 200 proof stock solution).

2.10 Circularization

1. CircLigase ssDNA ligase (*see* **Note 7**).
2. 10× CircLigase reaction buffer (supplied with enzyme).
3. 1 mM ATP (supplied with enzyme).
4. 50 mM $MnCl_2$ (supplied with enzyme).

2.11 rRNA Subtraction

1. 100 μM solution of hybridization oligonucleotides dissolved in 10 mM Tris, pH 8 (*see* **Note 8**):
   mix 77 μL of 100 μM oEO1055, /5Biosg/TCATCTCCGGGGGTAGAGCACTGTTTCG;
   4 μL of 100 μM oEO1056, /5Biosg/GGCTAAACCATGCACCGAAGCTGCGGCAG;
   17 μL of 100 μM oEO1057, /5Biosg/AAGGCTGAGGCGTGATGACGAGGCACT;
   and 2 μL of 100 μM oEO1058, /5Biosg/CGGTGCTGAAGCAACAAATGCCCTGCTT.
2. 20× saline-sodium citrate (SSC). Contains 3 M NaCl and 0.4 M sodium citrate buffered to pH 7 with HCl.
3. PCR thermocycler.
4. MyOne streptavidin C1 dynabeads (*see* **Note 7**).
5. Magnetic stand compatible for 1.5 mL tubes.
6. 1× binding and wash (B&W) buffer: 5 mM Tris, pH 7.5, 0.5 mM EDTA, 1 M NaCl, and 0.01% Tween.
7. 2× B&W buffer: 10 mM Tris, pH 7.5, 1 mM EDTA, 2 M NaCl, and 0.01% Tween.
8. 10 mM Tris, pH 8.
9. 5 M NaCl.

10. 0.5 M EDTA, pH 8.
11. GlycoBlue coprecipitant (15 mg/mL stock).
12. Isopropanol.
13. Microcentrifuge.
14. 80% ethanol in nuclease-free water (prepare from a 200 proof stock solution).

2.12 Library Amplification by PCR

1. High-Fidelity (HF) Phusion DNA polymerase (*see* **Note 7**).
2. 5× HF buffer (supplied with enzyme).
3. 10 mM dNTPs.
4. 10 μM solution of oCJC161 oligonucleotide resuspended in 10 mM Tris, pH 8:
   AATGATACGGCGACCACCGAGATCTACACTCTTTCCC TACACGACGCTCTTCCGATCT.
5. 10 μM solution of oCJC60–71 indexing oligonucleotides resuspended in 10 mM Tris, pH 8:
   oCJC60: CAAGCAGAAGACGGCATACGAGATCGAGTAA TGTGACTGGAGTTCAGACG, index: ATTACTCG.
   oCJC61: CAAGCAGAAGACGGCATACGAGATTCTCCG GAGTGACTGGAGTTCAGACG, index: TCCGGAGA.
   oCJC62: CAAGCAGAAGACGGCATACGAGATAATGAGC GGTGACTGGAGTTCAGACG, index: CGCTCATT.
   oCJC63: CAAGCAGAAGACGGCATACGAGATGGAATCT CGTGACTGGAGTTCAGACG, index: GAGATTCC.
   oCJC64: CAAGCAGAAGACGGCATACGAGATTTCTGAA TGTGACTGGAGTTCAGACG, index: ATTCAGAA.
   oCJC65: CAAGCAGAAGACGGCATACGAGATACGAATT CGTGACTGGAGTTCAGACG, index: GAATTCGT.
   oCJC66: CAAGCAGAAGACGGCATACGAGATAGCTT CAGGTGACTGGAGTTCAGACG, index: CTGAAGCT.
   oCJC67: CAAGCAGAAGACGGCATACGAGATGCGCAT TAGTGACTGGAGTTCAGACG, index: TAATGCGC.
   oCJC68: CAAGCAGAAGACGGCATACGAGATCATAGCC GGTGACTGGAGTTCAGACG, index: CGGCTATG.
   oCJC69: CAAGCAGAAGACGGCATACGAGATTTCGCG GAGTGACTGGAGTTCAGACG, index: TCCGCGAA.
   oCJC70: CAAGCAGAAGACGGCATACGAGATGCGCGA GAGTGACTGGAGTTCAGACG, index: TCTCGCGC.
   oCJC71: CAAGCAGAAGACGGCATACGAGATCTATCGC TGTGACTGGAGTTCAGACG, index: AGCGATAG).
6. 6× DNA gel loading dye.

7. Novex 8% TBE-polyacrylamide gel, 12 wells.
8. 1× TBE running buffer.
9. SYBR gold nucleic acid gel stain.
10. 18 gauge needle, 0.5 mL tubes, and 1.5 mL tubes.
11. Microcentrifuge.
12. 10 mM Tris, pH 8.
13. Costar Spin-X centrifuge filters.
14. 5 M NaCl.
15. 0.5 M EDTA, pH 8.
16. GlycoBlue coprecipitant (15 mg/mL).
17. Isopropanol.
18. 80% ethanol in nuclease-free water (prepare from a 200 proof stock solution).

2.13 Quantify, Sequence, and Analyze

1. Fragment analyzer or equivalent.
2. Read1 oligonucleotide sequence: ACACTCTTTCCCTACAC GACGCTCTTCCGATCT.
3. Indexing oligonucleotide sequence: GATCGGAAGAGCA CACGTCTGAACTCCAGTCAC.

3 Methods

3.1 Cell Harvesting by Rapid Filtration (~10–15 min for Harvesting)

1. Inoculate a single bacterial colony in 5 mL of growth medium. Use the appropriate growth medium for your strain of interest and/or experimental condition. Use of non-rich media has been shown to deplete specific amino acids more readily and will affect ribosome occupancy measurements. Culture cells overnight at 37 °C (or at a growth temperature required for your strain of interest) in a 15 mL culture tube.
2. Dilute overnight culture in 250 mL of growth medium (pre-warmed to 37 °C) in a 1 L baffled flask. Make sure cells go through more than five doublings. Starting OD_{600} should be less than 0.005.
3. Grow cells at 37 °C until culture reaches log-phase growth (or an OD_{600} of ~0.3–0.4). Culture conditions for log-phase growth should be optimized for each strain.
4. Connect filtration apparatus to a vacuum line in a 37 °C warm room. Make sure to use a 0.2 μm filter disc made of nitrocellulose. Filter discs made of different materials and pore sizes have been tested and do not filter as well. Cultures should be filtered at their growth temperature, if possible. Use a portable vacuum pump if a vacuum line is not set up in your warm room.

5. Filter liquid culture (*see* **Note 9**). Do not wait for liquid culture to filter fully. Immediately disassemble filtration apparatus once ~90–95% of culture has passed through the filter. This will minimize ribosome runoff, which occurs as soon as cells are deprived of growth medium.
6. Firmly scrape cells off filter disc with a Scoopula spatula. These spatulas are preferred because they have a long edge. Avoid scraping cells more than once, as this will help minimize ribosome runoff.
7. Quickly plunge Scoopula spatula into a 50 mL conical filled with liquid nitrogen. A second spatula (prechilled in liquid nitrogen) may be needed to dislodge cells that are firmly adhered to the Scoopula.
8. Remove excess liquid nitrogen. Pierce a 50 mL conical cap five times with an 18 gauge needle. Cap conical and invert to remove excess liquid nitrogen. Make sure vents face away from you.
9. Store frozen cells at −80 °C [STOP POINT] or continue to next section.

3.2 Cell Pulverization (~1 h)

1. Fill a Styrofoam box with liquid nitrogen. You may need to refill as liquid nitrogen evaporates.
2. Place a 12 mm grinding ball in a 10 mL grinding jar and submerge in a liquid nitrogen bath until liquid nitrogen stops boiling.
3. Prepare 1 mL of lysis buffer.
4. Fill a 50 mL conical with liquid nitrogen and add 650 μL of lysis buffer dropwise.
5. Remove excess liquid nitrogen. Pierce a 50 mL conical cap five times with an 18 gauge needle. Cap conical and invert to remove excess liquid nitrogen. Make sure vents face away from you.
6. Remove grinding jar (grinding ball included) from liquid nitrogen bath. Place frozen cells (from Subheading 3.1, **step 9**) and frozen lysis buffer pellets in grinding jar. Make sure liquid nitrogen has fully evaporated prior to assembling grinding jar.
7. Submerge closed jar in a liquid nitrogen bath until liquid nitrogen stops boiling.
8. Pulverize cells in a mixer mill at 15 Hz for 3 min.
9. Submerge jar in a liquid nitrogen bath until liquid nitrogen stops boiling.
10. Repeat **steps 8** and **9** four more times.
11. Transfer pulverized cell powder into a 50 mL conical filled with liquid nitrogen. Fill conical with liquid nitrogen up to the

25 mL line. Do not overfill. Use a rounded-edge spatula (pre-chilled in liquid nitrogen) to scrape out the pulverized cell powder and gently deposit cell powder into liquid nitrogen.

12. Remove excess liquid nitrogen. Pierce a 50 mL conical cap five times with an 18 gauge needle. Cap and tap the conical until excess liquid nitrogen escapes through vents. Do not invert or you will lose your pulverized cell powder. Make sure vents face away from you.
13. Store pulverized cell powder at −80 °C [STOP POINT], or continue to next section. If storing cell powder, store with a vented cap. If genome-wide translation efficiency measurements are desired, save one-third of pulverized cell powder for Subheading 3.3 and two-thirds of cell powder for Subheading 3.4 (*see* **Note 10**). If only ribosome density measurements are needed, proceed with Subheading 3.4.

3.3 mRNA Enrichment (~4–5 h) and Fragmentation (~1.5 h)

1. For total RNA extraction, dissolve pulverized cell powder in 0.7 mL of resuspension buffer.
2. Proceed with Subheading 3.5, **steps 1–18** and return.
3. Remove 16S and 23S ribosomal RNAs by subtractive hybridization using the MICROBExpress bacterial mRNA enrichment kit (follow manufacturer's instructions). Begin with 20 μg of total RNA. Isopropanol precipitate rRNA-subtracted RNAs. Resuspend in 40 μL of 10 mM Tris, pH 7.
4. Remove small RNAs less than 100 nucleotides using the MEGAclear clean-up kit (follow manufacturer's instructions). Isopropanol precipitate enriched mRNAs. Resuspend in 25 μL of 10 mM Tris, pH 7.
5. Fragment enriched mRNA by alkaline hydrolysis. To 25 μL of enriched mRNA, add 25 μL of freshly prepared 2× alkaline hydrolysis buffer.
6. Incubate at 95 °C for 23 min and transfer to ice.
7. Precipitate fragmented RNA by adding 450 μL of 10 mM Tris, pH 7, 55 μL of 3 M NaOAc pH, 5.5, 2 μL of GlycoBlue coprecipitant, and 0.55 mL of 100% isopropanol. Vortex and incubate at −80 °C for 30 min or longer.
8. Pellet at 20,000 × *g* or top speed in a microcentrifuge for 30 min.
9. Wash pellet twice with 0.5 mL of ice-cold 80% ethanol.
10. Dry pellet for 5 min in a chemical fume hood.
11. Resuspend pellet in 50 μL of 10 mM Tris, pH 7.
12. Proceed with Subheading 3.6, **step 3**.

3.4 Extract Preparation (~30 min), MNase Footprinting (~1.5 h), and Monosome Isolation (~3.5 h)

1. Thaw pulverized cell powder in a room temperature water bath for 2 min (or until completely thawed).
2. Incubate on ice for 10 min.
3. Spin down 50 mL conical at 4000 × *g* in a tabletop centrifuge for 1 min at 4 °C. This is to collect the extract adhering to the conical wall.
4. Transfer thawed extract to a prechilled 1.5 mL tube.
5. Spin down insoluble debris at 20,000 × *g* or top speed in a microcentrifuge for 10 min at 4 °C.
6. Transfer clarified supernatant to a prechilled 1.5 mL tube. Do not disrupt pellet.
7. Measure RNA concentration by Nanodrop by diluting extract 1:100 in nuclease-free water. Measure A_{260} and calculate concentration. 1 A_{260} unit equals 40 μg/mL of total RNA.
8. Digest 0.5 mg of total RNA in a 0.2 mL reaction volume. To 0.5 mg of total RNA, add 750 units of MNase, 2.5 μL of Superase•In, and 10 μL of 100 mM $CaCl_2$. Bring up reaction volume to 0.2 mL with lysis buffer (*see* recipe in Subheading 2.2).
9. Incubate reaction at 25 °C for 1 h.
10. Quench reaction with 2 μL of 0.5 M EGTA and leave on ice.
11. Prechill SW 41 Ti swinging buckets and rotor at 4 °C.
12. Prepare 15 mL of 10% sucrose solution.
13. Prepare 15 mL of 50% sucrose solution.
14. Add 6 mL of 50% sucrose solution to an open-top polyclear tube. SETON brand tubes are less prone to cracking.
15. Layer 6 mL of 10% sucrose solution with a motorized pipette controller. Use the slowest setting available to avoid mixing the two layers.
16. Make a 10–50% gradient. Use the preset 10–50% (w/v) short program on a Gradient Master.
17. Carefully load gradients into prechilled buckets. Gradients should be stored at 4 °C until quenched reactions are ready to be loaded.
18. Load samples without disrupting the gradients. Balance with lysis buffer, if necessary (*see* recipe in Subheading 2.2).
19. Carefully attach the loaded buckets to the prechilled rotor. Set ultracentrifuge to spin an SW 41 Ti rotor and spin at 35,000 rpm (151,000 × *g*) for 2.5 h at 4 °C.
20. Fractionate using a Piston Gradient Fractionator. Set piston speed to 0.2 mm/s.

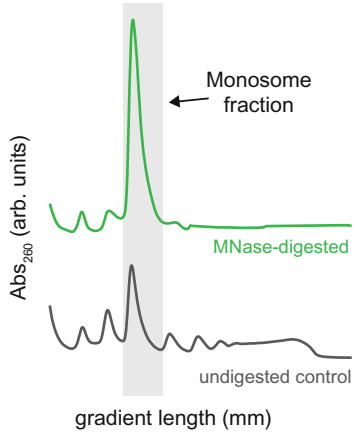

Fig. 1 Sucrose gradient profiles of mock versus MNase-digested polysomes. Polysomes were harvested from DH5α cells and run on a 10–50% sucrose gradient. Gradient lengths (mm) are plotted as a function of absorbance at 260 nm (in arbitrary units)

21. Manually collect monosome peak in a 2 mL screw cap tube (Fig. 1).
22. Flash freeze monosomes in liquid nitrogen and store at −80 °C [STOP POINT] or continue to next section.

3.5 RNA Extraction (~2 h)

1. To 0.7 mL of monosomes, add 40 μL of 20% SDS and 0.7 mL of acid phenol, pH 4.5 (prewarmed to 65 °C).
2. Vortex and incubate at 65 °C for 5 min.
3. Chill on ice for 5 min.
4. Spin at 20,000 × *g* or top speed in a microcentrifuge for 2 min.
5. Transfer aqueous layer to a new 1.5 mL tube.
6. Add 0.7 mL of room temperature acid phenol, pH 4.5.
7. Vortex and incubate at room temperature for 5 min.
8. Spin at 20,000 × *g* or top speed for 2 min.
9. Transfer aqueous layer to a new 1.5 mL tube.
10. Add 0.6 mL of chloroform.
11. Vortex and immediately spin at 20,000 × *g* or top speed for 1 min.
12. Transfer aqueous layer to new 1.5 mL tube.
13. Precipitate total RNA by adding 78 μL of 3 M NaOAc, pH 5.5, and 0.77 mL of 100% isopropanol. Vortex and incubate at −80 °C for 30 min or longer.
14. Pellet at 20,000 × *g* or top speed for 30 min.
15. Wash pellet twice with 0.5 mL of ice-cold 80% ethanol.

16. Dry pellet for 5 min in a chemical fume hood.
17. Resuspend pellet in 20 μL of 10 mM Tris, pH 7.
18. Store extracted RNA at −20 °C [STOP POINT] or continue to next section.

3.6 Footprint Size Selection (~1.5 h)

1. Quantify extracted RNA by Nanodrop.
2. Dilute 10 μg of extracted RNA in 50 μL of 10 mM Tris, pH 7.
3. Add 90 μL of AMPure XP beads (or SPRIselect beads). This method is based on a strategy for cloning microRNA libraries [31]. Make sure AMPure XP beads are fully resuspended prior to use. AMPure XP and SPRIselect beads are identical except SPRIselect beads are certified as RNAse-free. Nonetheless, AMPure XP beads have been used to purify RNA without observable degradation.
4. Mix by pipetting until beads are fully resuspended and incubate at room temperature for 5 min.
5. Place tube in a magnetic stand, and allow beads to settle until supernatant clears. RNA fragments greater than ~200 nucleotides will adhere to beads.
6. Carefully transfer supernatant to a new tube. This fraction contains RNA fragments that range in length from ~18 to 200 nucleotides.
7. Add 70 μL of AMPure XP beads and 90 μL of 100% isopropanol. Final concentrations: 10.6% PEG, 30% isopropanol.
8. Mix by pipetting until beads are fully resuspended and incubate at room temperature for 5 min.
9. Place tube in a magnetic stand, and allow beads to settle until supernatant clears.
10. Carefully transfer supernatant to a new tube.
11. Add 90 μL of AMPure XP beads and 270 μL of 100% isopropanol. Final concentrations: 7.5% PEG, 58% isopropanol.
12. Mix by pipetting until beads are fully resuspended, and incubate at room temperature for 5 min. RNA fragments ranging from ~18 to 100 nucleotides will adhere to beads (Fig. 2). RNA fragments outside the desired range of ~18–45 nucleotides can be removed by gel extraction following the reverse transcription reaction (*see* Subheading 3.9).
13. Place tube in a magnetic stand, and allow beads to settle until supernatant clears.
14. Discard supernatant.
15. Wash twice with 0.5 mL of room temperature 80% ethanol. Do not disrupt beads.
16. Dry beads for 5 min in a chemical fume hood.

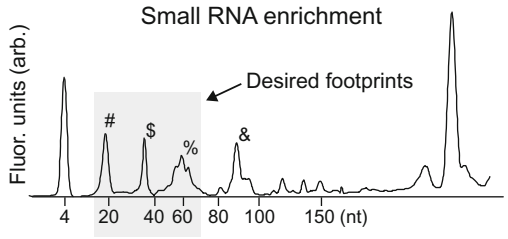

Fig. 2 RNA bioanalyzer trace of SPRI-selected small RNA footprints. RNA was quantified in fluorescence units (*y*-axis). #, $, %, and & signs reflect discrete, contaminating rRNA bands (at indicated sizes), which are used as internal sizing standards. The desired footprint lengths are enclosed within the gray box

17. Resuspend beads with 30 μL of 10 mM Tris, pH 7.
18. Mix by pipetting until beads are fully resuspended and incubate at room temperature for 2 min.
19. Place tube in a magnetic stand and allow beads to settle until supernatant clears.
20. Transfer eluate into a new 1.5 mL tube and store at −20 °C [STOP POINT] or continue to next section.

3.7 Dephosphorylation (~2.5 h)

1. To 15 μL of eluate, add 2 μL of 10× T4 PNK buffer (supplied with enzyme) and 1 μL of Superase•In. Make a master mix if performing many reactions. To monitor cloning efficiency, you can perform the same reaction with the control oligo oNTI199 in parallel (*see* **Note 11**).
2. Add 2 μL of T4 PNK.
3. Mix by pipetting.
4. Incubate reaction at 37 °C for 1 h.
5. Heat kill enzyme at 75 °C for 10 min.
6. Precipitate by adding 480 μL of 10 mM Tris, pH 7, 55 μL of 3 M NaOAc, pH 5.5, 2 μL of GlycoBlue coprecipitant, and 0.55 mL of 100% isopropanol. Vortex and incubate at −80 °C for 30 min or longer.
7. Pellet at 20,000 × *g* or at top speed in a microcentrifuge for 30 min at 4 °C.
8. Wash pellet twice with 0.5 mL of ice-cold 80% ethanol.
9. Dry pellet for 5 min in a chemical fume hood.
10. Resuspend pellet in 7 μL of 10 mM Tris, pH 7.
11. Store at −20 °C [STOP POINT] or continue to next section.

3.8 Linker Ligation (~3 h)

1. Denature 7 μL of dephosphorylated RNA at 80 °C for 2 min and return to ice.
2. Add 8 μL of 50% (w/v) PEG 8000 (supplied with enzyme), 2 μL of 10× T4 RNA ligase reaction buffer (supplied with

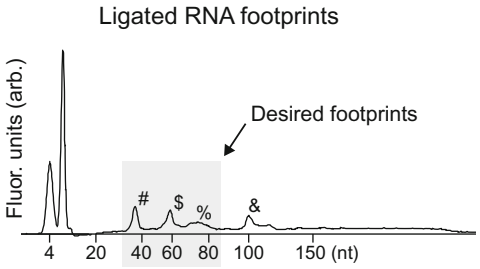

Fig. 3 Small RNA bioanalyzer trace of RNA footprints ligated with linker. RNA was quantified in fluorescence units (*y*-axis). #, $, %, and & signs reflect discrete, contaminating rRNA bands (at indicated sizes), which are used as internal sizing standards. RNA footprints will increase by ~18 nt, which is the length of the linker. The desired footprint lengths are enclosed within the gray box

enzyme), 1 μL of Superase•In, and 1 μL of 20 μM oCJC88 oligonucleotide. Make a master mix if performing many reactions.

3. Add 1 μL of T4 RNA ligase 2, truncated K227Q.
4. Mix by pipetting.
5. Incubate at 25 °C for 2 h.
6. To 20 μL of ligation reaction, add 29 μL of nuclease-free water, 75 μL of AMPure XP beads, and 129 μL of 100% isopropanol. Final concentrations: 7.5% PEG, 51% isopropanol.
7. Mix until beads are fully resuspended by pipetting and incubate at room temperature for 5 min. RNA fragments greater than ~35 nucleotides will adhere to beads (Fig. 3).
8. Discard supernatant.
9. Resuspend beads in 11.5 μL of 10 mM Tris, pH 7.
10. Place tube in a magnetic stand and allow beads to settle until supernatant clears.
11. Transfer eluate into a fresh 1.5 mL tube and store at −20 °C [STOP POINT] or continue to next section.

3.9 Reverse Transcription (~3.5 h)

1. To 11.5 μL of ligated RNA, add 1 μL of 10 mM dNTP and 1 μL of 20 μM oCJC160 oligonucleotide.
2. Denature at 65 °C for 5 min and return to ice.
3. Add 4 μL of 5× FSB buffer (supplied with enzyme), 1 μL of 0.1 M DTT (supplied with enzyme), and 1 μL of Superase•In. Make a master mix if performing many reactions.
4. Add 1 μL of SuperScript III reverse transcriptase.
5. Mix by pipetting.
6. Incubate at 50 °C for 30 min.
7. Add 2.3 μL of 1 N NaOH.

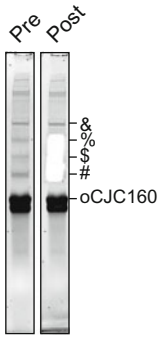

Fig. 4 Gel analysis of reverse transcription reaction. Reverse-transcribed RNA was resolved on a 15% TBE-urea gel (however, a 10% TBE-urea is recommended). Size selection of your desired fragment lengths should occur at this step. #, $, %, and & signs reflect discrete, contaminating rRNA bands, which are used as internal sizing standards, rather than DNA ladders. Left panel indicates gel before excision of bands (pre), while right panel indicates gel following excision of bands (post)

8. Hydrolyze RNA at 95 °C for 15 min.
9. Add 23 μL of 2× TBE-urea sample buffer.
10. Denature at 80 °C for 2 min and return to ice.
11. Set up a 10% TBE-urea gel in 1× TBE. Pre-run gel at 200 V for 1 h. Wash lanes prior to use.
12. Load samples and run at 200 V until loading dye runs out of gel. Instead of relying on loading controls, I now use the contaminating rRNA bands to approximate sizes (*see* **Note 12**).
13. Stain gel with a 1:10,000 dilution of SYBR gold reagent in 1× TBE for 2 min.
14. Size select desired bands. Cut below the lowest contaminating rRNA band (#) but well above the free oCJC160 oligonucleotide to below the highest contaminating rRNA band (&) (Fig. 4) (*see* **Note 13**). To maximize the recovery of all ribosome-protected mRNA footprints, cutting a larger band length is preferred. Use blue light source for detection, if possible. Avoid using UV as light source to prevent UV-induced crosslinking of library.
15. To recover DNA, pierce an 18 gauge needle through a 0.5 mL tube. Insert gel slice in pierced tube and nest inside a 1.5 mL tube.
16. Spin down at 20,000 × *g* or top speed in a microcentrifuge for 2 min to crush gel.
17. Add 0.5 mL of 10 mM Tris, pH 8. Incubate at −20 °C for 30 min.

18. Incubate at 70 °C for 10 min with shaking. Transfer gel slurry to a Spin-X cellulose acetate column using a wide-bore pipette (or cut a pipette tip with a razor blade).
19. Spin at 20,000 × *g* or top speed for 1 min. Transfer flow through to a new 1.5 mL tube.
20. Add 32 μL of 5 M NaCl, 1 μL of 0.5 M EDTA, 2 μL of GlycoBlue coprecipitant, and 0.55 mL of 100% isopropanol. Mix by vortexing and incubate at −80 °C for 30 min or longer.
21. Pellet at 20,000 × *g* or top speed for 30 min at 4 °C.
22. Wash pellet twice with 0.5 mL of ice-cold 80% ethanol.
23. Dry pellet for 5 min in a chemical fume hood.
24. Resuspend pellet in 15 μL of 10 mM Tris, pH 8.
25. Store at −20 °C [STOP POINT] or continue to next section.

3.10 Circularization (~2.5 h)

1. To 15 μL of reverse-transcribed ssDNA, add 2 μL of 10× CircLigase buffer (supplied with enzyme), 1 μL of 1 mM ATP (supplied with enzyme), and 1 μL of 50 mM $MnCl_2$ (supplied with enzyme). Make a master mix if performing many reactions.
2. Add 1 μL of CircLigase.
3. Mix by pipetting.
4. Incubate at 60 °C for 1 h. Use a heated lid, if possible.
5. Dope in 1 μL of CircLigase and incubate at 60 °C for 1 h.
6. Heat kill enzyme at 80 °C for 10 min.
7. Store reaction at −20 °C [STOP POINT] or continue to next section.

3.11 rRNA Subtraction (~3 h)

1. To 5 μL of circularized DNA, add 1 μL of 100 μM mix of hybridization oligonucleotides, 1 μL of 20× SSC, and 3 μL of water. Make a master mix if performing many reactions. Hybridization oligonucleotide sequences were designed for *E. coli* ribosomal RNAs and must be empirically determined for other bacterial species.
2. Incubate at 98 °C for 2 min.
3. Ramp down temperature to 37 °C over a span of 1 h in a PCR thermocycler.
4. Incubate at 37 °C for 20 min.
5. Prepare 25 μL of MyOne streptavidin C1 dynabeads using magnetic stand.
6. Wash dynabeads three times with 25 μL of 1× B&W buffer.
7. Resuspend in 10 μL of 2× B&W buffer.
8. Incubate dynabeads at 37 °C until needed.

9. Add 10 μL of hybridization reaction to pre-washed dynabeads.
10. Incubate at 37 °C for 15 min.
11. Place in a magnetic stand and recover supernatant.
12. Precipitate DNA by adding 0.48 mL of 10 mM Tris, pH 8, 32 μL of 5 M NaCl, 1 μL of 0.5 M EDTA, 2 μL of GlycoBlue coprecipitant, and 0.55 mL of 100% isopropanol. Mix by vortexing and incubate at −80 °C for 30 min or longer.
13. Pellet at 20,000 × *g* or top speed in a microcentrifuge for 30 min at 4 °C.
14. Wash pellet twice with 0.5 mL of ice-cold 80% ethanol.
15. Dry pellet for 5 min in a chemical fume hood.
16. Resuspend in 5 μL of 10 mM Tris, pH 8.
17. Store at −20 °C [STOP POINT] or continue to next section.

3.12 Library Amplification by PCR (~2.5 h)

1. Prepare a master mix containing 16.7 μL of 5× HF buffer (supplied with enzyme), 1.7 μL of 10 mM dNTPs, 4 μL of 10 μM oCJC161, 4 μL of 10 μM indexing oligonucleotide, 52 μL of water, and 0.8 μL of HF Phusion polymerase.
2. Add 79.2 μL of PCR master mix to 5 μL of rRNA-subtracted circularized DNA and vortex.
3. Aliquot 16 μL into five separate PCR tubes.
4. Perform PCR reaction. Set initial denaturation at 98 °C for 30 s. Cycle 14 times using following conditions: 98 °C for 10 s, 60 °C for 10 s, 72 °C for 5 s.
5. Remove a tube after cycle 6, 8, 10, 12 and 14.
6. Add 3.5 μL of 6× DNA gel loading dye.
7. Set up an 8% TBE-polyacrylamide gel in 1× TBE.
8. Load samples and run at 180 volts for 40 min.
9. Stain gel with a 1:10,000 dilution of SYBR gold reagent in 1× TBE for 2 min.
10. Excise desired bands (Fig. 5).
11. To precipitate library, pierce an 18 gauge needle through a 0.5 mL tube. Insert gel slice in pierced tube and nest inside a 1.5 mL tube.
12. Spin down at 20,000 × g or top speed in a microcentrifuge for 2 min to crush gel.
13. Add 0.5 mL of 10 mM Tris, pH 8. Incubate at −20 °C for 30 min.
14. Incubate at 70 °C for 10 min with shaking. Transfer gel slurry to a Spin-X cellulose acetate column using a wide-bore pipette.

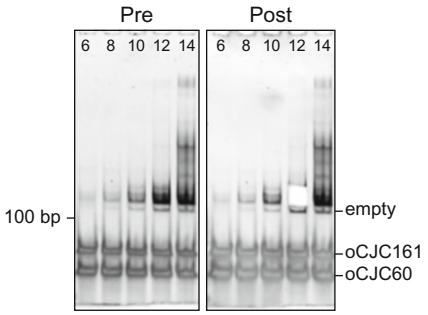

Fig. 5 Gel analysis of PCR amplification reaction. Circularized ssDNA was directly amplified without rRNA subtraction. PCR reactions were removed after the indicated cycles and resolved on an 8% TBE-polyacrylamide gel. Left panel indicates gel before excision of bands (pre), while right panel indicates gel following excision of bands (post)

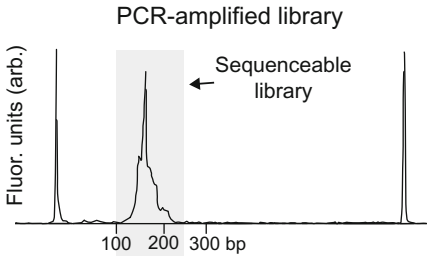

Fig. 6 High sensitivity small DNA fragment analyzer trace of amplified DNA library

15. Spin at 20,000 × *g* or top speed for 1 min. Transfer flow through to a fresh tube.
16. Add 32 μL of 5 M NaCl, 1 μL of 0.5 M EDTA, 2 μL of GlycoBlue coprecipitant, and 0.55 mL of 100% isopropanol. Mix by vortexing and incubate at −80 °C for 30 min or longer.
17. Pellet at 20,000 × *g* or top speed for 30 min at 4 °C.
18. Wash pellet twice with 0.5 mL of ice-cold 80% ethanol.
19. Dry pellet for 5 min in a chemical fume hood.
20. Resuspend pellet in 10 μL of 10 mM Tris, pH 8.
21. Store library at −20 °C [STOP POINT] or continue to next section.

3.13 Quantify, Sequence, and Alignment

1. Quantify library using a fragment analyzer (Fig. 6). The library should be between ~140 and 180 bp in length. Concentrations will vary based on cycling times and gel extraction efficiencies but should be greater than 2 nM. If more is needed, repeat PCR with additional cycling times or use more rRNA-subtracted circularized DNA as input.

2. Sequence using an Illumina sequencing platform.
3. Trim reads with cutadapt version 1.16.

```
cutadapt -m 15 -u 1 -a GATCGGAAGAGCACACGT -o $output_name
$input_fastq_file
```

4. Remove rRNA sequences and align with bowtie v.1.2.2. Use aligned reads for quantifying translation rates.

```
bowtie rRNA_sequences -v 2 -m 1 $input_fastq_file >
$output_aligned2rRNA -un %output_unaligned
```

```
bowtie reference_genome -v 2 -m 1 $output_unaligned >
$output_aligned2genome
```

5. Quantifying translation rates. Translation rates can be measured using various metrics [29, 30]. Studies on ribosome pausing (i.e., local translation) require a more refined analysis [16] when compared with quantifying bulk (or gene level) translation rates. Briefly, to measure gene level translation rates, the sum of aligned reads to an open reading frame (ORF) should be normalized to the length of the ORF and the total number of reads aligned to all ORFs. This gives the units reads per kilobase million (RPKM) for each gene. To account for variability in read length (~15–45 nucleotides), footprints can be trimmed from each side by a constant length (e.g., 7 nucleotides), with the remaining nucleotides given a score of 1/N (where N is the number of positions leftover after discarding each end).
6. Quality control metrics. To assess the extent of ribosome run-off, perform a metagene analysis. Generate a ribosome density profile for each ORF and scale each position by the mean density for that ORF. Exclude genes with less than 128 aligned reads. This gives differentially expressed genes equal weighting. Align each normalized ribosome density profile from the start codon and average across each position. If the average ribosome density is lower near the beginning of the message compared to the middle, then there is ribosome runoff. Nutrient deprivation and delays in cell harvesting are major causes of this. To overcome these issues, cells should be harvested during early-log to log-phase growth, and cell harvesting must be performed as quickly as possible.

4 Notes

1. Cell pulverization has been optimized using the Retsch MM301 mixer mill. If a different system is used, cell pulverization should be reoptimized such that cells are fully lysed while polyribosome complexes remain mostly intact.
2. This protocol uses DNase I, RNase-free (Roche, catalog no. 04716728001). It is essential to use RNase-free DNase I to preserve the integrity of polyribosome complexes. While RNase-free DNase I from other sources may be suitable for use, this may require further optimization, as each company uses non-standardized unit definitions for enzymatic activity.
3. For MICROBExpress bacterial mRNA enrichment and MEGAclear clean-up kits, follow manufacturer's instructions. Perform standard isopropanol precipitations to concentrate.
4. This protocol utilizes MNase from Sigma, catalog no. 10107921001. The source of MNase has been specifically optimized for this protocol. I have found that MNase from different sources show drastically differing activities (unpublished results).
5. Superase•In is a propriety RNase inhibitor that has been specifically validated to inhibit RNase I, RNase A, but not MNase (unpublished results).
6. While other gradient makers and fraction collectors can be employed, this protocol describes sucrose gradients made and collected using the BIOCOMP Gradient Master and Piston Gradient Fractionator. It is essential to use the open-top polyclear tubes from SETON when using the BIOCOMP Piston Gradient Fractionator, as the gaskets seal poorly with other brands.
7. Only the indicated reagents and enzymes have been used. While other sources might be compatible, these should be independently tested.
8. Special codes for oligonucleotides: /5rApp/, 5′ adenylation; /3ddC/, 3′ dideoxycytidinylation; /5Phos/, 5′ phosphorylation; /iSp18/, an 18-atom hexa-ethyleneglycol spacer; /5Biosg/, 5′ biotinylation, /3phos/, 3′ phosphorylation.
9. It is strongly advised to avoid centrifugation as a method of harvesting, as cells harvested by centrifugation versus rapid filtration show measurably different ribosome occupancy profiles [25]. Updated protocols even recommend directly freezing the liquid culture as a harvesting method, particularly for evaluating ribosome pause sites [16].

10. Purifying and sequencing mRNAs are needed for translation efficiency measurements, particularly when comparing genes encoded on distinct operons. For mRNA enrichment, save one-third of the pulverized cell powder, purify total RNA by acid phenol extraction, and prepare sequencing library.
11. The oligonucleotide oNTI199 AUGUACACGGAGUC GACCCGCAACGCGA/3phos/ (*see* **Note 8**) is often used as a control to gauge reaction efficiency.
12. 10 bp DNA ladder commonly used in most ribosome profiling protocols is now discontinued. As a replacement, other DNA ladders can be readily substituted, or specific oligonucleotides of defined lengths can be synthesized and used as a sizing standard.
13. All gel extraction steps can be replaced by an automated size selection approach (Pippin Prep, Sage Science). However, this requires a dedicated machine and may not be cost effective for some laboratories.

Acknowledgments

I thank Emily Powers and Gloria Brar for use of reagents and equipment.

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