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An Integrated Polysome Profiling and Ribosome Profiling Method to Investigate In Vivo Translatome

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Abstract

Recent advances in global translatome analysis technologies enable us to understand how translational regulation of gene expression modulates cellular functions. In this chapter, we present an integrated method to measure various aspects of translatome by polysome profiling and ribosome profiling using purified B cells. We standardized our protocols to directly compare the results from these two approaches. Parallel assessment of translatome with these two approaches can generate a comprehensive picture on how translational regulation determines protein output.

Keywords

Translatome; Ribosome profiling; Ribo-seq; Polysome profiling; Translational regulation of gene expression

1 Introduction

Over the past few decades, quantification of mRNA abundance in polysome fractionation by sucrose density gradient centrifugation (polysome profiling) has been used to investigate translation activity of individual genes. Recently, this was combined with high-throughput technologies, such as microarray or RNA-seq, to reveal genome-wide landscape of translatome [1, 2]. Essentially, global profiling of mRNAs in each fraction of polysome gradient allows the estimation of ribosome occupancy on all mRNAs expressed in a cell. Based on the assumption that ribosome density (the number of ribosomes associated with an mRNA molecule) highly correlates with protein production, the translational efficiency of individual genes can be calculated from these results.

Ribosomes leave ~30 nt footprints (ribosome-protected RNA fragment, RPF) when they are bound to mRNAs [3]. This, in combination with advanced sequencing technology, led to the development of ribosome profiling [4]. In this method, deep sequencing of RPFs would globally quantify the abundance of ribosome footprints on individual genes and pinpoint the position of ribosomes on mRNAs.

Both technologies can be utilized to investigate global translational activity, but they have distinct strengths over each other [5]. Polysome profiling measures ribosome density of each mRNA, so that translational activity can be directly captured by a single measurement. The readout of ribosome profiling, ribosome footprint abundance, is a sum of mRNA changes and ribosome density changes, so that translational activity is indirectly estimated by subtracting mRNA abundance changes from ribosome footprint abundance changes [5]. On the other hand, ribosome profiling captures positional information of ribosome footprints at the subcodon level while polysome profiling does not, and is therefore more suitable for investigating alternative start codons or open reading frames [5]. The analysis of RPF distribution also reveals codon-specific regulation of gene expression during the translational elongation stage [6]. The size of ribosome footprint, which can be measured only by ribosome profiling, may indicate differential conformation of ribosomes or the presence of disomes [7, 8]. A comparison of these two technologies is summarized in Table 1.

The most immediate use of these technologies is to measure global gene expression. Historically, mRNA abundance was often used as a proxy for gene expression. However, it has been frequently shown that mRNA abundance and protein level lack sufficient correlation due to the regulation of gene expression at the level of translation [9]. Therefore, faithful measurement of translome is essential for investigating the end point of gene expression, protein abundance, in a cell. Indeed, quantifying translomes by ribosome profiling and polysome profiling led to the discovery of novel genes that are mainly regulated at the translational level in specific cellular contexts [10, 11].

Despite the significance of these technologies, both approaches exhibit technical limitations (Table 1). In polysome profiling, the separation of heavy polysomes starts to lose its accuracy when the number of ribosomes associated with an mRNA exceeds seven or eight. In ribosome profiling, the length of footprints is much smaller (~30 nts) than that of RNA fragments generated by standard RNA-seq methods. This increases the propensity to align the footprints to multiple genomic locations and makes the dataset intrinsically noisy. Moreover, a small fraction of ribosome footprints are aligned to genomic locations outside of coding sequences, such as 5'UTR and 3'UTR, independent of their protein coding capacity [8, 12]. This may result in underestimation of the differential translational regulation occurring in the coding sequences.

To compensate for these limitations, it may be critical to study translome with these two approaches in parallel and cross-validate each other. We recently combined these two approaches to investigate primary B cells with transgenic miR-17~92 expression or complete deletion of the miR-17~92 family miRNAs and revealed that the predominant miRNA effect on their target genes occurs at the translational level [13]. We noticed that the current protocols for ribosome profiling and polysome profiling are from different labs and therefore different in terms of sample preparation and detailed procedures. This may hinder the direct comparison between the results from these two approaches. Here, we provide an integrated ribosome profiling and polysome profiling method to directly compare the results from these two approaches (Fig. 1).

2 Materials

2.1 Generation of Sucrose Gradients

1. RNase inhibitor.
2. 100× Cyclohexamide (CHX): 10 mg/mL.
3. 5× sucrose base buffer: 0.4 M NaCl, 25 mM MgCl₂, 100 mM Tris-HCl, pH 7.4, 5 mM DTT, 20 units of RNase inhibitor/mL.
4. Thin wall polyallomer centrifuge tubes, 14 × 89 mm.

2.2 B Cell Purification and Activation

1. MACS LD column (Miltenyi Biotech, San Diego, CA, USA).
2. B cell medium: DMEM-GlutaMAX, 50 mL FCS, 5 mL of 100× Nonessential amino acids, 5 mL of 1 M HEPES, 5 mL of 100× Penn/Strep, 0.6 mL of 55 mM β-Mercaptoethanol.
3. Lipopolysaccharides from *Escherichia coli* 055:B5 (LPS).
4. IL-4.
5. Ficoll solution.

2.3 Cytosolic Fractionation

1. Hypotonic buffer: 1.5 mM KCl, 10 mM MgCl₂, 5 mM Tris-HCl, pH 7.4, 100 µg/mL CHX.
2. Hypotonic lysis buffer: 2% sodium deoxycholate, 2% Triton X-100, 2.5 mM DTT, 100 µg/mL CHX, 10 units of RNase inhibitor/mL.

2.4 Polysome Profiling and Total RNA Extraction from Each Fraction

1. Solaris control RNA (Dharmacon-GE Healthcare, Lafayette, CO, USA) or ERCC control RNA (ThermoFisher Scientific, Waltham, MA, USA).
2. Trizol-LS (ThermoFisher Scientific).

2.5 Ribosome Profiling Library Preparation

1. RNase decontamination wipes and reagent (*see* Note 1).
2. RNase-free tubes with a low-binding affinity for nucleic acids (*see* Note 2).
3. Hypotonic lysis buffer without RNase Inhibitor: 2% sodium deoxycholate, 2% Triton X-100, 2.5 mM DTT, 100 µg/mL CHX.
4. RNase I.

¹-Maintain RNase-free working conditions: Routine use of RNase decontamination wipes, e.g., RNaseZAP or RNase Displace (ThermoFisher Scientific, Waltham, MA, USA) to wipe down the work area and rinse gel apparatuses is recommended.

²-Non-stick, RNase-free tubes are recommended throughout protocol, e.g., Eppendorf® DNA LoBind microcentrifuge tubes (Eppendorf, Hamburg, Germany) or Nonstick, RNase-free Microfuge Tubes (ThermoFisher Scientific, Waltham, MA, USA).

5. RNase inhibitor.
6. miRNeasy RNA purification kit (Qiagen, Valencia, CA, USA) (*see* Note 3).
7. 2× denaturing loading buffer: 98%(vol/vol) formamide with 10 mM EDTA and 300 µg/mL bromophenol blue.
8. 5× TBE solution: Combine; 27 g Tris Base, 13.7 g Boric acid, pH 8.0, 10 mL 0.5 M EDTA and fill up to 500 mL RNase-free water.
9. 10% polyacrylamide TBE-Urea gel: Combine; 16.8 g urea, 4 mL of 5× TBE and 16 mL RNase-free water. Heat to dissolve urea for 20 min. Cool down the solution to room temperature and add 10 mL of 40% acrylamide/bis solution (37.5:1), 240 µL of 10% APS, and 32 µL of TEMED. For more detailed condition, *see* ref. 14.
10. 10 bp DNA ladder.
11. 26–34 nt RNA marker: 10 µM mix of NI-NI-19 and NI-NI-20. For the detailed sequence information, *see* ref. 15.
12. SYBR gold, 10,000× (ThermoFisher Scientific).
13. Isopropanol (99.5%).
14. Glycoblu, 15 mg/mL (ThermoFisher Scientific).

2.5.1 Ribo-Zero rRNA Depletion

1. Ribo-Zero rRNA removal kit (Illumina, San Diego, CA, USA).
2. Zymo RNA Clean and Concentrator-5 kit (Zymo Research, Irvine, CA, USA).

2.5.2 Dephosphorylation, Linker Ligation, and Purification of Linker Ligated Products

1. T4 Polynucleotide Kinase.
2. Linker-1 (/5rApp/CTGTAGGCACCATCAAT/3ddC/) (*see* Note 4).
3. T4 RNA ligase 2, truncated (New England Biolabs, Ipswich, MA, USA), supplied with PEG 8000 50% (wt/vol) and 10× T4 Rnl2 buffer.

2.5.3 Reverse Transcription and Circularization

1. RT primer (*see* ref. 15):
 - (a) 5'-(phos)-agatcggagagcgtcgttagggaaagagttagatctcgggtgcgc-(iSp18)-cactca-(iSp18)-ttcagacgtgtgctctccgatctattgatgtgcctacag-3'.
The designation (Phos) indicates 5'-phosphorylation and -(iSp18)- indicates a 18 atom hexa-ethyleneglycol spacer.
2. 3 M sodium acetate, pH 5.5.

³.Other RNA purification columns may be substituted at this step.

⁴.There is a pre-made version available from Integrated DNA Technologies (Coralville, IA, USA) or a custom synthesis can be done.

- 1 N NaOH.
- 0.1 M Dithiothreitol (DTT).
- 10 mM dNTP mix.
- Phusion polymerase (New England Biolabs) (*see* Note 5).
- 7.5% polyacrylamide TBE-Urea gel: Combine; 16.8 g urea, 4 mL of 5× TBE and 18.5 mL RNase-free water. Heat to dissolve urea for 20 min. Cool down the solution to room temperature and add 7.5 mL of 40% acrylamide/bis solution (37.5:1), 240 µL of 10% APS and 32 µL of TEMED.
- DNA gel extraction buffer: 300 mM NaCl, 10 mM Tris–HCl, pH 8.0, and 10 mM EDTA.
- CircLigase (Illumina).

2.5.4 Indexed Library Generation

- Index library primers (*see* ref. 15):
- Phusion polymerase (New England Biolabs).
- 8% polyacrylamide TBE gel: Combine; 27.8 mL distilled water, 4 mL of 5× TBE, 8 mL of 40% acrylamide:bis solution (37.5:1), 200 µL of APS and 20 µL of TEMED.
- 6× DNA gel loading dye.¹

Barcode_Forward	5'-aatgatacggcgaccaccgagatctacac-3'
Barcode_Reverse_01_ACGACT	5'-caagcagaagacggcatacagatAGTCGTgtgactggagttcagacgtgtgctcttccgatct-3'
Barcode_Reverse_02_ATCAGT	5'-caagcagaagacggcatacagatACTGATgtgactggagttcagacgtgtgctcttccgatct-3'
Barcode_Reverse_03_CAGCAT	5'-caagcagaagacggcatacagatATGCTGgtgactggagttcagacgtgtgctcttccgatct-3'
Barcode_Reverse_04_CGACGT	5'-caagcagaagacggcatacagatACGTCGgtgactggagttcagacgtgtgctcttccgatct-3'
Barcode_Reverse_05_GCAGCT	5'-caagcagaagacggcatacagatAGCTGCgtgactggagttcagacgtgtgctcttccgatct-3'
Barcode_Reverse_06_TACGAT	5'-caagcagaagacggcatacagatATCGTAgtgactggagttcagacgtgtgctcttccgatct-3'
Barcode_Reverse_07_CTGACG	5'-caagcagaagacggcatacagatCGTCAGgtgactggagttcagacgtgtgctcttccgatct-3'
Barcode_Reverse_08_GCTACG	5'-caagcagaagacggcatacagatCGTAGCgtgactggagttcagacgtgtgctcttccgatct-3'
Barcode_Reverse_09_TGCAGC	5'-caagcagaagacggcatacagatGCTGCAgtgactggagttcagacgtgtgctcttccgatct-3'

2.6 Illumina Sequencing

- Standard Illumina cluster generation and sequencing kits (minimum 50 bases plus indexing reads) appropriate for the sequencer used (HiSeq, MiSeq, or NextSeq).

⁵.Other commercially available thermostable high-fidelity polymerases can be substituted for Phusion (e.g., KAPPA Hi-Fi, Herculase II Fusion).

3 Methods

3.1 Generation of Sucrose Gradients

1. Generate 15% sucrose solution (50 mL of 5× sucrose base buffer, 37.5 g of sucrose, and fill up to 250 mL).
2. Generate 45% sucrose solution (50 mL of 5× sucrose base buffer, 112.5 g of sucrose, and fill up to 250 mL).
3. (Optional) Filter the sucrose solution using 0.45 µm vacuum filter.
4. Next, generate five different concentrations of gradients by mixing 15% and 45% sucrose solution.
5. Add 2.2 mL of 45% sucrose gradient to thin wall polyallomer centrifuge tubes and freeze it down for 20 min at −80 °C. Once it is frozen, overlay with 2.2 mL of 37.5% sucrose gradient and freeze it down for 20 min. Repeat it with 30%, 22.5% and 15% sucrose gradient. This will generate frozen 15–45% gradient stocks. They can be thawed in a cold room (4 °C) for a few hours to up to 12 h before centrifugation is conducted.

	15% sucrose solution	45% sucrose solution
Final 15% sucrose solution	50 mL	0 mL
Final 22.5% sucrose solution	37.5 mL	12.5 mL
Final 30% sucrose solution	25 mL	25 mL
Final 37.5% sucrose solution	12.5 mL	37.5 mL
Final 45% sucrose solution	0 mL	50 mL

3.2 B Cell Purification and Activation

1. 100×10^6 follicular B cells were purified from total splenocytes by depleting cells positive for CD5, CD43 or CD93 using MACS LD column according to the manufacturer's instruction. The purified cells were in vitro activated with LPS (25 µg/mL) and IL-4 (5 ng/mL) in B cell medium for aimed time points [16].
2. Before harvest, add CHX directly to the culture medium and incubate for 15 min. This will freeze the ribosomes on mRNAs.
3. (*Optional*) At later time points of B cell activation, a significant fraction of cells undergo apoptosis. Purifying live cells from the culture may improve the results. Live cells can be purified using Ficoll solution according to the manufacturer's instruction. During this process, add same final concentration of CHX to Ficoll solution to maintain ribosome association to mRNAs.
4. Split samples into two groups at this point. One for polysome profiling (*see* Subheading 3.3) and the other for ribosome profiling (*see* Subheading 3.5.1).

3.3 Cytosolic Fractionation

1. Wash cells with 10 mL of ice-cold hypotonic buffer.

2. Swell cells for 10 min on ice.
3. Spin down with $500 \times g$ for 5 min at 4 °C, aspirate the hypotonic buffer, and gently resuspend the cells using 300 μ L hypotonic buffer.
4. Transfer the resuspended cells to new tube and add 300 μ L hypotonic lysis buffer (total 600 μ L).
5. Incubate samples on ice for 20 min.
6. Spin down the cells with $2300 \times g$ at 4 °C for 10 min and collect supernatant. This removes nuclei and collects the cytosolic fraction.
7. (*Optional*) Confirm the cytosolic fractionation efficiency (*see Fig. 2*).

3.4 Polysome Profiling and Total RNA Extraction from Each Fraction

1. Carefully overlay the cytosolic fractions on the thawed 15–45% sucrose gradient at 4 °C.
2. Centrifuge at 40,000 rpm ($274,000 \times g$ at r-max and $121,000 \times g$ at r-min) for 1.5 h at 4 °C, using SW41 rotor.
3. Fractionate the sucrose into 20 tubes using automated fractionation machine and simultaneously measure A254 values to estimate the overall translation profile. Each fraction will contain roughly 540 μ L of sucrose after fractionation. Flow rates of 0.75 mL/min and collection exchange speed of 0.73 min/tube are recommended.
4. (*Optional*) Add equal amounts of Solaris RNA (2 μ L) to each collection as a normalization control. Alternatively, ERCC RNA can be used.
5. Add 1.5 mL of Trizol-LS to each tube (total ~2 mL solution) and extract total RNA following the manufacturer's instruction.
6. Conduct standard qRT-PCR or RNA-seq of each fraction to reveal polysome distribution of individual genes or global polysome distribution, respectively.

3.5 Ribosome Profiling Library Preparation

Ribosome footprint library is generated as previously described with modifications to make the conditions as comparable to that of polysome profiling as possible [4, 15]. Major modifications are: (1) Used total RNA purified from cytosolic fractions instead of whole cell lysate with DNase treatment method [15]. (2) RNase I treated footprints are purified using the same sucrose gradient as polysome profiling. This method was originally proposed in the initial ribosome profiling protocol [4], but later modified to either sucrose cushion method [15] or Sephacryl S-400 column purification method [17, 18]. (3) rRNAs are depleted using Ribo-zero rRNA depletion kit instead of biotinylated rRNA depletion oligos after footprint recovery and before the dephosphorylation and linker ligation steps [15].

3.5.1 Cytosolic Fractionation

1. Extract the cytosolic fraction (final volume of 600 μ L) as described in Subheading 3.3, using the hypotonic lysis buffer without RNase Inhibitor. Maintain 4 °C in cold room to avoid unwanted mRNA degradation during the process.

3.5.2 RNase I Footprinting and Ribosome Recovery

1. Take the 600 μ L of cytosolic fraction and add 7.5 μ L of RNase I (100 U/ μ L).
2. Incubate for 45 min at room temperature with gentle mixing on nutator.
3. To stop the RNase I digestion, add 10 μ L of SUPERase Inhibitor and tap the tubes gently.
4. Run 15–45% sucrose gradients as described in Subheading 3.4 and confirm efficient RNase I digestion (*see* Fig. 3).
5. Collect and combine fractions 6, 7, and 8. Fractions 9–10 include disome-protected mRNA fragments which may represent ribosome stalling [8], and are excluded from our analysis (*see* Fig. 3).
6. Add 4.5 mL of Trizol-LS to the combined fractions (total 6 mL) and extract total RNA. To increase yield, the miRNeasy RNA purification kit is recommended. Expected amounts of RNA from 50 million B cells in each step are listed in Table 2.

3.5.3 Footprint Gel Purification

1. Add 2 $\times$ denaturing loading buffer to each sample.
2. Prepare 1 μ L of 26 nt and 34 nt RNA marker and 1 μ L of 10 bp DNA ladder as control. Add 4 μ L of 10 mM Tris-HCl (pH 8.0) and 5 μ L of 2 $\times$ denaturing loading buffer to each.
3. Denature the sample at 80 °C for 90 s and transfer the sample to ice directly.
4. Separate the sample using 10% polyacrylamide TBE-Urea gel in 0.5 $\times$ TBE buffer with constant power (5 W) for 80 min until the bromophenol blue dye reaches two third of the gel. Flushing each well with 0.5 $\times$ TBE solution with syringe before sample loading helps to generate nicely shaped bands.
5. Carefully disassemble the gel cassette and stain the gel for 3 min with 1 $\times$ SYBR gold in 0.5 $\times$ TBE buffer (*see* Fig. 4).
6. Excise the ribosome footprint (24–36 nt region) and 26 nt/34 nt marker (*see* Fig. 4). 26 nt/34 nt footprints extracted here serve as a control for the remaining steps.
7. Overnight gel extraction. Add 400 μ L of RNA gel extraction buffer and freeze the sample for 30 min on dry ice. Leave the sample overnight (15 h is recommended) with gentle mixing. Collect all solution and transfer to non-stick RNase-free eppitubes. Precipitate the footprints by adding 2 μ L Glycoblue and 500 μ L isopropanol. Carry out precipitation for 30 min or more on dry ice.

Centrifuge at maximum speed at 4 °C for 30 min using a tabletop centrifuge. Footprints frequently end up on the side of the tubes. If this happens, resuspend footprints and centrifuge again. Completely remove the supernatant and allow air dry for 5 min. Dissolve footprints in 10 µL RNase-free water.

3.5.4 Ribo-Zero rRNA Depletion

1. Conduct the rRNA depletion using Ribo-zero rRNA removal kit following the manufacturer's protocol.
2. Concentrate rRNA-depleted sample using Zymo RNA Clean and Concentrator kit according to the manufacturer's protocol. At the final elution step, use 8 µL of RNase-free water, which will give 7 µL final elute.

3.5.5 Dephosphorylation, Linker Ligation, and Purification of Linker Ligated Products

1. Dephosphorylation reaction. Mix the 7 µL of rRNA depleted footprint and 1 µL PNK buffer. Incubate at 75 °C for 1 min and cool on ice. Add 1 µL of SUPERase Inhibitor and 1 µL T4 PNK. Incubate for 1 h at 37 °C (total volume: 10 µL). Upon completion, heat-inactivate samples at 65 °C for 3 min and cool samples on ice.
2. Linker ligation reaction. Mix the 10 µL of dephosphorylated products and 1 µL of linker-1. Denature the sample for 90 s at 80 °C and immediately cool on ice for 10 min. Add 1 µL of T4 Rnl2 buffer, 1 µL of SUPERase inhibitor, 6 µL of PEG8000, and 1 µL of T4 RNA ligase 2 (truncated). Incubate for 3 h at 25 °C.
3. Add 30 µL of RNase-free water to each sample and purify ligated product using Zymo RNA Clean and Concentrator-5 kit. Note that the kit efficiently removes un-ligated linker (*see* Fig. 5). At the final step, elute the ligated product in 11 µL of 10 mM Tris-HCl (pH 8.0) solution. Final elute is ~10 µL.

3.5.6 Reverse Transcription and Circularization

1. Add 2 µL of 0.25 µM reverse transcription primer to 10 µL of linker-ligated product.
2. Denature the sample for 2 min at 80 °C and immediately cool on ice.
3. Add 8 µL of reverse transcription master mix to each sample to make a 20 µL reaction (Master mix: 4 µL of first-strand buffer, 1 µL of 10 nM dNTP, 1 µL of 0.1 M DTT, 1 µL of SUPERase inhibitor, and 1 µL of Super Script III).
4. Hydrolyze the RNA template by adding 2.2 µL of 1 N NaOH and incubate at 90 °C for 20 min.
5. Precipitate the RT product. Add 20 µL of 3 M sodium acetate (pH 5.5), 2 µL of Glycoblue, 156 µL distilled water, 300 µL isopropanol and precipitate on dry ice for 30 min. Pellet the DNA for 30 min as described and resuspend the RT product in 10 µL distilled water.

6. Prepare 2 μL of 0.25 μM RT primer (mixed with 3 μL of 10 nM Tris-HCl, pH 8.0 and 5 μL of 2 $\times$ denaturing loading buffer) and 1 μL of 10 bp DNA ladder as control. Add 10 μL of 2 $\times$ loading buffer to each sample. Denature the sample at 80 $^{\circ}\text{C}$ for 90 s and immediately cool on ice.
7. Separate the sample using 7.5% polyacrylamide TBE-Urea gel in 0.5 $\times$ TBE buffer with constant power (5 W) for 1 h and 30 min until the bromophenol blue dye reaches the end of the gel (*see* Fig. 6).
8. Excise the reverse transcription product and transfer to non-sticky eppi-tubes. 2 mL tubes are recommended for DNA extraction. Avoid any contamination from un-extended RT primer.
9. Overnight gel extraction. Add 400 μL of DNA gel extraction buffer and freeze the sample for 30 min on dry ice. Leave the sample overnight (15 h is recommended) with gentle mixing. Collect the entire solution and transfer it to non-stick RNase-free tubes (*see* Note 2). Precipitate the footprints by adding 2 μL Glycoblue and 500 μL isopropanol. Carry out precipitation for 30 min or more on dry ice. Centrifuge at maximum speed at 4 $^{\circ}\text{C}$ for 30 min using tabletop centrifuge. Completely remove the supernatant and allow air dry for 5 min. Dissolve the footprint in 15 μL of 10 mM Tris-HCl (pH 8.0).
10. Circularization reaction according to the manufacturer's protocol. Mix 15 μL of gel extracted product, 2 μL of CircLigase buffer, 1 μL of 1 mM ATP, 1 μL of 10 nM MnCl_2 and 1 μL of CircLigase per sample, incubate the reaction for 1 h at 60 $^{\circ}\text{C}$ and heat-inactivate for 10 min at 80 $^{\circ}\text{C}$.
11. Recover the final DNA product. Add 74 μL distilled water, 6 μL of 5 M NaCl, 2 μL of Glycoblue and 150 μL of isopropanol and precipitate DNA as described in **step 9** in this session.
12. Resuspend the circularized DNA product in 5 μL of 10 mM Tris-HCl (pH 8.0).

3.5.7 Indexed Library Generation

1. PCR amplification of circularized library using Phusion polymerase according to the manufacturer's instruction. Run several different cycles (i.e., 4, 6, and 8 cycles) for comparison. Use different barcode reverse primers (Barcode_Reverse_##) for different samples with a single forward primer (Barcode_Forward).
2. Add 6 $\times$ DNA loading dye to each PCR product and prepare 1 μL 10 bp DNA ladder as control.
3. Separate the PCR products using 8% polyacrylamide TBE gel, for 90 min at 180 V in 0.5 $\times$ TBE buffer, or until the bromophenol blue dye reaches to two-third of the gel (*see* Fig. 7).
4. Stain the gel for 3 min in 1 $\times$ SYBR Gold in 0.5 $\times$ TBE buffer.

5. Excise the ~175 nt PCR product from the gel and carefully exclude slowly migrating smeared band (above 200 nt), bands from un-extended reverse transcription (~145 nt), template and primer (less than 100 nt) (see Fig. 7).
6. Overnight gel extraction. Add 400 μ L of DNA gel extraction buffer and freeze the sample for 30 min on dry ice. Leave the sample overnight (15 h is recommended) with gentle mixing. Collect the entire solution and transfer it to non-stick RNase-free eppi tubes. Precipitate the footprints by adding 2 μ L Glycoblue and 500 μ L isopropanol. Carry out precipitation for 30 min or more on dry ice. Centrifuge at maximum speed at 4 °C for 30 min using tabletop centrifuge. Completely remove the supernatant and air dry for 5 min. Dissolve the footprint in 15 μ L of 10 mM Tris–HCl (pH 8.0).
7. Measure DNA concentration and combine equal amount from each sample for Illumina sequencing.

3.6 Illumina Sequencing

1. The end product of this protocol results in a fully functional sequencing library compatible with Illumina HiSeq, MiSeq, and NextSeq sequencing platforms. The user should follow standard procedures recommended by Illumina for adjusting the final library concentration for loading onto an Illumina flow-cell and sequencing appropriate for the sequencer used. We recommend a minimum of single-end 50 bp read lengths with six base indexing reads for downstream demultiplexing as described here. Sequencing protocols for polysome fraction and ribosome profiling are identical.

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Fig. 1.
Concurrent polysome profiling and ribosome profiling to investigate translome in vivo

Fig. 2.

Western blot analysis showing the efficient separation of cytosolic from nuclear fractions. After the cytosolic fractions are harvested, remaining pellets (nuclear fractions) were further lysed with 1% NP-40 lysis buffer with standard procedure. Note that the nuclear marker, HDAC1, is mainly present in the nucleus while the cytosolic marker, GAPDH, is present exclusively in cytosolic fractions

Fig. 3. A254 profiles before and after RNase I digestion. Profiles are from 25.5h activated wild type B cells

Fig. 4. Size selection of ribosome footprints from RNase I treated monosome fractions. Representative gel images before and after gel cutting are shown. Brackets indicate the location of excised gel bands

Fig. 5.

Confirm the efficiency of linker ligation. Zymo RNA Clean and Concentrator-5 purified linker ligated RNA marker or footprints. Un-ligated linkers are completely removed by the Zymo kit, therefore do not need this gel running steps for experimental samples

Fig. 6. Representative gel picture showing RT product of linker-ligated 26nt/34nt RNA marker and footprint

Fig. 7.
Representative gel pictures of barcoded PCR products

Table 1

The strength and weakness of polysome profiling and ribosome profiling

Method	Strength	Weakness
Polysome profiling	<ul style="list-style-type: none">• Direct measurement of ribosome density per mRNA.• Well-established protocol.	<ul style="list-style-type: none">• Low resolution for heavy polysome fractions.• Presence of pseudopolysomes
Ribosome profiling	<ul style="list-style-type: none">• Retain positional information of ribosome at the sub-codon level. Suitable for revealing alternative translation initiation sites or codon-specific translational regulation.• Retain footprint length information, which can indicate different conformations of ribosome or the presence of disomes.	<ul style="list-style-type: none">• Indirect estimation of translation efficiency. Results need to be normalized against mRNA abundance.• Short read length (~30 nts). Ribosome footprints can be aligned to multiple genetic locations.• Footprints present in 5' UTR or 3' UTR may diminish the differential footprint abundance in coding region and underestimate the contribution of translational regulation.

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Table 2

Expected RNA amount at each step, starting with 50 million 25.5 h activated B cells

Step	Method	Expected RNA amount
3.5.1	Total RNA from cytosolic fractions and miRNeasy purification	~200 µg
3.5.2	RNase I treated, selection of ribosome protected monosome from sucrose gradient and miRNeasy purification	~15 µg
3.5.3	26–32 nt RNA gel purification	~1 µg
3.5.4	rRNA depletion using Ribo-zero kit	~200 ng
3.5.5	Linker ligation	90% efficiency