HHS Public Access

Author manuscript

Methods Mol Biol. Author manuscript; available in PMC 2021 December 15.

Published in final edited form as:

Methods Mol Biol. 2021 ; 2252: 27–55. doi:10.1007/978-1-0716-1150-0_2.

Identification of translation start sites in bacterial genomes

Sezen Meydan[1], **Dorota Klepacki**[2], **Alexander S. Mankin**[2], **Nora Vázquez-Laslop**[2]

[1]National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD, 20892, USA

[2]Center for Biomolecular Sciences, University of Illinois at Chicago, Chicago, IL, 60607, USA

Abstract

The knowledge of translation start sites is crucial for annotation of genes in bacterial genomes. However, systematic mapping of start codons in bacterial genes has mainly relied on predictions based on protein conservation and mRNA sequence features which, although useful, are not always accurate. We recently found that the pleuromutilin antibiotic retapamulin (RET) is a specific inhibitor of translation initiation that traps ribosomes specifically at start codons and we used it in combination with ribosome profiling to map start codons in the *Escherichia coli* genome. This genome-wide strategy, that was named Ribo-RET, not only verifies the position of start codons in already annotated genes but also enables identification of previously unannotated open reading frames and reveals the presence of internal start sites within genes. Here, we provide a detailed Ribo-RET protocol for *E. coli*. Ribo-RET can be adapted for mapping the start codons of the protein coding sequences in a variety of bacterial species.

Keywords

Ribosome profiling; Ribo-seq; Translation initiation; Start codons; Bacterial translation; Retapamulin; Pleuromutilin; Alternative proteome

1 Introduction

Translation initiation in bacteria ensues with the small (30S) ribosomal subunit recognizing a start codon of the protein coding region in mRNA [1]. The recognition of a translation initiation site (TIS) and recruitment of the initiator formylmethionyl-tRNA^{Met} (fMet-tRNA) to the P site of the 30S subunit are assisted by the three initiation factors IF1, IF2, and IF3 . Binding of the fMet-tRNA at the initiation codon, defines the starting point of translation and sets up the reading frame. Upon dissociation of the IFs, and binding of the large (50S) ribosomal subunit 70S initiation complex is formed at the start codon. Accommodation of the first elongator tRNA into the ribosomal A site, catalysis of the first peptide bond, and subsequent translocation of the ribosome to the second codon denotes the transition to the elongation step of translation [2,3].

*Corresponding authors: Alexander Mankin: shura@uic.edu; Nora Vázquez-Laslop: nvazquez@uic.edu.

AUG and GUG are the most commonly used start codons in the bacterial genes. However, some other triplets, such as UUG, CUG, AUU and AUC, which can be decoded by the initiator fMet-tRNA can be also employed with varying efficiency for translation initiation [4]. In most of the bacterial genomes, the start codons of the ORFs are often preceded by a Shine-Dalgarno (SD) sequence (with the consensus GGAGG), which is fully or partially complementary to a stretch of nucleotides at the 3' end of the 16S rRNA of the 30S subunit [5]. The presence of a SD sequence, however, is not a prerequisite for the start codon recognition or for efficient translation initiation [6-9] and it may play only a supportive role [10,11]. Accessibility of the TIS to the ribosome, which depends on mRNA folding, is another important factor that affects initiation [12,6,13,14]. Additional, yet still poorly understood, features help the ribosome to find and distinguish the start codon from similar ones within or outside of the ORFs [15,16].

Accurate identification of TISs is crucial for correct annotation of the genomes, mapping the boundaries of the ORFs and gaining comprehensive information about the proteome. Several proteomics approaches based on identifying the N-terminal peptides of the proteins have been developed for experimental mapping of TISs in bacteria [17-19]. The best-suited proteomics technique for identifying the authentic N-terminal peptides relies on treating bacteria with actinonin, whose ability to inhibit peptide deformylase leads to retention of the formyl-methionine [17,18]. Alternative approaches based on bottom-up mass spectrometry also hold potential for revealing TISs of unannotated ORFs [19]. These proteomics techniques, however, are limited by the size and abundance of the proteins and may not always report the authentic TIS of a gene or detect the presence of alternative start sites.

Computational approaches have also been widely employed for identifying boundaries of bacterial ORFs. Various algorithms that analyze codon usage, biases in nucleotide periodicity, the presence of known TIS signatures or the conservation of the encoded proteins can relatively accurately predict the start codons of many genes [20-26]. However, these approaches often fail to distinguish between closely spaced putative start codons and are poorly applicable for identifying small ORFs [27,28]. Furthermore, identification of alternative TISs that are utilized for expression of more than one protein product from a single coding sequence, or detecting in-frame or out-of-frame ORFs within ORFs [29,30] represent a formidable problem for the available computational techniques.

More sophisticated analysis of TISs has been carried out using ribosome profiling (Ribo-Seq), a genome-wide technique employing deep-sequencing of ribosome protected mRNA fragments. Ribo-Seq shows the distribution of ribosomes along the translated mRNAs [31]. Ribo-Seq, in combination with computational algorithms and proteomics, has been utilized to map TISs, re-annotate prokaryotic genomes and detect N-terminal protein extensions in various bacterial species [32-34]. Nevertheless, the direct use of Ribo-Seq for mapping ORF boundaries faces specific challenges. The mRNA coverage and resolution of the Ribo-Seq data near start codons vary between different genes. Furthermore, direct Ribo-Seq is poorly applicable for mapping internal TISs (iTISs) located within the coding regions because the footprints originated from elongating ribosome may obscure the footprints that come from the ribosomes engaged at an iTIS (Fig. 1). One solution to circumvent these limitations is to specifically capture ribosomes at start codons by arresting them at the translation initiation

step while clearing the mRNAs from the elongating ribosomes. Several such approaches have been carried out for eukaryotic systems [35-38]. When Ribo-Seq was performed with bacteria treated with the translation inhibitor tetracycline, it was noted that high peaks of ribosome density accumulated specifically at the start codons of the genes [39]. However, interpretation of the tetracycline-based Ribo-Seq data is not straightforward because this antibiotic can bind to elongating ribosomes. To be able to more reliably map translation start sites in bacteria, we used the bacterial translation initiation inhibitor retapamulin (RET) to develop RET-assisted Ribo-Seq or Ribo-RET [30]. RET binds to the ribosomal peptidyl transferase center, where it overlaps with both the A and P sites [40-42]. RET can readily bind to the initiating ribosome that carries fMet-tRNA in its P site but its association with the elongating ribosome is barred due to the steric clash with the growing protein chain. Bound to the initiating ribosome, RET precludes placing of the aminoacyl-tRNA in the A site. As the result, it blocks the first peptide bond formation and arrests the ribosome at the start codon [30]. The use of Ribo-RET allowed for comprehensive mapping of TISs throughout the *E. coli* genome, revealing numerous unannotated genes outside of the coding regions [27] and identifying many unknown iTISs [30].

Ribo-RET consists of three main steps: 1) Optimizing the RET treatment of bacterial cells in order to ensure high enrichment of translating ribosomes arrested at the start codons of the mRNAs (Fig. 1). 2) Applying the Ribo-Seq protocol to prepare Next Generation Sequencing (NGS)-compatible cDNA libraries of the ribosomal footprints from untreated and RET-treated cells (Fig. 2). 3) Mapping the ribosomal footprints to the genome and identifying start sites.

Because Ribo-RET relies on a brief treatment of bacterial cells with RET, the minimum inhibitory concentration of RET (MIC_{RET}) should be first estimated, as it can greatly vary between different bacterial strains [43]. RET is highly active against many Gram-positive bacteria but shows relatively low activity against Gram-negative bacterial species. Genetic manipulation of the target strain, for example, inactivation of the drug efflux pumps (e.g. TolC in *E. coli*) or increasing permeability of the outer membrane may be required to achieve sufficient level of translation inhibition by RET. The procedures described here were optimized for the RET-hypersusceptible *E. coli* strain BL21 Δ*tolC* for which MIC_{RET} is 0.06-0.12 μg/mL [30]. Newer antibiotics of the same class, e.g. lefamulin [44], are reported to have higher activity and potentially could be used directly against some bacteria that are naturally resistant to retapamulin. The subsequent Ribo-Seq steps have been largely adapted from published procedures [45,46] and minimally modified using our own experience.

2. Materials

2.1 Metabolic labeling with RET

1. Growth medium M9AA-minus-Met: M9 minimal medium containing 19 amino acids (all natural amino acids except methionine), at a final concentration of 40 μg/mL each, supplemented with 3 μM thiamine (added right before use from the thiamine stock solution).

2. Thiamine stock solution: Dissolve thiamine in ddH_2O to the concentration of 3 mM and store at −20°C.
3. Retapamulin: Dissolve in 100 % ethanol to the concentration of 10 mg/mL and store at −20°C.
4. [^{35}S]-L-Methionine with specific activity of ~1,000 Ci/mmol at ~ 10 mCi/mL (*see* Note 1).
5. Trichloroacetic acid (TCA): Prepare 100% (weight/volume) TCA solution by adding 22.7 ml of ddH_2O to 50 g TCA, then dilute to a final concentration of 5% with ddH_2O and store it at 4°C.
6. Filter discs: Whatman Grade 3 MM Chr Cellulose 0.34 mm thick, ⊘ 2.5 cm circle discs.
7. Acetone: 99.9% ACS reagent grade.
8. Scintillation vials, scintillation cocktail, and scintillation counter.

2.2 Preparation of cell lysates for Ribo-RET

1. MOPS growth medium: prepare from MOPS EZ Rich Defined Medium kit (*see* Note 2).
2. Retapamulin: prepare stock solution (*see* #3 of Section 2.1).
3. Glass filtration system capable of accommodating ⊘ 90 mm filters, connected to a vacuum pump.
4. Filter discs: Millipore Express PLUS Membrane hydrophilic polyethersulfone filters, ⊘ 90 mm, 0.22 μm pore size.
5. Liquid nitrogen.
6. Stainless-steel Scoopula spatulas (scoopulas) ~ 15 cm long; container tubes compatible with holding liquid nitrogen and deep enough to encase 90% of the length of the scoopula; 50 mL conical tubes whose lids have been pierced 5-8 times with a 20-gauge needle.
7. Lysis buffer: 20 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 100 mM NH_4Cl, 5 mM $CaCl_2$, 0.4% Triton X-100, 0.1% NP-40. Store at −20°C.
8. DNase I (RNase free), 10 U/μL.
9. GMPPNP: Dissolve in ddH_2O to the concentration of 100 mM and store at −20°C.
10. SUPERase_In RNase inhibitor, 20 U/μL.

2.3 Pulverization of cells

1. Mixer mill, 10 mL jar, 12 mm diameter grinding ball.
2. Spatulas, 50 mL conical tubes with pierced lid (*see* #6 of Section 2.2).

3. Liquid nitrogen.

2.4 Preparation of cell lysate and of ribosome protected mRNAs fragments

1. Tris solution: 10 mM Tris-HCl, pH 7.0.
2. SUPERase_In, 20 U/μL.
3. Micrococcal nuclease (MNase) solution: take MNase from the original vial and dilute it with 10 mM Tris pH 8.0 to 75 U/μL (*see* Note 3). Store at −80°C.
4. EGTA solution: 0.5 mM EGTA at pH 8.0.
5. Nanodrop spectrophotometer.

2.5 Monosome isolation by sucrose gradient centrifugation

1. Gradient buffer: 20 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 100 mM NH_4Cl, prepared with DEPC-treated ddH_2O.
2. Sucrose solutions: 10% and 40% (weight/volume) solutions prepared in 20 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 100 mM NH_4Cl, prepared with DEPC-treated ddH_2O. Store at 4°C.
3. Beckman SW41 rotor and open-top polyclear ultracentrifuge tubes.
4. Sucrose gradient maker.
5. Gradient fractionation system.

2.6 Phenol-chloroform extraction of ribosome protected mRNAs fragments

1. SDS solution: 20% (weight/volume) SDS dissolved in RNase free ddH_2O.
2. Acidic phenol solution: phenol:chloroform:isoamyl alcohol 125:24:1 at pH 4.5, molecular biology grade.
3. Chloroform, molecular biology grade.
4. 3 M NaOAc, pH 5.5 prepared in RNase free ddH_2O.
5. Isopropanol, molecular biology grade.
6. 80% ethanol, ice cold.
7. Tris solution: 10 mM Tris-HCl pH 7.0.

2.7 Size selection of ribosome protected mRNAs fragments

1. Tris solution: 10 mM Tris-HCl pH 7.0.
2. Novex 2X TBE-Urea Sample Buffer.
3. Control RNA oligonucleotides solutions: from 100 μM stock solutions, dilute each RNA oligonucleotide to 20 μM in DEPC-treated ddH_2O and store at −20°C. The names and sequences of the control RNA oligos are as follows:

   o15, 5'_AUGUACACGGAGUCG_3'

o28, 5’_AUGUACACGGAGUCGACCCGCAACGCGA_3’

o45, 5’_AUGUACACGGAGUCGACCCGCAACGCGAUGUACACGGAGUCGAC_3’

4. 15% TBE-Urea gel: 15% denaturing polyacrylamide TBE-Urea gel, 8 cm x 8 cm, 1 mm thick.
5. 10X TBE running buffer.
6. SYBR Gold.
7. RNA elution buffer: 300 mM NaOAc pH 5.5, 1 mM EDTA pH 8.0. Store at room temperature.
8. Superase_In, 20 U/μL
9. Spin-X columns: Spin-X centrifuge tube filters with cellulose acetate membrane, pore size 0.22 μm.
10. Glycoblue.

2.8 Dephosphorylation

1. T4 polynucleotide kinase (PNK), 10 U/μL.
2. SUPERase_In, 20 U/μL.
3. Tris solution: 10 mM Tris-HCl pH 7.0.
4. 3 M NaOAc, pH 5.5.
5. Glycoblue.

2.9 Enzymatic pre-adenylation of linkers and linker ligation

1. 5′ DNA Adenylation Kit: Mth RNA ligase.
2. Linker oligonucleotides [46] (linker-specific bar code is underlined)

   NI-810, 5’Phos/NNNNN<u>ATCGT</u>AGATCGGAAGAGCACACGTCTGAA/3’ddC

   NI-811, 5’Phos/NNNNN<u>AGCTA</u>AGATCGGAAGAGCACACGTCTGAA/3’ddC

   NI-812, 5’Phos/NNNNN<u>CGTAA</u>AGATCGGAAGAGCACACGTCTGAA/3’ddC

   NI-813, 5’Phos/NNNNN<u>CTAGA</u>AGATCGGAAGAGCACACGTCTGAA/3’ddC

   NI-814, 5’Phos/NNNNN<u>GATCA</u>AGATCGGAAGAGCACACGTCTGAA/3’ddC

   NI-815, 5’Phos/NNNNN<u>GCATA</u>AGATCGGAAGAGCACACGTCTGAA/3’ddC

   NI-816, 5’Phos/NNNNN<u>TAGAC</u>AGATCGGAAGAGCACACGTCTGAA/3’ddC

   NI-817, 5’Phos/NNNNN<u>TCTAG</u>AGATCGGAAGAGCACACGTCTGAA/3’ddC
3. Oligo Clean & Concentrator kit.
4. T4 RNA ligase kit: T4 RNA ligase 2, truncated K227Q.

5. 5’ deadenylase, 50 U/μL.
6. $RecJ_f$ DNA exonuclease, 30 U/μL.

2.10 Reverse transcription

1. dNTP mix: a mix of dATP, dCTP, dGTP, and dTTP each at 10 mM.
2. NI-802 DNA oligo: dilute with ddH_2O to 25 μM from a 100 μM stock solution of NI-802 DNA oligo [46] and store at −20°C.

   NI-802, 5’Phos/NNAGATCGGAAGAGCGTCGTGTAGGGAAAGAG/iSp18/GTGACTGGAGTTCAGACGTGTGCTC
3. Reverse transcriptase (RT) kit: SuperScript III Reverse Transcriptase, 200 U/μL.
4. SUPERase_In, 20 U/μL.
5. NaOH solution: 1 M NaOH.
6. Oligo Clean & Concentrator kit.
7. Tris solution: 10 mM Tris-HCl, pH 8.0.
8. 10% TBE-Urea gel: 10% denaturing polyacrylamide TBE-Urea gel, 8 cm x 8 cm, 1 mm thick.
9. 10X TBE running buffer.
10. SYBR Gold.
11. 20-gauge needles.
12. DNA Elution Buffer. To prepare 50 mL, mix the following:

    5 M NaCl 3 mL

    0.5 M EDTA pH 8.0 100 μL

    1 M Tris-HCl pH 8.0 500 μL

    DEPC-treated ddH_2O 46.4 mL

    Store at room temperature.
13. Spin-X columns: Spin-X centrifuge tube filters with cellulose acetate membrane, pore size 0.22 μm.
14. Glycoblue.

2.11 Circularization

1. CircLigase ssDNA Ligase, 100 U/μL.

2.12 PCR amplification

1. Phusion High Fidelity DNA polymerase, 2000 U/mL.
2. 10 mM dNTP mix.

3. PCR primers. Prepare 100 μM stock solutions and store at −20°C. The DNA primer pairs are as follows (Illumina unique dual indexes (UDI) are underlined):

AM-i51,
5’_AATGATACGGCGACCACCGAGATCTACACAGCGCTAGACACTCTTTCCCTACACGACGCTC_3’

AM-i71,
5’_CAAGCAGAAGACGGCATACGAGATAACCGCGGGTGACTGGATTCAGACGTGTG_3’

AM-i52,
5’_AATGATACGGCGACCACCGAGATCTACACGATATCGAACACTCTTTCCCTACACGACGCTC_3’

AM-i72,
5’_CAAGCAGAAGACGGCATACGAGATGGTTATAAGTGACTGGAGTTCAGACGTGTG_3’

AM-i53,
5’_AATGATACGGCGACCACCGAGATCTACACCGCAGACGACACTCTTTCCCTACACGACGCTC_3’

AM-i73,
5’_CAAGCAGAAGACGGCATACGAGATCCAAGTCCGTGACTGGAGTTCAGACGTGTG_3’

AM-i54,
5’_AATGATACGGCGACCACCGAGATCTACACTATGAGTAACACTCTTTCCCTACACGACGCTC_3’

AM-i74,
5’_CAAGCAGAAGACGGCATACGAGATTTGGACTTGTGACTGGAGTTCAGACGTGTG_3’

AM-i55,
5’_AATGATACGGCGACCACCGAGATCTACACAGGTGCGTACACTCTTTCCCTACACGACGCTC_3’

AM-i75,
5’_CAAGCAGAAGACGGCATACGAGATCAGTGGATGTGACTGGAGTTCAGACGTGTG_3’

AM-i56,
5’_AATGATACGGCGACCACCGAGATCTACACGAACATACACACTCTTTCCCTACACGACGCTC_3’

AM-i76,
5’_CAAGCAGAAGACGGCATACGAGATTGACAAGCGTGACTGGAGTTCAGACGTGTG_3’

4. 6X DNA loading dye.

5. 10 bp DNA ladder, ready-to-use 10-150 bp
6. 8% TBE gel, 8 x 8 cm, 1 mm thick.
7. 1X TBE buffer.
8. SYBR gold.
9. Tris solution: 10 mM Tris-HCl, pH 8.0.
10. DNA Elution Buffer, described in point #12 of Section 2.10.

3. Methods

3.1 Optimization of RET treatment

The goal of this experiment is to optimize the treatment of the bacterial cells with RET to achieve complete inhibition of protein synthesis (as estimated by incorporation of [^{35}S]-Met into polypeptides) in a short period of time (2-5 min) (Fig. 3) (*see* Notes 4-5).

1. Grow an overnight cell culture at 37°C in an incubator shaker in M9AA-minus-Met medium freshly supplemented with thiamine.

   Steps 2-9 are optimally carried out in a 37°C temperature room:

2. Dilute the overnight culture 1:200 into 5 mL of M9AA-minus-Met medium freshly supplemented with thiamine and grow it with constant shaking until it reaches an A_{600} ~0.2. While the culture is growing prepare the materials described in steps 3-6 (*see* Note 1).
3. Dilute 1 μL (~ 10 μCi) of [^{35}S]-Met into 65 μL of M9AA-minus-Met freshly supplemented with thiamine. Prepare a set of 1.5 mL Eppendorf tubes labeled with the pre-chosen time points for the RET treatment of the cell culture (for example, 0, 1, 2.5, 5 and 10 min). Pipette 2 μL of the diluted [^{35}S]-Met into each of the tubes.
4. Prepare a glass beaker with 500 mL of the 5% TCA (cover the beaker with aluminum foil to minimize exposure to the TCA fumes).
5. Label a set of filter discs with pencil (as the discs are going to be submerged in the TCA solution and solvent based labels would be washed out) and place them in a shallow container, e.g. a Petri dish. Pre-soak each disc with ~25 μL of TCA solution and let them air-dry for 1-2 min.
6. Prepare a control sample for non-specific binding of [^{35}S]-Met to the filter: Add 28 μL of 5% TCA and to one of the tubes with 2 μL of the diluted [^{35}S]-Met (Step 4). Pipette 25 μL of this mixture on one of the filter discs from step 5. Immediately place the disc in the beaker with 5% TCA.
7. Prepare the sample for 0-time RET treatment: Place a 28 μL aliquot of the culture at A_{600} ~0.2 (Step 2) into one of the tubes with 2 μL of the diluted [^{35}S]-Met (Step 3). Incubate for 1 min and then pipette 25 μL of this mixture

onto one of the filter discs. Immediately place the disc in the beaker with 5% TCA.

8. Prepare the samples for the time course of RET treatment: pipette 350 μL aliquot of the culture at A_{600} ~0.2 (Step 2) into an Eppendorf tube. Add RET stock solution to reach a concentration 50-100X the MIC_{RET} (5 μg/mL for *E. coli* BL21 Δ*tolC*), vortex and immediately start the timer.

9. At the required times, transfer 28 μL to the tubes with 2 μL of the diluted [^{35}S]-Met (Step 3). Incubate for 1 min and then pipette 25 μL of this mixture on one of the filter discs (Step 4). Immediately place the disc in the beaker with 5% TCA.

10. Once all the sample-containing filter discs are in the beaker, bring its content to boil under a fume hood and keep boiling for 5 min. Decant and discard the TCA, being careful to keep the filter discs inside the beaker. Add a fresh batch of 500 mL of 5% TCA. Boil for additional 5 min under a fume hood and discard the TCA solution. Add ~100 mL of acetone to the beaker, swirl it for ~3 min. Dry the filter discs for ~5 min under a fume hood.

11. Place the discs into scintillation vials, add scintillation liquid. Measure radioactivity in a scintillation counter.

12. Plot [^{35}S]-Met incorporation in the RET-treated samples relative to the 100% incorporation control sample (Step 7) (Fig. 2). For the RiboRet procedure, choose the shortest RET treatment time where maximum protein synthesis inhibition was achieved (Fig. 3).

3.2 Collection of cells treated with RET

The aim of this procedure is to collect bacterial cells enriched with ribosomes stalled at initiation codons by the action of RET. Cells are exposed to RET under conditions optimized in Section 3.1. A control culture devoid of RET treatment is recommended to be processed in parallel.

1. Grow an overnight culture in MOPS medium (*see* Note 6) at 37°C in an incubator shaker.

   Steps 2-4 are optimally carried out in a 37°C temperature room. The scoopulas for scraping cells and the filtration apparatus connected to a vacuum line should be placed in the 37°C room at least 30 min before cell harvesting takes place.

2. Dilute the overnight culture from step 1 to A_{600} ~ 0.05 in MOPS media pre-warmed to 37°C. Grow the diluted culture, shaking, until it reaches an A_{600} ~0.3 (*see* Notes 6-8).

3. Add RET to the cell culture according to the antibiotic concentration and time of exposure determined by the metabolic labeling experiments described in Section 3.1 (*see* Note 4) and continue shaking.

4. Filter the cells as rapidly as possible and immediately scrape them off the filter using a scoopula (*see* Notes 6-8). Quickly submerge the scoopula with the cell pellet in the container tube filled with liquid nitrogen.

5. Using another scoopula pre-chilled in liquid nitrogen, dislodge the frozen cells in a 50-mL conical tube (with pierced lid) containing ~10 mL of liquid nitrogen. Cell pellets can be stored at −80°C (liquid nitrogen will evaporate through the lid holes). Otherwise, continue to Section 3.3.

3.3 Preparation of cell lysates

1. Freshly supplement 750 μL of lysis buffer with: 7.5 μL DNase I (RNase free), 22.5 μL GMPPNP, 12 μL Superase_In.

2. Take the 50 mL tubes with the frozen cell pellets (Section 3.2) and add ~10 mL of liquid nitrogen, re-filling to this volume with liquid nitrogen as needed. Slowly drip 650 μL of lysis buffer (step 1) in order to form small, discrete droplets.

3. Pre-chill the jar and grinding ball of the mixer mill in liquid nitrogen.

4. Take the frozen cell pellets and drops of frozen lysis buffer (care should be taken to keep them frozen at all times) and immediately transfer them into the pre-chilled jar with the grinding ball in it.

5. Lyse the frozen cells in the mixer mill by carrying 5 cycles of 3 min each at 15 Hz, re-chilling the jar in liquid nitrogen following each cycle.

6. Use a pre-chilled spatula to transfer the pulverized frozen cells into a 50-mL conical tube containing 10 mL of liquid nitrogen. Close with a pierced lid and either store at −80°C or proceed to Section 3.4.

3.4 Preparation of ribosome-protected mRNA fragments

Treatment of cell lysate with MNase results in degradation of the mRNA fragments not protected by stalled ribosomes and conversion of polysomes to monosomes (*see* Note 9).

1. Collect the lysates (Section 3.3) to the bottom of the tubes by briefly spinning in a 4°C centrifuge.

2. Thaw the lysates by placing the tubes for 2 min in a 30°C water bath for 20 min and transfer them to pre-chilled Eppendorf tubes.

3. Pellet insoluble debris at 20,000 x g for 10 min in a 4°C microfuge.

4. Carefully transfer supernatants to pre-chilled Eppendorf tubes.

5. Blank the Nanodrop spectrophotometer with an aliquot of lysis buffer (from step 1 of Section 3.3) diluted 1:100 with 10 mM Tris. Mix 2 μL of clarified lysate from step 5 with 198 μL of 10 mM Tris and measure A_{260}.

6. Determine the concentration (A_{260} units/μL) of the lysates.

7. Dilute 22 A_{260} units of lysate into a final volume of 220 μL of lysis buffer (step 1 of Section 3.3) (*see* Note 10).
8. Add 6 μL of SUPERase_In and 4.4 μL of the MNase solution (*see* Note 3). Incubate at 25°C for 1 hr with shaking at 1400 rpm in a thermomixer.
9. Quench the reactions by addition of 2 μL of EGTA solution and immediately place the tubes on ice.

3.5 Isolation of monosomes by sucrose gradient fractionation

1. Pre-chill the SW41 rotor and buckets to 4°C.
2. Prepare a 10-40% (weight/vol) sucrose gradients in centrifuge tubes for the SW41 rotor using a gradient maker.
3. Weigh the tubes to ensure proper balance. If further balancing is required, remove or add small volumes of the 10% sucrose solution.
4. Carefully pipette the MNase-treated lysates (~230 μL) and, if desired, MNase-free control samples (Fig. 4) (*see* Note 9) (Section 3.4) on top of the gradients.
5. Centrifugate at 190,000 xg (39,000 rpm) in SW41 rotor for 2 hrs at 4°C.
6. Fractionate the gradients using an automated fractionation system with continuous monitoring of A_{254} optical density.
7. Collect the fractions corresponding to the 70S monosome peak, whose final volume typically ranges between 1.8 to 2 mL.
8. Flash-freeze the monosome fractions in liquid nitrogen. Fractions can be stored at −80°C or can be immediately processed as described in Section 3.6.

3.6 Phenol-chloroform extraction of ribosome-protected mRNA fragments

1. To be able to perform the extraction in 1.5 mL Eppendorf tubes, divide the ~2 mL monosome fractions (Section 3.5) in aliquots of ~700 μL. In the following steps, we describe the extraction for one of the aliquots but all ~700 μL aliquots from the collected ~2-mL samples should be processed in parallel.
2. Add 40 μL of 20% SDS to the ~700 μL aliquot of monosome fraction.
3. Add 700 μL of acidic phenol pre-warmed to 65°C. Mix by brief vortexing and incubate at 65°C for 5 min with shaking at 1400 rpm in a thermomixer. Chill on ice for 5 minutes.
4. Spin at 20,000 x g for 2 min at room temperature in a microfuge and transfer the top aqueous phase to a fresh tube. Add 700 μL of room-temperature acidic phenol. Mix by brief vortexing and incubate with shaking at room temperature for 5 min.
5. Spin at 20,000 x g for 2 min at room temperature and transfer the top aqueous phase to a fresh tube. Add 600 μL chloroform and mix by vortexing.

6. Spin at 20,000 x g for 1 min at room temperature and transfer the top aqueous phase to a fresh tube. Add 75 μL of 3 M NaOAc, pH 5.5 and mix. Add 800 μL of 100% isopropanol and briefly vortex. Chill at −80°C for 30 min.

7. Pellet the extracted RNA at 20,000 x g for 1 hr at 4°C in a microfuge. Wash pellet with 800 μL of ice-cold 80% ethanol. Air-dry pellet for 5 min.

8. Use a total of 20 μl of Tris solution to resuspend all RNA pellets originated from the same monosome fraction. Snap-freeze and store at −80°C or proceed to Section 3.7.

3.7 Size selection of ribosome-protected mRNA fragments

1. To quantify the concentration of RNA fragments, dilute 1 μL of RNA (Section 3.6) with 9 μL of Tris solution and estimate the concentration in a Nanodrop spectrophotometer. Optical density of 1 A_{260} equals ~ 40 μg/ml of RNA.

2. Prepare RNA for the sizing electrophoresis by placing 28 μg RNA into a final volume of 10 μL of Tris solution. Add 10 μL of 2X TBE-Urea sample buffer.

3. Prepare three individual samples of control RNA oligos for electrophoresis by combining 2 μL of the 20 μM oligo solutions (Section 2.7) with 3 μL of Tris solution and 5 μL of 2X TBE-Urea sample buffer (*see* Note 11).

4. Pre-run the 15% TBE-Urea gel for 1 hr at 200 V.

5. Denature samples from steps 2 and 3 by incubating them at 80°C for 2 min and immediately place them on ice.

6. Wash the wells of the gel and load the RNA oligo controls (step 3) into individual lanes. To avoid overloading the lane, we recommend splitting the 20 μL RNA samples (step 2) into two 10 μL aliquots and loading them into two adjacent lanes. Run the gel at 200 V for 65 min. Stain the gel with 5 μL of SYBR gold in 50 mL of 1X TBE for 5 min.

7. Excise desired bands whose sizes range between ~17-42 nt (Fig. 5). In parallel, excise the three control RNA oligos. Note that all the subsequent steps up to step 13 of Section 3.12, described here for the ribosome-protected mRNA fragments, are carried out also with the control RNA oligos (*see* Note 11).

8. Recover the size selected RNA fragments as follows. Place the gel slices in a 0.5 mL PCR tube whose bottom has been pierced with a 20-gauge needle. Nest the tube into a 2 mL collection tube. Spin at 20,000 x *g* for 2 min or until most of the gel has extruded into the collection tube.

9. Add to the crushed gel pieces 500 μL of RNA elution buffer supplemented with 2.5 μL SUPERase_In. Shake overnight in a thermomixer at 1400 rpm at 4°C.

10. Spin samples for 10 sec in a microfuge and transfer the gel suspension to a Spin-X column using a wide bore pipette tip. Spin at 20,000 x g for 3 min. Transfer eluate to a fresh tube.

11. Perform a second elution step by adding 200 μL of RNA elution buffer preheated to 70°C to the Spin-X column. Shake in a thermomixer for 5 min at 1400 rpm at 70°C. Spin the column for 3 min at 20,000 x g and combine the eluate with that obtained in step 10.

12. Add to the extracted RNA 2 μL of Glycoblue and briefly vortex. Add 750 μL of 100% isopropanol and vortex. Chill at −80°C for 30 min.

13. Pellet RNA at 20,000 x g for 1 hr at 4°C in a microfuge. Aspirate supernatant. Wash the pellet by adding 800 μL of ice cold 80% ethanol, spinning the tubes for 30 sec at 20,000 x g in a microfuge, and aspirating the supernatants. Air-dry pellets for 5 min.

14. Resuspend the pellet in 15 μL of Tris solution.

3.8 Dephosphorylation of ribosome-protected mRNA fragments

1. Prepare buffer master mix. For a single reaction:

   10x T4 PNK buffer 2μL

   SUPERase_In 1 μL

2. Add 3 μL of buffer master mix to 15 μL of the RNA isolated in Section 3.7. Add 2 μL of T4 PNK to each tube. Incubate reaction at 37°C for 1 hr.

3. Heat inactive T4 PNK at 75°C for 10 min.

4. Prepare precipitation buffer master mix. For a single reaction:

   Tris solution 448 μL

   3M NaOAc, pH 5.5 50 μL

   Glycoblue 2 μL

5. Add 500 μL of precipitation buffer master mix to each tube. Precipitate dephosphorylated RNA by adding 600 μL of 100% isopropanol and mix by vortexing. Chill at −80°C for 30 min.

6. Pellet RNA at 20,000 x g for 1 hr at 4 °C in a microfuge. Aspirate supernatant and wash pellet with 800 μL of ice cold 80% ethanol as described in steps 12-13 of Section 3.7. Air-dry pellet for 5 min.

7. Resuspend the RNA pellet in 5 μL of Tris solution.

3.9 Enzymatic adenylation of the linkers and linker ligation

Adenylation of the linkers (*see* Note 12):

1. For each linker (Section 2.9), combine the following in a 0.2 mL PCR tube:

   Linker (100 μM) 1.2 μL

   5’ DNA adenylation 10x buffer 2 μL

   ATP (1 mM) 2 μL

ddH_2O 12.8 μL

Mth RNA Ligase 2 μL

2. Incubate at 65°C for 1 hr.
3. Heat inactive the Mth RNA Ligase at 85°C for 5 min.
4. Add 30 μL of ddH_2O to the sample and clean the sample using the Oligo Clean & Concentrator kit according to the manufacturer's instructions, except that the elution step is carried out with only 6 μL of ddH_2O. Store 5'-adenylated linkers at −20°C or proceed immediately with the ligation steps.

Linker ligation:

5. Prepare the ligation reaction. For a single reaction:

   50% PEG-8000 3.5 μL

   T4 RNA ligase 10x buffer 1 μL

   Pre-adenylated linker (20 μM) (steps 1-4) 0.5 μL

   T4 RNA ligase 0.5 μL
6. Add 5 μL of dephosphorylated RNA (Section 3.8) to the ligation reaction.
7. Incubate at 22°C for 3 hrs.
8. Add 0.5 μL of 5-deadenylase and 0.5 μL of 5-$RecJ_f$. Incubate at 30°C for 45 min.
9. Clean the ligation reaction using Oligo Clean & Concentrator kit. Elute with 10 μL ddH_2O. Store at −80°C or proceed to Section 3.10.

3.10 Reverse transcription

1. Prepare the RT oligo master mix. For a single reaction:

   10 mM dNTP 1 μL

   25 μM NI-802 1 μL

   ddH_2O 1.5 μL
2. Add 3.5 μL of RT oligo master mix to 10 μL of the ligated RNA (Section 3.9). Denature at 65°C for 5 min and chill it on ice for 5 min.
3. Prepare the RT buffer master mix. For a single reaction:

   5X FSB Buffer 4 μL

   0.1 M DTT 1 μL

   SUPERase_In 1 μL
4. Add 6 μL of the buffer master mix to the RNA from step 2. Add 1 μL of RT.
5. Incubate at 55°C for 30 min.

6. Quench the reaction by hydrolyzing RNA templates: Add 2.3 μL of NaOH solution and incubate at 95°C for 15 min. Note that this solution will turn pink in color.

7. Add 27.2 μL of ddH_2O to bring the reaction volume to 50 μL. Recover cDNA using Oligo & Clean Concentrator kit. Elute the purified cDNA in 8 μL of ddH_2O. Store at −80°C or proceed with gel electrophoresis.

8. Pre-run the 10%TBE-Urea gel in 1X TBE buffer for 1 hr at 200 V.

9. Prepare for electrophoresis the RT primer control:

   NI-802 primer diluted to 1.25 μM 2 μL

   Tris solution 6 μL

   2x TBE-Urea Sample Buffer 8 μL

10. Prepare the cDNA samples from step 7 for electrophoresis (as well as the cDNA samples generated using the processed control RNA oligos o15, o28, and o45; *see* Note 11) by adding 8 μL of 2x TBE-Urea sample buffer.

11. Denature the samples at 80°C for 2 min and chill them on ice. Load samples onto the wells of the pre-run gel and run electrophoresis at 200 V for 70 min (during this time the bromophenol blue dye may run out from the gel).

12. Stain the gel with 5 μL of SYBR gold in 50 mL of 1X TBE for 5 min and excise the desired bands (Fig. 6). In parallel, from the control RNA oligos, we recommend excising only the cDNA band corresponding to o15 (*see* Note 11).

13. Recover the size selected cDNA products as follows. Place the gel slices in a 0.5 mL tube whose bottom has been pierced with a 20-gauge needle. Nest the tube into a 2 mL tube. Spin at 20,000 x *g* for 2 min or until most of the gel has extruded into the collection tube.

14. Add 500 μL DNA elution buffer. Shake overnight in a thermomixer at 1000 rpm at 25°C.

15. Briefly spin samples and transfer the gel suspension to a Spin-X column using a wide bore pipette tip. Spin at 20,000 x g for 3 min. Transfer the eluate to a fresh tube and keep on ice.

16. Perform a second elution step by adding to the Spin-X column 200 μL of DNA elution buffer pre-heated to 70°C. Shake Spin-X column in a thermomixer for 5 min at 1400 rpm at 70°C. Spin at 20,000 x g for 3 min and combine the eluate with that obtained in step 15.

17. Add to the extracted cDNA 2 μL of Glycoblue and 750 μL of 100% isopropanol and vortex. Chill at −80°C for 30 min.

18. Pellet cDNA at 20,000 x g for 1 hr at 4°C in microfuge. Aspirate supernatant and wash the pellet with 800 μL of ice cold 80% ethanol as described in step 13 of Section 3.7. Air-dry cDNA pellet for 5 min.

19. Resuspend the pellet in 15 μL of Tris solution.

3.11 Circularization of cDNA

1. Prepare circularization master mix. For one reaction:

   10X CircLigase Buffer 2 μL

   1 mM ATP 1 μL

   50 mM $MnCl_2$ 1 μL

2. Add 4 μL of circularization master mix to 15 μL of cDNA samples (Section 3.10). Add 1 μL of CircLigase.

3. Incubate at 60°C for 1 hr.

4. Heat inactivate CircLigase by incubating the reaction at 80°C for 10 min. Chill on ice.

5. Clean the circularized cDNA (*see* Note 13) by adding 500 μL DNA elution buffer, 2 μL of Glycoblue and 600 μL of isopropanol. Incubate at 80°C for 30 min. Spin at 20,000 x g for 1 hr at 4°C in a microfuge. Aspirate supernatant and wash the pellet with ice cold 80% ethanol as described in step 14 of Section 3.7. Air dry the pellets for 5 minutes.

6. Resuspend the pellet in 10 μL of Tris solution.

3.12 PCR amplification

Pilot PCR:

1. Prepare the PCR amplification mix. For the following single reaction, we have used the primer pair AM-i51/AM-i71 (also see Fig. 2) as an example (*see* Notes 14-15):

   5X HF buffer 16.7 μL

   10 mM dNTP 1.7 μL

   100 μM AM-i51 primer 0.4 μL

   100 μM AM-i71 primer 0.4 μL

   ddH_2O 58.8 μL

   HF Phusion 0.8 μL

2. Add 4.5 μL of Tris solution to 0.5 μL of circularized cDNA (Section 3.11). Add 79.2 μL of PCR amplification mix and mix by vortexing.

3. Aliquot 17 μL of the PCR mix into four separate PCR tubes.

4. Set up 12 cycles of the following PCR program:

   Initial denaturation 30 sec at 98°C

   Denaturation 10 sec at 98°C

Annealing 10 sec at 65°C

Extension 5 sec at 72°C

5. Sequentially remove individual PCR tubes after 6, 8, 10, or 12 cycles and place on ice.

6. Add 3.5 μL of 6X DNA loading dye to each tube.

7. Prepare 10 bp DNA ladder:

   10 bp ladder 1 μL

   Tris solution 9 μL

   6X DNA loading dye 2 μL

8. Set up a 8% TBE gel in 1X TBE.

9. Load samples and run for 55 min at 180 V.

10. Stain the gel with 5 μL of SYBR gold in 50 mL of 1X TBE for 5 minutes and visualize.

11. Identify the number of PCR cycles that results in high yield of the DNA fragments with the target size range of 150-170 bp but the lack of high molecular weight products (Fig. 7).

12. Preparative PCR: Set up reactions for preparative PCR exactly as described in steps 1-3. Run the PCR reaction for the optimal number of cycles determined in step 11.

13. Run the samples in an 8% TBE gel as described in steps 6 and 9-10, along with the 10 bp DNA ladder (step 7).

14. Excise the desired bands (Fig. 7) and recover the double-stranded DNA products as described in Steps 13-18 of Section 3.10.

15. Resuspend the pellet in 10 μL of Tris solution.

3.13 Preparing samples for NGS

1. Quantify samples using 1 μL of the prepared DNA (Section 3.12) mixed with 2 μL of ddH_2O using Agilent TapeStation system or an equivalent platform.

2. Depending on the number of samples and sequencing platform to be used, samples can be sequenced individually or combined into a single sample. The latter is possible if different pairs of PCR primers listed in Section 2.12 were used for each sample in Section 3.12. We routinely combine 4 to 8 samples in the same tube so that the final amount of DNA is 10 nmol. This is enough for a single lane on Illumina HiSeq 4000 or NextSeq platforms (*see* Note 15). We usually request an SR75 or SR100 runs (single-end reading of 75 or 100 bases, respectively). In some instances, we have obtained sufficiently good results with SR50 runs (single-end 50 bases sequencing). We usually target to obtain 40 to 60 million raw reads per sample.

3.14 Computational processing of ribosome profiling reads

The data processing and analysis is performed using custom scripts and publicly available software packages available in the GALAXY platform [47]. Main steps of the recommended data processing flow are as follows:

1. Remove the adapter sequence from the raw sequencing reads using Cutadapt algorithm [48].
2. Align the processed reads to ribosomal and other non-coding RNA sequences using Bowtie algorithm [49] (parameters: -n 1 -l 20 -m 1) and discard them. Align remaining reads to genome using the same parameters.
3. Assign the 15th nucleotides upstream of the 3' end of each read as the first nucleotide of the P-site codon. Divide the number of reads assigned to each genomic position by the total number of mapped reads and then divide by 1,000,000. This normalization results in reads per million (RPM) value for every nucleotide and can be assembled in a Wiggle track format (Wig file). Wig files are used to visualize the Ribo-RET peaks in a genome browser (e.g. MochiView [50]) and to analyze genome-wide start sites using custom scripts (*see* Note 16).
4. Identify potential start sites by searching for a start codon (AUG, GUG, CUG, UUG, AUU, AUC) within 3 nucleotides upstream or downstream of the Ribo-RET peaks. For annotated start sites, we define "Ribo-RET peak" as any read in the Ribo-RET data that has density of >1 RPM. However, for different analysis, such as alternative TIS searching, we suggest using more stringent cut-off for Ribo-RET peak definition (*see* step 5).
5. If the start codon is within the annotated coding regions, we classify the corresponding site as in-frame or out-of-frame iTIS. If TIS identifies a start codon upstream of the genes, we classify it as 'N-terminal extension' or 'upstream TIS' if no stop codon is present between such TIS and the TIS of the annotated ORF. For the Ribo-RET peaks that are outside of the annotated ORFs and are out-of-frame relative to the first downstream ORF or that are in frame with the downstream ORF but a stop codon is present prior to the annotated start site, we consider them as potential TISs of the unannotated novel ORFs (Fig. 8). For pTIS classification, we only consider the peaks with minimum of 1 RPM. For alternative start sites, however, we use more stringent cut-off (RPM > 5) (*see* Note 17).

4 Notes

1. Proper practices for handling of radioactive material and waste should be followed for the procedures described in Section 3.1, steps 3-11.
2. We recommend to not autoclave or filter sterilize the complete MOPS medium as these procedures may deplete it from specific components [51].
3. Because of the heterogeneity in activity of different lots of MNase, we recommend to carry out the following steps prior to performing the RiboRET

experiments: i) pool together several different lots of the enzyme; ii) prepare a mock lysate from cells not treated with RET by following the procedure outlined in Sections 3.2 to 3.4; carry out the MNase treatment (steps 9 and 10 of Section 3.4) with different number of units of the enzyme (include an aliquot of lysate not treated with MNase); iii) assess the MNase-mediated disruption of polysomes by the fractionation procedure described in Section 3.5. Choose the amount of MNase that converts polysomes into monosomes without affecting the 70S monosome peak. Prepare, aliquot, and store the MNase stock solution (step 3 of Section 2.4) according to this optimization procedure.

4. As an alternative to radioactive metabolic labeling, cell-permeable methionine analogs such as 4-Azido-L-homoalanine (L-AHA) or L-Homopropargylglycine (HPG) can be used [52,53].

5. It is recommended to first optimize the concentration of RET by exposing cells for the same amount of time (e.g. 2 min) to different concentrations of the drug, and then carry out the time-course experiment described in Section 3.1 at the chosen concentration of RET. If optimization of RET treatment by metabolic labeling cannot be performed, exposure of cells to 100X MIC_{RET} for 5 min will likely afford a nearly 100% inhibition of protein synthesis [30]. Longer exposure times of bacterial cells to RET are not recommended as they can lead to undesirable secondary effects [54,55] .

6. For the procedure described here, we found that 150 mL of an early exponential *E. coli* BL21 culture is the optimal volume to be filtered (*see* Note 7). The optimal conditions for the collection/filtering steps may vary between strains or growth characteristics of the cultures.

7. It is important to keep the filtering time of the cells as brief as possible in order to preserve the position of ribosomes on mRNA [51]. For shortening the filtering time: i) pay attention that the shiny side of the filter is facing up; ii) swirl the filtering flask while the culture is going through the filter disc; iii) After completion of filtration, the filter can be transferred quickly onto a chilled-glass plate to more rapidly and evenly scrape off the cell pellet (*see* also Note 8).

8. We highly recommend pre-optimizing the filtering step using a mock culture. Strains of some bacterial species may not be filterable and thus other collection methods (such as centrifugation) may be considered. Alternatively, with the availability of the appropriate mixer mill equipment, the cell collection conditions could be adjusted for the filtering step to be skipped altogether [51].

9. Comparing MNase-untreated samples from the control and RET-treated cultures shows whether the optimized RET treatment (Section 3.1) resulted in the expected enrichment of monosomes and concomitant depletion of polysomes (Fig. 4). Furthermore, including MNase-untreated samples of the control cell culture helps to evaluate the completeness of conversion of polysomes to monosomes following MNase treatment.

10. The remaining lysates can be stored at −80°C. If desired, an aliquot of the lysates can be saved separately for RNA-seq.

11. We found it beneficial to separately process each of the RNA control oligos o15, o28, and o45 (Section 2.7) in parallel to the experimental samples throughout the procedures described in Sections 3.7 to 3.10 (step 12). Processing the oligos separately allows for loading them in different lanes in the size-selection gels (Fig. 5 and Fig. 6) which facilitates a more accurate selection of the desired size range of the bands in the experimental samples. Starting from step 13 of Section 3.10, through all the steps in Section 3.11, and up to step 13 of Section 3.12, we process only the control oligo o15, as it is the most helpful marker to evaluate the size of PCR amplification products (Section 3.11).

12. If the number of samples is less than 6, the same linker can be ligated to RNA from all the samples because multiplexing can be achieved by using different pairs of PCR primers for each sample (Section 3.12). If the number of samples exceeds 6 it is beneficial to use different ligation linkers, each contributing its own individual bar code (see Section 2.9).

13. Cleaning circularized cDNA is optional, but we routinely carry out this step in order to improve reproducibility of the subsequent steps.

14. Using different PCR primer pairs (point 3 of Section 2.12) for each sample allows for combining them together for sequencing the pooled samples a single lane of the Illumina sequencing platforms (also *see* Note 15).

15. Inform the sequencing facility that your library is double-bar coded and indicate the Illumina barcodes that were present in the primers used in Section 3.12.

16. We recommend confirming global RET-induced ribosome arrest at start codons by generating a metagene plot of averaged Ribo-RET occupancy in the vicinity of all start codons.

17. We observed that the absolute height (RPM value) of the RET peaks at the TISs of the annotated genes does not correlate with the expression of a gene in no-drug conditions and may vary between experiments. Therefore, when comparing RET peaks in different conditions, data from replicates should be diligently analyzed to ensure reproducibility. For example, for our alternative TIS assignment in the *E. coli* genome [30], we only considered the sites that were common in two different strains of *E. coli*. Depending on the research question and the bacterial strain of interest, different thresholds and adjustments to this computational analysis should be considered.

Acknowledgements

The work in Mankin/Vazquez-Laslop laboratory is supported by grants from the National Institutes of Health (R35 GM127134, to A.S.M.) and National Science Foundation (MCB 1951405, to N. V.-L.).

References

1. Laursen BS, Sorensen HP, Mortensen KK, Sperling-Petersen HU (2005) Initiation of protein synthesis in bacteria. Microbiol Mol Biol Rev 69:101–123 [PubMed: 15755955]
2. Ramakrishnan V (2002) Ribosome structure and the mechanism of translation. Cell 108:557–572 [PubMed: 11909526]
3. Rodnina MV (2018) Translation in Prokaryotes. Cold Spring Harb Perspect Biol. doi:10.1101/cshperspect.a032664
4. Villegas A, Kropinski AM (2008) An analysis of initiation codon utilization in the Domain Bacteria - concerns about the quality of bacterial genome annotation. Microbiology 154:2559–2661 [PubMed: 18757789]
5. Shine J, Dalgarno L (1975) Determinant of cistron specificity in bacterial ribosomes. Nature 254:34–38 [PubMed: 803646]
6. Gualerzi CO, Pon CL (2015) Initiation of mRNA translation in bacteria: structural and dynamic aspects. Cell Mol Life Sci 72 (22):4341–4367 [PubMed: 26259514]
7. Moll I, Grill S, Gualerzi CO, Blasi U (2002) Leaderless mRNAs in bacteria: surprises in ribosomal recruitment and translational control. Mol Microbiol 43:239–246 [PubMed: 11849551]
8. Nakagawa S, Niimura Y, Miura K, Gojobori T (2010) Dynamic evolution of translation initiation mechanisms in prokaryotes. Proc Natl Acad Sci USA 107:6382–6387 [PubMed: 20308567]
9. Baez WD, Roy B, McNutt ZA, Shatoff EA, Chen S, Bundschuh R, Fredrick K (2019) Global analysis of protein synthesis in Flavobacterium johnsoniae reveals the use of Kozak-like sequences in diverse bacteria. Nucleic Acids Res 47:10477–10488 [PubMed: 31602466]
10. Li GW, Burkhardt D, Gross C, Weissman JS (2014) Quantifying absolute protein synthesis rates reveals principles underlying allocation of cellular resources. Cell 157:624–635 [PubMed: 24766808]
11. Woolstenhulme CJ, Guydosh NR, Green R, Buskirk AR (2015) High-precision analysis of translational pausing by ribosome profiling in bacteria lacking EFP. Cell Rep 11:13–21 [PubMed: 25843707]
12. de Smit MH, van Duin J (1990) Secondary structure of the ribosome binding site determines translational efficiency: a quantitative analysis. Proc Natl Acad Sci US 87:7668–7672
13. Espah Borujeni A, Channarasappa AS, Salis HM (2014) Translation rate is controlled by coupled trade-offs between site accessibility, selective RNA unfolding and sliding at upstream standby sites. Nucleic Acids Res 42:2646–2659 [PubMed: 24234441]
14. Kozak M (2005) Regulation of translation via mRNA structure in prokaryotes and eukaryotes. Gene 361:13–37 [PubMed: 16213112]
15. Vimberg V, Tats A, Remm M, Tenson T (2007) Translation initiation region sequence preferences in Escherichia coli. BMC Mol Biol 8:100 [PubMed: 17973990]
16. Osterman IA, Evfratov SA, Sergiev PV, Dontsova OA (2013) Comparison of mRNA features affecting translation initiation and reinitiation. Nucleic Acids Res 41:474–486 [PubMed: 23093605]
17. Bienvenut WV, Giglione C, Meinnel T (2015) Proteome-wide analysis of the amino terminal status of Escherichia coli proteins at the steady-state and upon deformylation inhibition. Proteomics 15:2503–2518 [PubMed: 26017780]
18. Impens F, Rolhion N, Radoshevich L, Becavin C, Duval M, Mellin J, Garcia Del Portillo F, Pucciarelli MG, Williams AH, Cossart P (2017) N-terminomics identifies Prli42 as a membrane miniprotein conserved in Firmicutes and critical for stressosome activation in Listeria monocytogenes. Nat Microbiol 2:17005 [PubMed: 28191904]
19. Yuan P, D'Lima NG, Slavoff SA (2018) Comparative Membrane Proteomics Reveals a Nonannotated E. coli Heat Shock Protein. Biochemistry 57:56–60 [PubMed: 29039649]
20. Gao T, Yang Z, Wang Y, Jing L (2010) Identifying translation initiation sites in prokaryotes using support vector machine. J Theor Biol 262:644–649 [PubMed: 19840808]

21. Makita Y, de Hoon MJ, Danchin A (2007) Hon-yaku: a biology-driven Bayesian methodology for identifying translation initiation sites in prokaryotes. BMC Bioinformatics 8:47 [PubMed: 17286872]
22. Salzberg SL, Delcher AL, Kasif S, White O (1998) Microbial gene identification using interpolated Markov models. Nucleic Acids Res 26:544–548 [PubMed: 9421513]
23. Lomsadze A, Gemayel K, Tang S, Borodovsky M (2018) Modeling leaderless transcription and atypical genes results in more accurate gene prediction in prokaryotes. Genome Res 28:1079–1089 [PubMed: 29773659]
24. Benson DA, Cavanaugh M, Clark K, Karsch-Mizrachi I, Lipman DJ, Ostell J, Sayers EW (2017) GenBank. Nucleic Acids Res 45 (D1):D37–D42 [PubMed: 27899564]
25. Hyatt D, Chen GL, Locascio PF, Land ML, Larimer FW, Hauser LJ (2010) Prodigal: prokaryotic gene recognition and translation initiation site identification. BMC Bioinformatics 11:119 [PubMed: 20211023]
26. Delcher AL, Bratke KA, Powers EC, Salzberg SL (2007) Identifying bacterial genes and endosymbiont DNA with Glimmer. Bioinformatics 23:673–679 [PubMed: 17237039]
27. Weaver J, Mohammad F, Buskirk AR, Storz G (2019) Identifying small proteins by ribosome profiling with stalled initiation complexes. MBio 10 (2)
28. Baek J, Lee J, Yoon K, Lee H (2017) Identification of Unannotated Small Genes in Salmonella. G3 7:983–989 [PubMed: 28122954]
29. Meydan S, Vázquez-Laslop N, Mankin AS (2019) Genes within genes in bacterial genomes. In: Storz G, Papenfort K. (ed) Regulating with RNA in Bacteria and Archaea. ASM Press, Washington, DC, pp 133–154
30. Meydan S, Marks J, Klepacki D, Sharma V, Baranov PV, Firth AE, Margus T, Kefi A, Vazquez-Laslop N, Mankin AS (2019) Retapamulin-assisted ribosome profiling reveals the alternative bacterial proteome. Mol Cell 74:481–493 [PubMed: 30904393]
31. Ingolia NT, Ghaemmaghami S, Newman JR, Weissman JS (2009) Genome-wide analysis in vivo of translation with nucleotide resolution using ribosome profiling. Science 324:218–223 [PubMed: 19213877]
32. Ndah E, Jonckheere V, Giess A, Valen E, Menschaert G, Van Damme P (2017) REPARATION: ribosome profiling assisted (re-)annotation of bacterial genomes. Nucleic Acids Res 45:e168 [PubMed: 28977509]
33. Giess A, Jonckheere V, Ndah E, Chyzynska K, Van Damme P, Valen E (2017) Ribosome signatures aid bacterial translation initiation site identification. BMC Biol 15:76 [PubMed: 28854918]
34. Schrader JM, Zhou B, Li GW, Lasker K, Childers WS, Williams B, Long T, Crosson S, McAdams HH, Weissman JS, Shapiro L (2014) The coding and noncoding architecture of the Caulobacter crescentus genome. PLoS Genet 10:e1004463 [PubMed: 25078267]
35. Gao X, Wan J, Liu B, Ma M, Shen B, Qian SB (2015) Quantitative profiling of initiating ribosomes in vivo. Nat Methods 12:147–153 [PubMed: 25486063]
36. Lee S, Liu B, Lee S, Huang SX, Shen B, Qian SB (2012) Global mapping of translation initiation sites in mammalian cells at single-nucleotide resolution. Proc Natl Acad Sci USA 109:E2424–2432 [PubMed: 22927429]
37. Ingolia NT, Lareau LF, Weissman JS (2011) Ribosome profiling of mouse embryonic stem cells reveals the complexity and dynamics of mammalian proteomes. Cell 147:789–802 [PubMed: 22056041]
38. Fritsch C, Herrmann A, Nothnagel M, Szafranski K, Huse K, Schumann F, Schreiber S, Platzer M, Krawczak M, Hampe J, Brosch M (2012) Genome-wide search for novel human uORFs and N-terminal protein extensions using ribosomal footprinting. Genome Res 22:2208–2218 [PubMed: 22879431]
39. Nakahigashi K, Takai Y, Kimura M, Abe N, Nakayashiki T, Shiwa Y, Yoshikawa H, Wanner BL, Ishihama Y, Mori H (2016) Comprehensive identification of translation start sites by tetracycline-inhibited ribosome profiling. DNA Res 23:193–201 [PubMed: 27013550]
40. Davidovich C, Bashan A, Auerbach-Nevo T, Yaggie RD, Gontarek RR, Yonath A (2007) Induced-fit tightens pleuromutilins binding to ribosomes and remote interactions enable their selectivity. Proc Natl Acad Sci USA 104:4291–4296 [PubMed: 17360517]

41. Poulsen SM, Karlsson M, Johansson LB, Vester B (2001) The pleuromutilin drugs tiamulin and valnemulin bind to the RNA at the peptidyl transferase centre on the ribosome. MolMicrobiol 41:1091–1099

42. Schlunzen F, Pyetan E, Fucini P, Yonath A, Harms JM (2004) Inhibition of peptide bond formation by pleuromutilins: the structure of the 50S ribosomal subunit from *Deinococcus radiodurans* in complex with tiamulin. Mol Microbiol 54:1287–1294 [PubMed: 15554968]

43. Paukner S, Riedl R (2017) Pleuromutilins: potent drugs for resistant bugs-mode of action and resistance. Cold Spring Harb Perspect Med 7:a027110 [PubMed: 27742734]

44. Paukner S, Gelone SP, Arends SJR, Flamm RK, Sader HS (2019) Antibacterial activity of lefamulin against pathogens most commonly causing community-acquired bacterial pneumonia: SENTRY antimicrobial surveillance program (2015-2016). Antimicrob Agents Chemother 63:e02161–18 [PubMed: 30670415]

45. Oh E, Becker AH, Sandikci A, Huber D, Chaba R, Gloge F, Nichols RJ, Typas A, Gross CA, Kramer G, Weissman JS, Bukau B (2011) Selective ribosome profiling reveals the cotranslational chaperone action of trigger factor in vivo. Cell 147:1295–1308 [PubMed: 22153074]

46. McGlincy NJ, Ingolia NT (2017) Transcriptome-wide measurement of translation by ribosome profiling. Methods 126:112–129 [PubMed: 28579404]

47. Afgan E, Baker D, van den Beek M, Blankenberg D, Bouvier D, Cech M, Chilton J, Clements D, Coraor N, Eberhard C, Gruning B, Guerler A, Hillman-Jackson J, Von Kuster G, Rasche E, Soranzo N, Turaga N, Taylor J, Nekrutenko A, Goecks J (2016) The Galaxy platform for accessible, reproducible and collaborative biomedical analyses: 2016 update. Nucleic Acids Res 44 (W1):W3–W10 [PubMed: 27137889]

48. Martin M (2011) Cutadapt removes adapter sequences from high-throughput sequencing reads. EMBnetjournal 17:10–12

49. Langmead B, Trapnell C, Pop M, Salzberg SL (2009) Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. Genome Biol 10:R25 [PubMed: 19261174]

50. Homann OR, Johnson AD (2010) MochiView: versatile software for genome browsing and DNA motif analysis. BMC Biol 8:49 [PubMed: 20409324]

51. Mohammad F, Green R, Buskirk AR (2019) A systematically-revised ribosome profiling method for bacteria reveals pauses at single-codon resolution. Elife 8:e42591 [PubMed: 30724162]

52. Dieterich DC, Link AJ, Graumann J, Tirrell DA, Schuman EM (2006) Selective identification of newly synthesized proteins in mammalian cells using bioorthogonal noncanonical amino acid tagging (BONCAT). Proc Natl Acad Sci USA 103:9482–9487. doi:10.1073/pnas.0601637103 [PubMed: 16769897]

53. Oulhen N, Swartz SZ, Laird J, Mascaro A, Wessel GM (2017) Transient translational quiescence in primordial germ cells. Development 144:1201–1210 [PubMed: 28235822]

54. Felden B, Cattoir V (2018) Bacterial adaptation to antibiotics through regulatory RNAs. Antimicrob Agents Chemother 62:e02503–17 [PubMed: 29530859]

55. Steinchen W, Bange G (2016) The magic dance of the alarmones (p)ppGpp. Mol Microbiol 101:531–544 [PubMed: 27149325]

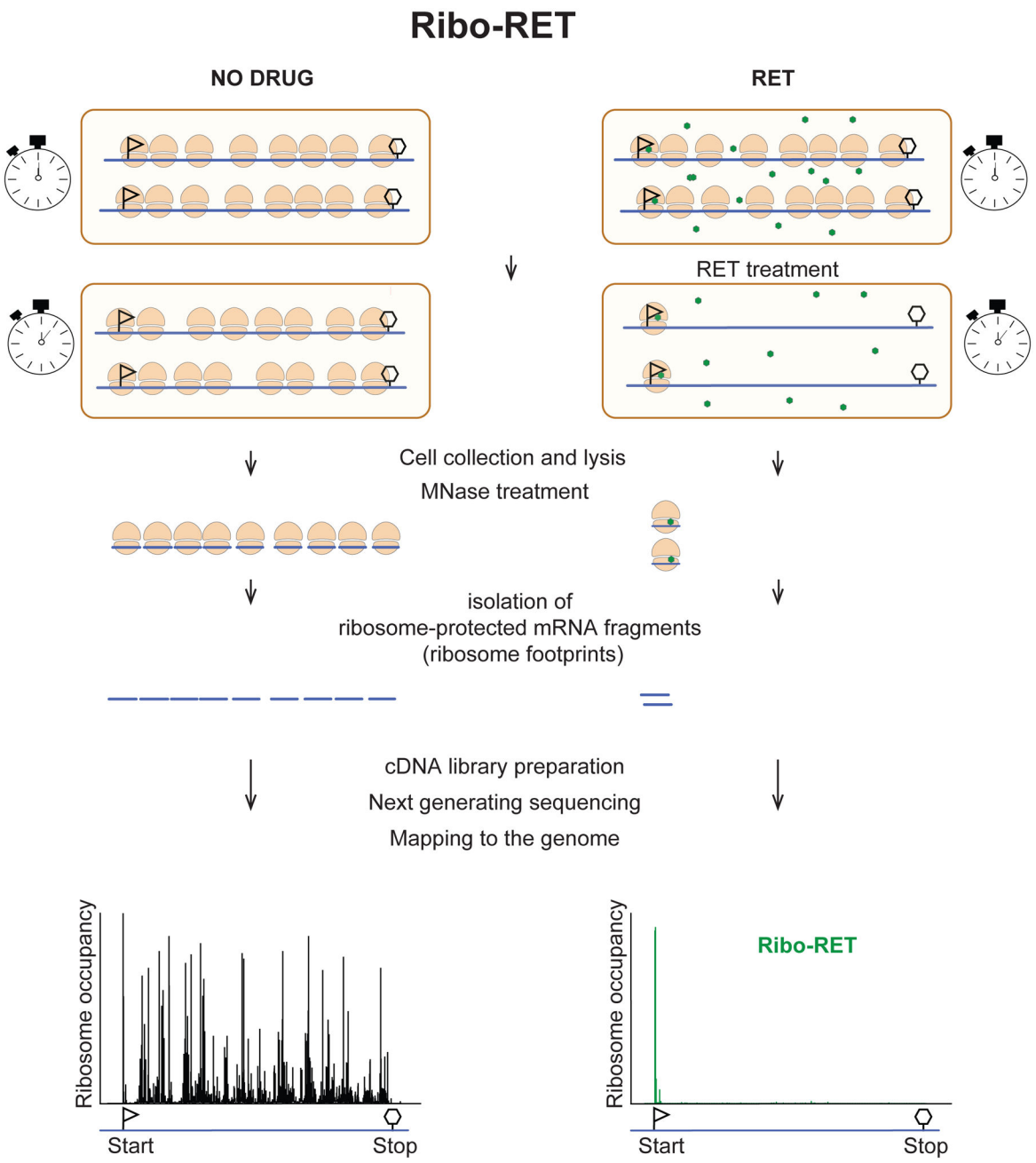

Fig. 1.

The Ribo-RET experimental pipeline. Bacterial cells are first treated with RET at a concentration and incubation time that are pre-determined by metabolic labeling experiments. Upon cell collection and lysis, conventional Ribo-seq experimental steps are performed. To assess the general translation status of the cells, a culture where the drug treatment is omitted (indicated as NO DRUG) is prepared and processed in parallel.

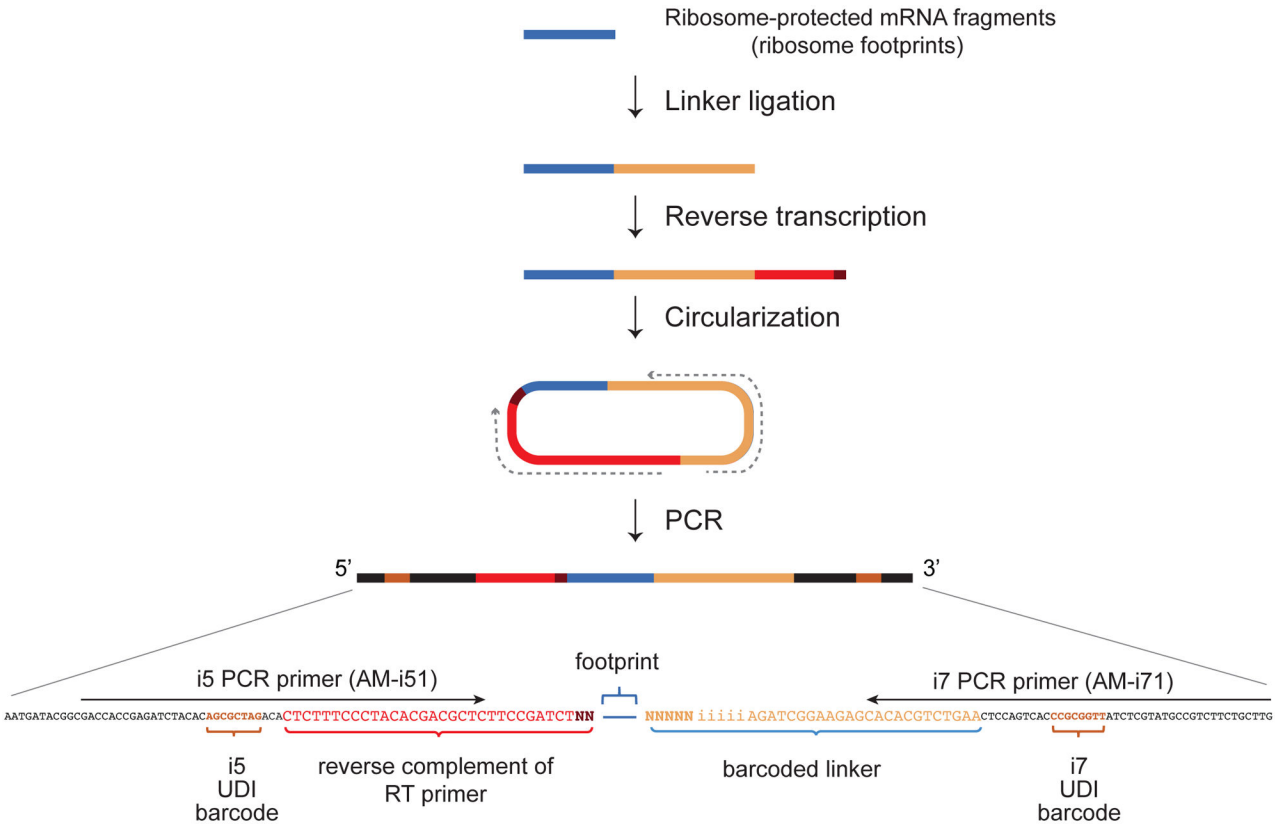

Fig. 2.

The steps of the Ribo-seq protocol for preparing samples amenable for next generation sequencing. The general structure of the final product for Illumina sequencing is shown (Section 3.12). Illumina unique dual indexes (UDI) (in this case derived from AM-i51 and AM-i71 PCR primers) (Sections 2.12 and 3.12) are indicated. "N" indicates a random nucleotide; "iiiii" indicates a unique barcode sequence that resides within NI-810 through NI-817 linkers (Sections 2.9 and 3.9).

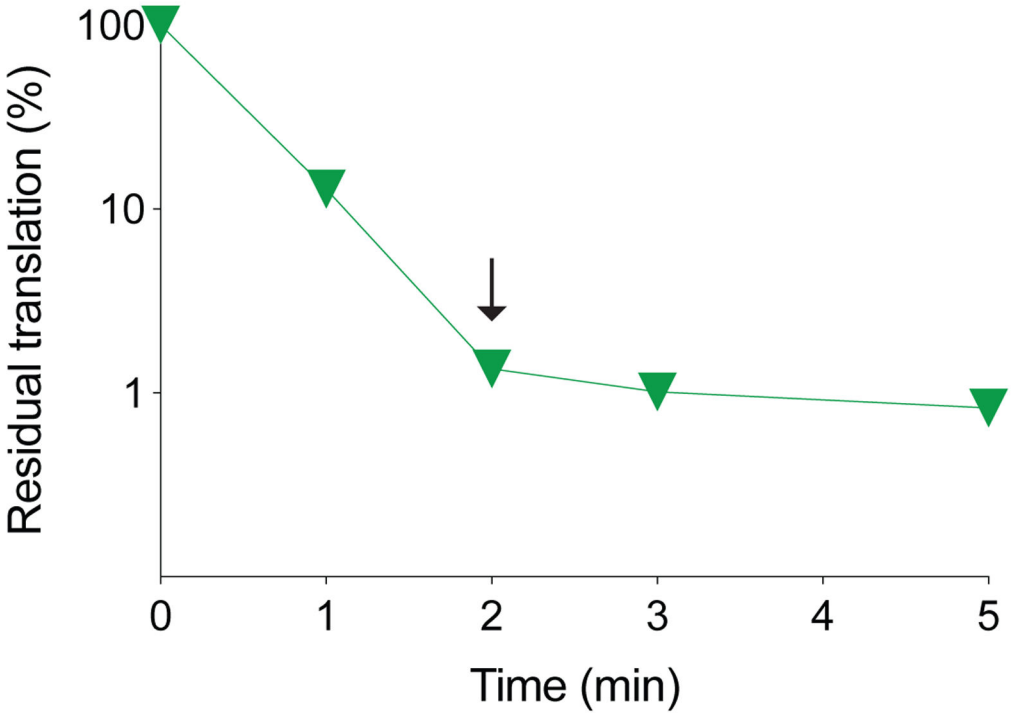

Fig. 3.

Metabolic labeling experiment determining the residual protein synthesis in *E. coli* BL21 *ΔtolC* cells treated for the indicated times with RET. The arrow indicates the shortest incubation time where maximum protein synthesis inhibition was achieved.

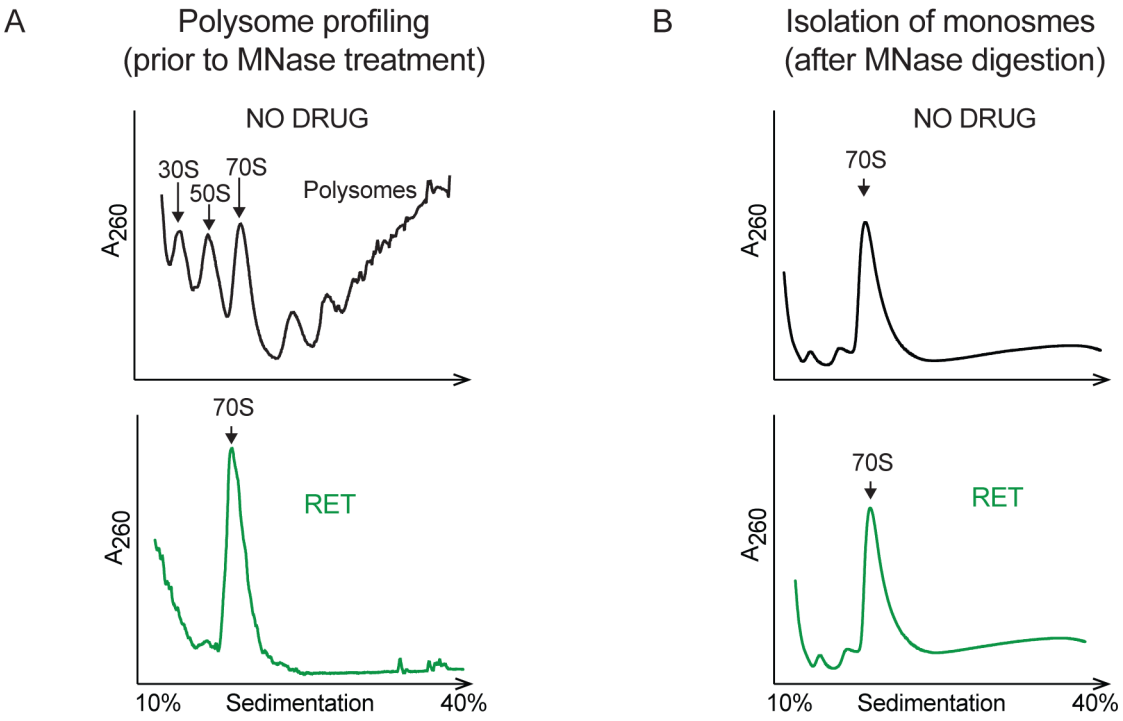

Fig. 4.

(**a**) Sucrose gradient fractionation of the lysates prior to MNase digestion. Note that polysomes are collapsed into 70S ribosomes in the RET sample. (**b**) Sucrose gradient fractionation of the samples following digestion with MNase. The treatment with MNase should be optimized to collapse polysomes (in the control sample) but preserve the integrity of the 70S peak.

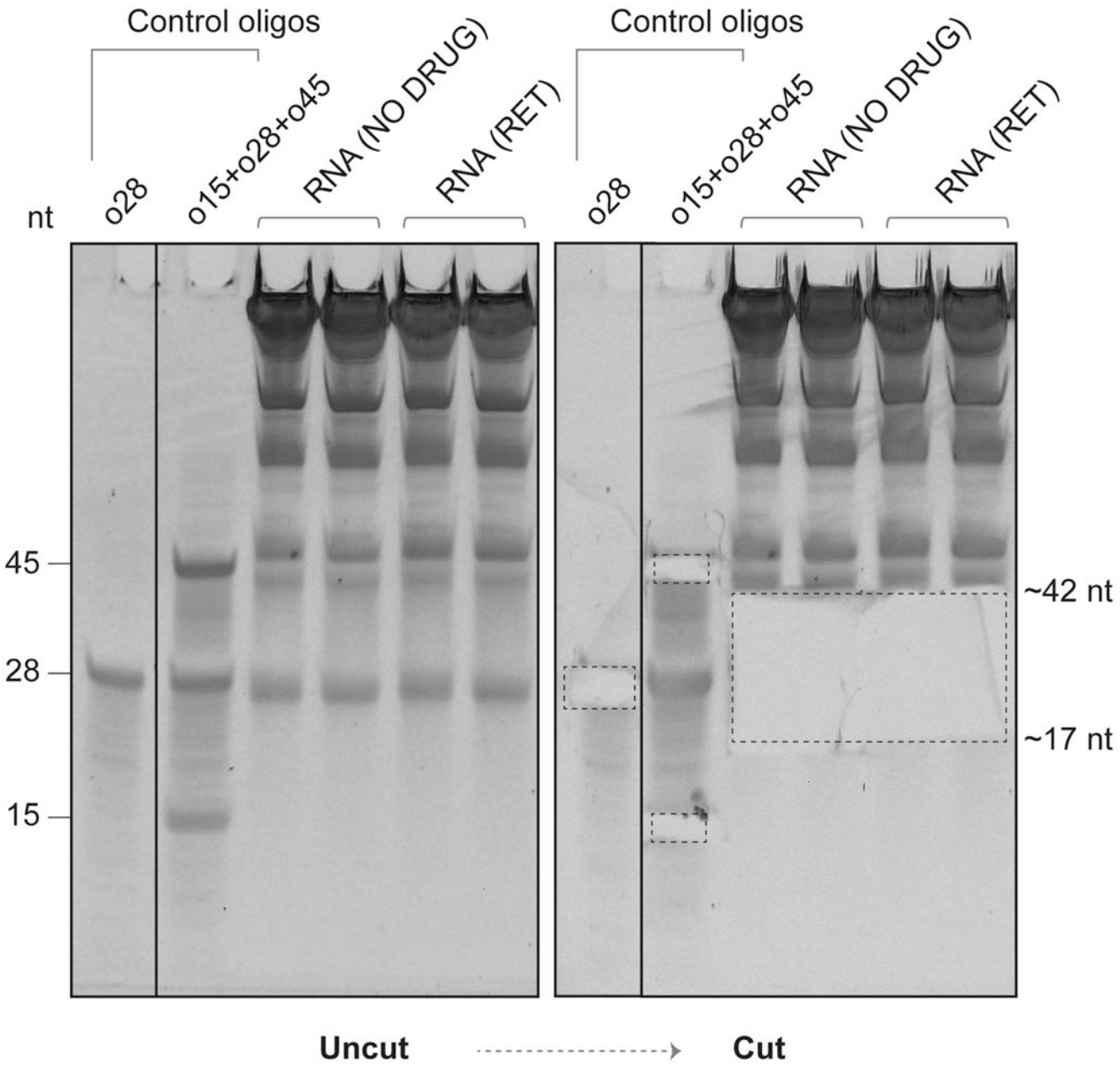

Fig. 5.

Selection of mRNA fragments of the desired size ranges. The excised gel areas (right panel) are indicated by dashed rectangles.

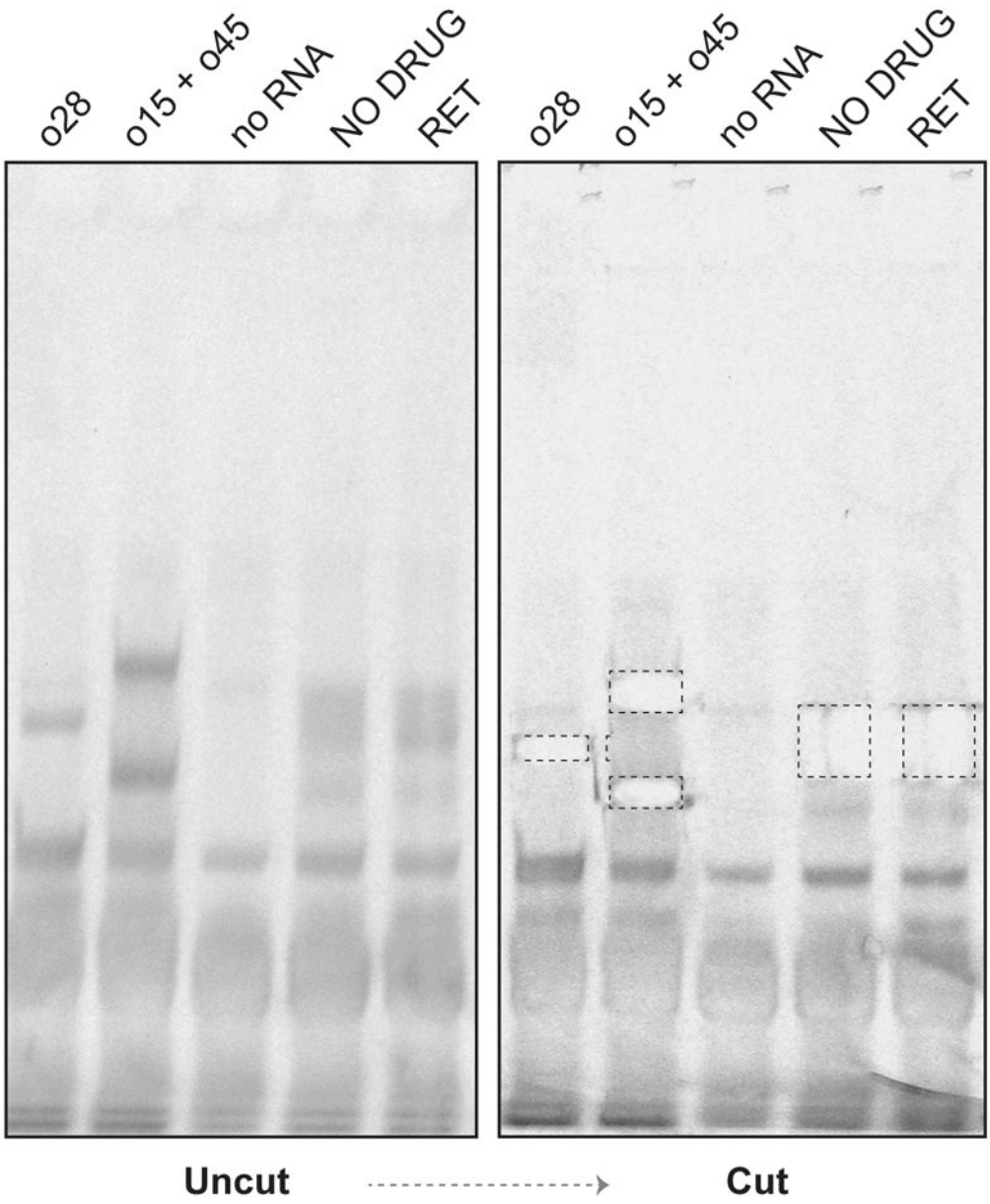

Fig. 6.

Selection of cDNA products after reverse transcription. The cDNA products originated from control RNA oligos o15, o28 and o45 were used as size markers. The excised gel areas are indicated by dashed rectangles.

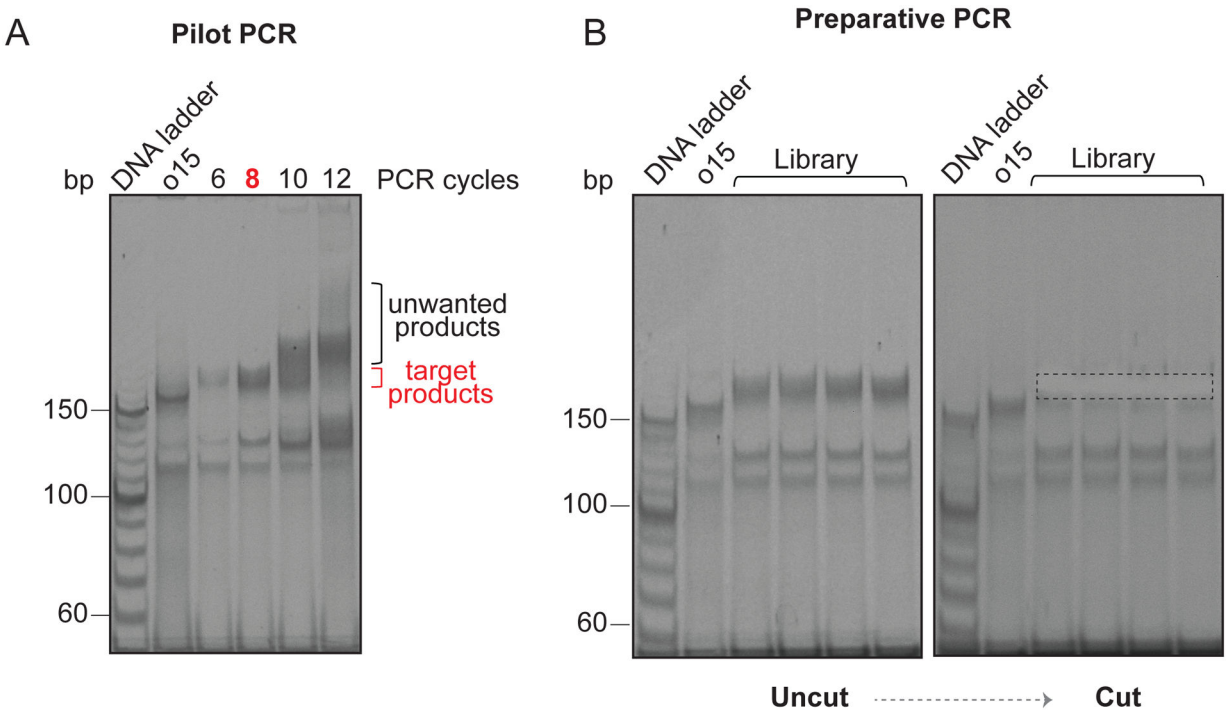

Fig. 7.

(**a**) Pilot PCR analysis for determining the optimal number of cycles required for generation of the PCR library. In this example, the 8 cycle-amplification (indicated in red) produced the desired result because it yielded a sufficient amount of the target products but lacked the undesirable higher molecular weight fragments appearing with increased number (10 and 12) of cycles. (**b**) Preparative gel for isolation of the PCR fragments obtained after 8 amplification cycles. The excised gel area is indicated with a dashed rectangle.

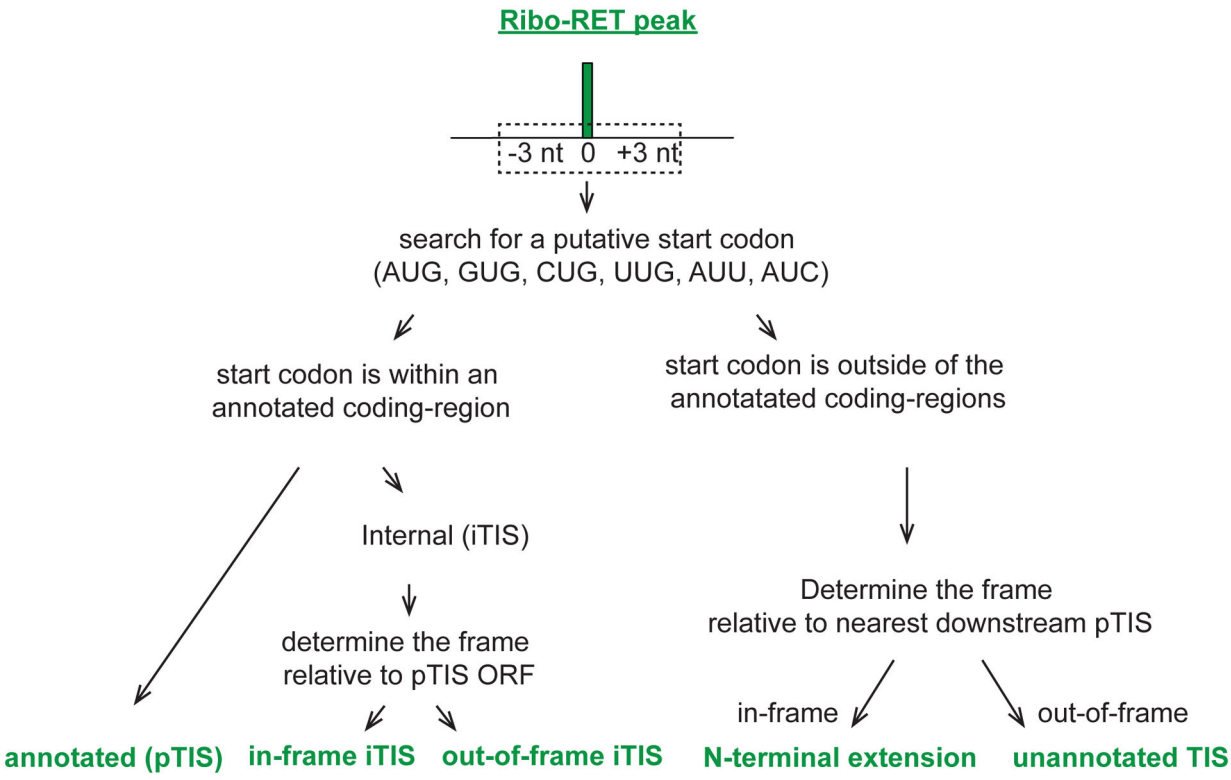

Fig. 8.

Schematics of the computational algorithm to find genome-wide start sites using Ribo-RET peaks.