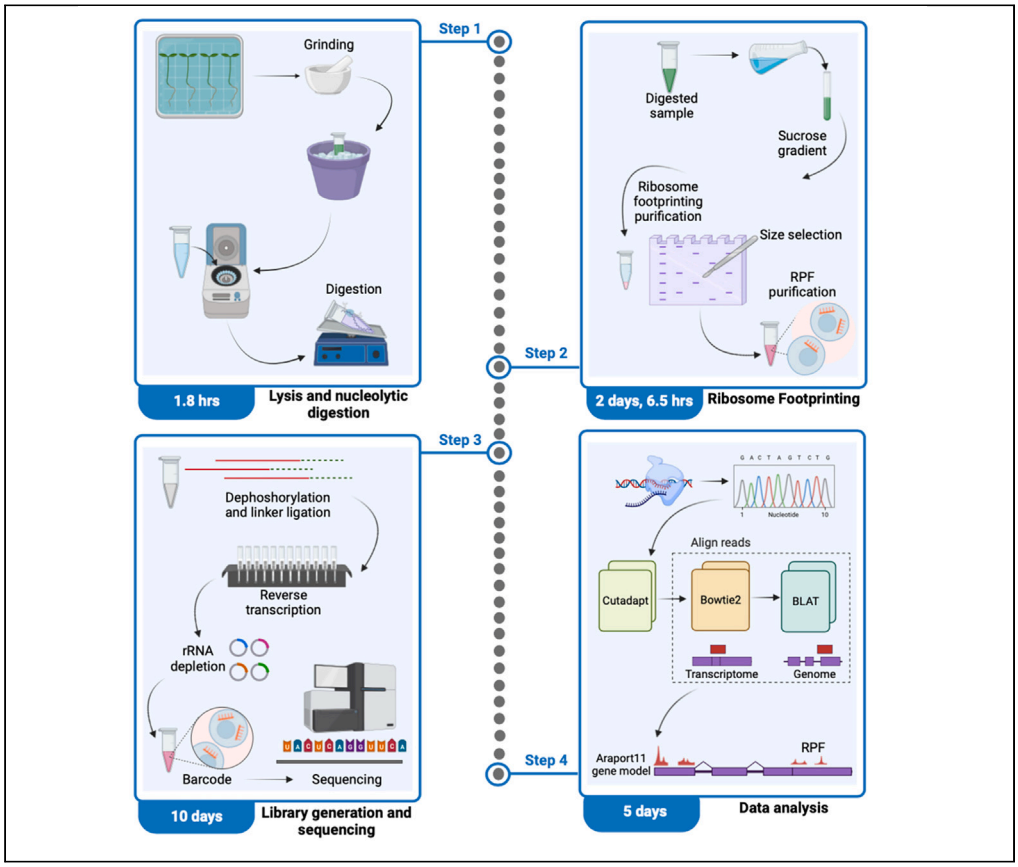
STAR Protocols

CellPress
OPEN ACCESS

Protocol

Protocol to measure ribosome density along mRNA transcripts of *Arabidopsis thaliana* tissues using Ribo-seq



Ribosome profiling (Ribo-seq) measures ribosome density along messenger RNA (mRNA) transcripts and is used to estimate the "translational fitness" of a given mRNA in response to environmental or developmental cues with high resolution. Here, we describe a protocol for Ribo-seq in plants adapted for the model plant *Arabidopsis thaliana*. We describe steps for lysis and nucleolytic digestion and ribosome footprinting. We then detail library construction, sequencing, and data analysis.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

Isabel Cristina Vélez-Bermúdez, Shu-Jen Chou, Ai-Ping Chen, Wen-Dar Lin, Wolfgang Schmidt

icvb@gate.sinica.edu.tw (I.C.V.-B.)
wosh@gate.sinica.edu.tw (W.S.)

Highlights

Steps to prepare lysates for mRNA isolation from *Arabidopsis thaliana* tissues

Monosome isolation and RNA purification for RNA footprinting

Circularization and rRNA depletion for Ribo-seq data

Library construction and sequencing for data analysis

Vélez-Bermúdez et al., STAR Protocols 4, 102520 September 15, 2023
https://doi.org/10.1016/j.xpro.2023.102520

STAR Protocols

CellPress
OPEN ACCESS

Protocol

Protocol to measure ribosome density along mRNA transcripts of *Arabidopsis thaliana* tissues using Ribo-seq

Isabel Cristina Vélez-Bermúdez,[1,4,*] Shu-Jen Chou,[2] Ai-Ping Chen,[2] Wen-Dar Lin,[3] and Wolfgang Schmidt[1,5,*]

[1]Institute of Plant and Microbial Biology, Academia Sinica, Taipei 11529, Taiwan

[2]Institute of Plant and Microbial Biology, Genomic Technology Core, Academia Sinica, Taipei 11529, Taiwan

[3]Institute of Plant and Microbial Biology, Bioinformatics Core Lab, Academia Sinica, Taipei 11529, Taiwan

[4]Technical contact

[5]Lead contact

*Correspondence: icvb@gate.sinica.edu.tw (I.C.V.-B.), wosh@gate.sinica.edu.tw (W.S.)
https://doi.org/10.1016/j.xpro.2023.102520

SUMMARY

Ribosome profiling (Ribo-seq) measures ribosome density along messenger RNA (mRNA) transcripts and is used to estimate the "translational fitness" of a given mRNA in response to environmental or developmental cues with high resolution. Here, we describe a protocol for Ribo-seq in plants adapted for the model plant *Arabidopsis thaliana*. We describe steps for lysis and nucleolytic digestion and ribosome footprinting. We then detail library construction, sequencing, and data analysis.

BEFORE YOU BEGIN

Introduction

Ribosome profiling (Ribo-seq), is a method based on next-generation sequencing of ribosome-protected mRNA fragments that measures translational regulation and allows for detailed and accurate *in vivo* analysis of protein production. Ribo-seq can provide the exact position of the ribosomes to be translated under precisely controlled conditions and determine which mRNAs are being actively translated, contributing to a better understanding of translation and its regulation.[1,2] One of the main and distinctive features of Ribo-seq compared to RNA-seq is its triplet periodicity. This pattern is not observable in data derived from RNA-seq experiments, and a strong framing preference is indicative of high quality Ribo-seq data. Among the particular strengths of the Ribo-seq methodology are the sensitivity and precision of the quantification, the provision of positional information, and the instantaneous, snapshot-like measurements of translation.

Since the development of ribosome profiling and the publication of the method—initially adapted to mammalian cells[3] —several Ribo-seq protocols have been published for the model plant *Arabidopsis thaliana*.[4–9] The main steps involved in performing ribosome profiling are harvesting and lysate preparation, ribonuclease digestion, size selection, rRNA depletion, linker ligation and reverse transcription, library preparation, and data analysis.

In Arabidopsis, Ribo-seq surveys have allowed for a better understanding of the regulation of gene expression during translation and generated insights into translational elongation. Ribo-seq has been used to study translation during plant development,[6,7,10–12] pathogen attack,[13,14] abiotic

stress,[15–17] and in organelles such as mitochondria.[18] Ribo-seq has also been employed to improve genome annotation.[6,19]

Prepare buffers

Timing: 4 h

The day before starting the protocol, the materials listed below should be prepared in advance.

1. Prepare 20% Triton X-100 by mixing 20 mL of Triton X-100 in 80 mL nuclease-free water and mix until obtaining a clear, transparent, and homogeneous solution using a magnetic stirrer.

   Note: Triton X-100 can be stored indefinitely at 4°C.

   ⚠ CRITICAL: Triton X-100 can cause serious skin and eye irritation. Use only in a well-ventilated area and wear eye/face protection.

2. Prepare 0.5 M EDTA (pH 8.0) by dissolving 18.61 g EDTA disodium salt in nuclease-free water until 100 mL, adjust the pH with NaOH, and autoclave to sterilize the solution.

   ⚠ CRITICAL: EDTA can cause serious eye irritation. Use only in a well-ventilated area and wear eye/face protection.

   Alternatives: In our hands, UltraPure 0.5 M EDTA pH 8.0 (Invitrogen, Cat#15575020) worked well for the protocol.

3. Prepare 2× denaturing loading buffer by dissolving 15 mg bromophenol blue in 1.0 mL of 0.5 M EDTA and add 200 μL to 9.8 mL formamide.

   Note: 2× Denaturing loading buffer can be stored indefinitely at room temperature.

   ⚠ CRITICAL: Formamide is highly toxic, suspected of causing cancer and fertility problems. It is recommended to work under a chemical hood. Do not inhale the substance and avoid the generation of vapor. Dispose contents to an approved waste disposal plant.

4. Prepare RNA gel extraction buffer as shown in the table below:

Reagent	Final concentration	Amount
NaOAc (pH 5.5; 3 M)	300 mM	1 mL
EDTA (0.5 M)	1.0 mM	0.02 mL
SDS (20%)	0.25%	0.125 mL
Nuclease-free water	N/A	8.855 mL
Total	**N/A**	**10 mL**

   Note: RNA gel extraction buffer can be stored indefinitely at room temperature.

   ⚠ CRITICAL: SDS is a flammable solid, harmful if swallowed or inhaled. Can cause serious eye damage as well as skin and respiratory irritation. Avoid breathing dust and wear eye/face protection.

5. Prepare DNA gel extraction buffer as shown in the table below:

Reagent	Final concentration	Amount
NaCl (5 M)	300 mM	0.6 mL
Tris-HCl (pH 8.0, 1 M)	10 mM	0.1 mL
EDTA (0.5 M)	1 mM	0.02 mL
Nuclease-free water	N/A	9.28 mL
Total	**N/A**	**10 mL**

Note: DNA gel extraction buffer can be stored indefinitely at room temperature.

6. Prepare 3 M NaOAc (pH 5.5) by mixing 246.1 g of sodium acetate in nuclease-free water to a final volume of 1 L and adjust the pH with glacial acetic acid.

Alternatives: In our hands, nuclease-free 3 M NaOAc (Ambion, Cat#AM9740) worked well for the protocol.

Note: NaOAc can be stored indefinitely at room temperature.

7. Prepare the NaOH solution (1 N) by dissolving 40 g of NaOH pellets in nuclease-free water to a final volume of 1 L with constant stirring.

Note: Since NaOH is hygroscopic and can react with atmospheric carbon dioxide, it is difficult to obtain NaOH pellets that are pure sodium hydroxide. We recommend to standardize a more concentrated solution and dilute as necessary. NaOH can be stored indefinitely at room temperature.

⚠ CRITICAL: NaOH is highly corrosive an can cause severe skin burns and eye damage. It is advised to wear gloves of impermeable material and resistant to the product as well as safety glasses with side shields or goggles.

8. Prepare the subtraction oligos at a concentration of 10 mM each.

Note: Subtraction oligos can be stored indefinitely at −20°C.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Arabidopsis roots and shoots (14-day-old)	TAIR	https://www.arabidopsis.org/
Chemicals, peptides, and recombinant proteins		
Cycloheximide (100 mg/mL)	Sigma-Aldrich	Cat#C4859-1ML
UltraPure 1 M Tris-HCl (pH 8.0)	Invitrogen	Cat#15568025
Trizma hydrochloride solution (pH 7.4)	Sigma-Aldrich	Cat#T2194-1L
NaCl (5 M), RNase-free	Invitrogen	Cat#AM9760G
$MgCl_2$ (1 M)	Ambion	Cat#AM9530G
Polyoxyethylene (10) tridecyl ether	Sigma-Aldrich	Cat#78330-21-9
Deoxycholic acid	Sigma-Aldrich	Cat#83-44-3
KCl (2 M), RNase-free	Invitrogen	Cat#AM9640G
TRITON X-100 detergent, molecular biology grade	Calbiochem	Cat#648466

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
DL-dithiothreitol solution	Sigma-Aldrich	Cat#43816
Ethanol. 200 proof (absolute), molecular biology grade	Sigma-Aldrich	Cat#E7023-500ML
Isopropanol, molecular biology grade	VWR	Cat#87000-048
TRIzol reagent	Ambion	Cat#15596-018
SUPERase*In RNase inhibitor (20 U/μL)	Ambion	Cat#AM2694
TURBO DNase (2 U/μL)	Ambion	Cat#AM2238
RNase I (cloned) 100 U/μL	Invitrogen	Cat#AM2294
UltraPure DNase/RNase-free distilled water	Invitrogen	Cat#10977-023
SDS solution, molecular biology grade (10% w/v)	Promega	Cat#V6551
UltraPure 0.5 M EDTA (pH 8.0)	Invitrogen	Cat#15575020
3 M sodium acetate (pH 5.5)	Ambion	Cat#AM9740
Formamide, molecular biology grade	Promega	Cat#H5051
Bromophenol blue	Bio-Rad	Cat#161-0404
TBE buffer, 10×, molecular biology grade	Promega	Cat#V4251
10,000× SYBR Gold	Invitrogen	Cat#S11494
Ultra Low Range DNA Ladder	Invitrogen	Cat#10597012
GlycoBlue coprecipitant (15 mg/mL)	Invitrogen	Cat#AM9515
Sucrose	Sigma-Aldrich	Cat#S0389
Diethyl pyrocarbonate (DEPC)	Sigma-Aldrich	Cat#D5758
RNaseZap RNase Decontamination Solution	Invitrogen	Cat#AM9780
T4 Polynucleotide kinase reaction buffer (10× T4 PNK buffer)	New England Biolabs	Cat#M0236S
T4 Polynucleotide kinase (10 U/μL)	New England Biolabs	Cat#M0201S
Linear acrylamide (5 mg/mL) (1 mL tube)	Ambion	Cat#AM9515
T4 RNA ligase 2, truncated (200 U/μL) T4 Rnl2(tr), supplied with PEG 8000 50% (wt/vol) and 10× T4 Rnl2 buffer	New England Biolabs	Cat#M0242L
Universal miRNA cloning linker (Linker #1)	New England Biolabs	Cat#S1315S
Novex TBE-urea sample buffer (2×)	Thermo Fisher Scientific	Cat#LC6876
Low-range ssRNA ladder	New England Biolabs	Cat#N0364S
dNTP mix (10 mM)	Invitrogen	Cat#18427-013
SuperScript III	Invitrogen	Cat#18080044
CircLigase	Epicentre Biotechnologies	Cat#CL4115K
Dynabeads MyOne Streptavidin C1	Invitrogen	Cat#65001
Critical commercial assays		
Qubit RNA HS assay kit	Thermo Fisher Scientific	Cat#Q32855
Agilent small RNA kit	Agilent Technologies	Cat#5067-1548
Qubit dsDNA HS assay kit	Thermo Fisher Scientific	Cat#Q33231
Agilent high-sensitivity DNA kit	Agilent Technologies	Cat#5067-4626
Oligonucleotides		
Upper size marker oligoribonucleotide NI-NI-19	Ingolia et al.[3]	5′- AUGUACACGGAGUCGAGC UCAACCCGCAACGCGA-(Phos)
Lower size marker oligoribonucleotide NI-NI-20	Ingolia et al.[3]	5′-AUGUACACGGAGUCGACC CAACGCGA-(Phos)
Reverse transcription primer	This study	5′-/5Phos/GATCGTCGGACTG TAGAACTCTGAACGTGTAGAT CTCGGTGGTCG-(SpC18)-CACT CA-(SpC18)-TTCAGACGTGTGC TCTTCCGATCTATTGATGGTGC CTACAG-3′
Subtraction oligo 1	This study	/5BioTEG/CATAAACGATGCC GACCAGGGATCAGCGG
Subtraction oligo 2	This study	/5BiotinTEG/CTCTGATGATTCA TGATAACTCGACGGATC GCATGG

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Subtraction oligo 3	This study	/5BiotinTEG/CATTAGCATGGG ATAACATCAT
Subtraction oligo 4	This study	/5BiotinTEG/TGCCAAGGATGT TTTCATTAATCAAGAACG
Forward library primer (Modified from Illumina)	Oligonucleotide sequences © 2007–2013 Illumina, Inc. All rights reserved. Derivative works created by Illumina customers are authorized for use with Illumina instruments and products only. All other uses are strictly prohibited.	5′ AATGATACGGCGA CCACCGAGATCTACACGTTC
Reverse indexed primer 3 (Modified from Illumina)	Oligonucleotide sequences © 2007–2013 Illumina, Inc. All rights reserved. Derivative works created by Illumina customers are authorized for use with Illumina instruments and products only. All other uses are strictly prohibited.	5′ CAAGCAGAAGACGGC ATACGAGATGCCTAAGT GACTGGAGTTCAGACG TGTGCTCTTCCGATC
Reverse indexed primer 4 (Modified from Illumina)	Oligonucleotide sequences © 2007–2013 Illumina, Inc. All rights reserved. Derivative works created by Illumina customers are authorized for use with Illumina instruments and products only. All other uses are strictly prohibited.	5′ CAAGCAGAAGACG GCATACGAGATTGGT CAGTGACTGGAGTTCAGA CGTGTGCTCTTCCGATC
Reverse indexed primer 6 (Modified from Illumina)	Oligonucleotide sequences © 2007–2013 Illumina, Inc. All rights reserved. Derivative works created by Illumina customers are authorized for use with Illumina instruments and products only. All other uses are strictly prohibited.	5′ CAAGCAGAAGACGGC ATACGAGATATTGGCGT GACTGGAGTTCAGACGT GTGCTCTTCCGATC
Reverse indexed primer 7 (Modified from Illumina)	Oligonucleotide sequences © 2007–2013 Illumina, Inc. All rights reserved. Derivative works created by Illumina customers are authorized for use with Illumina instruments and products only. All other uses are strictly prohibited.	5′ CAAGCAGAAGACGG CATACGAGATGATCTG GTGACTGGAGTTCAGA CGTGTGCTCTTCCGATC
Reverse indexed primer 12 (Modified from Illumina)	Oligonucleotide sequences © 2007–2013 Illumina, Inc. All rights reserved. Derivative works created by Illumina customers are authorized for use with Illumina instruments and products only. All other uses are strictly prohibited.	5′ CAAGCAGAAGACGG CATACGAGATTACAAG GTGACTGGAGTTCAGA CGTGTGCTCTTCCGATC
Reverse indexed primer 23 (Modified from Illumina)	Oligonucleotide sequences © 2007–2013 Illumina, Inc. All rights reserved. Derivative works created by Illumina customers are authorized for use with Illumina instruments and products only. All other uses are strictly prohibited.	5′CAAGCAGAAGACGGCA TACGAGATCCACTCGTGA CTGGAGTTCAGACGTGTG CTCTTCCGATC
Reverse indexed primer 27 (Modified from Illumina)	Oligonucleotide sequences © 2007–2013 Illumina, Inc. All rights reserved. Derivative works created by Illumina customers are authorized for use with Illumina instruments and products only. All other uses are strictly prohibited.	5′ CAAGCAGAAGACGG CATACGAGATAGGAAT GTGACTGGAGTTCAGA CGTGTGCTCTTCCGATC
Deposited data		
Packaged programs	This study	https://maccu.project.sinica.edu.tw/20230330/
Software and algorithms		
Cutadapt	Martin[20]	https://github.com/marcelm/cutadapt
Bowtie2	Langmead and Salzberg[21]	https://bowtie-bio.sourceforge.net/bowtie2/index.shtml
Samtools	Li et al.[22]	http://www.htslib.org/
RackJ	This study	https://rackj.sourceforge.net/
Araport11	Cheng et al.[23]	https://www.arabidopsis.org/download/index-auto.jsp?dir=%2Fdownload_files%2FGenes%2FAraport11_genome_release
Other		
Nonstick, RNase-free microfuge tubes, 1.5 mL	Applied Biosystems	Cat#AM12450 Cat#AM12450

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Polycarbonate centrifuge tubes (13 mm × 56 mm)	Beckman Coulter	Cat#362305
Pipette filter tips, 10 µL	Labcon	Cat#1051-800-000-9
Pipette filter tips, 200 µL	Labcon	Cat#1059-800-000-9
Pipette filter tips, 1000 µL	Labcon	Cat#1097-965-008-9
Eppendorf centrifuge	Eppendorf	Cat#5417R
Novex TBE-urea gels, 10%, 12 well	Invitrogen	Cat#EC68752BOX
Novex TBE-urea gels, 15%, 12 well	Invitrogen	Cat#EC68852BOX
Mini gel tank	Thermo Fisher	Cat#A25977
Premium sterile surgical blades	Kiato	Cat#18111
Supertek Scientific porcelain mortars and pestles (10 cm)	Fisher Scientific	Cat#S09570B
Liquid nitrogen	N/A	N/A
Labnet Prism R refrigerated microcentrifuge	Merck	Cat#Z723762
Vortex-Genie 2	Scientific Industries, Inc.	Cat#SI-0236
ELMI Intelli-Mixer RM-2L	ELMI	N/A
BioVoltTM power supply	BioProducts	Cat#SBE250
Optima TLX ultracentrifuge	Beckman	Cat#361545
TLA 100.3 rotor	Beckman	Cat#349481
Transilluminator LED MaestroGen - model LB-16	DIVERS DUTSCHER	Cat#887005
Spin-X cellulose acetate filter column	Corning	Cat#8162
Thermal cycler	Bio-Rad	Cat#170-9713
MF-Millipore membrane filter, 0.22 µm pore size	Millipore	Cat#GSWP04700
DynaMag-2 magnet	Invitrogen	Cat#12321D
ThermoMixer	VWR	Cat#21516-170
Qubit Flex fluorometer	Thermo Fisher Scientific	Cat#Q33327
2100 BioAnalyzer	Agilent Technologies	Cat#G2940CA
HiSeq 3000/HiSeq 4000 sequencing system	Illumina	N/A
Computer hardware (a 64-bit linux server with at least 16 GB of RAM)	N/A	N/A

MATERIALS AND EQUIPMENT

Polysome buffer

Reagent	Final concentration	Amount
NaCl (5 M)	150 mM	0.6 mL
$MgCl_2$ (1 M)	5 mM	0.1 mL
Tris-HCl (pH 7.4; 1 M)	20 mM	0.4 mL
DTT (1 M)	1 mM	0.02 mL
Cycloheximide (100 mg/mL)	100 µg/mL	0.02 mL
Nuclease-free water	N/A	18.86 mL
Total	**N/A**	**20 mL**

Note: Prepare fresh with RNase-free reagents and keep on ice.

⚠ CRITICAL: Cycloheximide is highly toxic, contact can cause skin and eye irritation. Use personal protective equipment as required, do not eat, drink, or smoke when using this product and dispose the contents to an approved waste disposal plant.

Lysis buffer D (Option A)[6]		
Reagent	Final concentration	Amount
Polyoxyethylene (10) tridecyl ether (20%)	2% (vol/vol)	1 mL
Deoxycholic acid (20%)	1%	0.5 mL
DTT (1 M)	1 mM	0.01 mL
Cycloheximide (100 mg/mL)	100 μg/mL	0.01 mL
Turbo DNase I (2 U/μL)	10 U/mL	0.05 mL
Tris-HCl (pH 8.0; 1 M)	100 mM	1 mL
KCl (2 M)	40 mM	0.2 mL
$MgCl_2$ (1 M)	20 mM	0.2 mL
Nuclease-free water	N/A	7.03 mL
Total	**N/A**	**10 mL**

Note: Prepare fresh with RNase-free reagents. Keep the lysis buffer on ice. The 20% polyoxyethylene (10) tridecyl ether should be prepared by dissolving 2 g in 10 mL of nuclease-free water, 20% deoxycholic acid should be prepared by mixing 2 g in 10 mL of nuclease-free water. Mix the compounds until the solutions are homogeneous using a magnetic stirrer.

⚠ CRITICAL: Polyoxyethylene (10) tridecyl ether can causes serious eye damage. Avoid contact with skin and eyes, do not breath mist, gas, or vapours. Wear protective gloves, protective clothing, eye protection, and face protection. Do not discharge into the environment. Deoxycholic acid is considered as acute oral toxicity. Do not eat, drink, or smoke when using this product. Wear personal protective equipment/face protection, ensure adequate ventilation and avoid dust formation.

Lysis buffer (Option B)[3]		
Reagent	Final concentration	Amount
Polysome buffer	N/A	9.375 mL
Triton X-100 (20%)	1%	0.5 mL
Turbo DNase I (2 U/μL)	25 U/mL	0.125 mL
Total	**N/A**	**10 mL**

Note: Prepare fresh with RNase-free reagents. Keep the lysis buffer on ice.

Sucrose cushion buffer		
Reagent	Final concentration	Amount
Polysome buffer	N/A	15.6 mL
Sucrose	1 M	6.8 g
SUPERase*In (20 U/μL)	20 U/mL	0.02 mL
Total	**N/A**	**20 mL**

Note: Prepare fresh with RNase-free reagents and keep the buffer on ice. The sucrose solution is ~34% (wt/vol).

2× bind/wash buffer		
Reagent	Final concentration	Amount
NaCl (5 M)	2 M	4 mL
Tris-HCl (pH 7.4; 1 M)	5 mM	0.05 mL
EDTA (0.5 M)	1 mM	0.02 mL
Triton X-100 (20%)	0.20%	0.1 mL
Nuclease-free water	N/A	5.83 mL
Total	**N/A**	**10 mL**

Note: 2× bind/wash buffer should be prepared fresh.

STEP-BY-STEP METHOD DETAILS

Preparation of lysates

Timing: 1 h

For this step, it is very critical to immobilize the ribosomes on the mRNA. To maintain the *in vivo* translational status, it is necessary to freeze the samples with liquid nitrogen, to conduct the lysis in the presence of the translation inhibitor cycloheximide and under conditions that inhibit the activity of RNases.

1. Collect the Arabidopsis tissue maintaining the integrity of the roots and shoots.
2. Grind 200 mg of Arabidopsis tissue with liquid nitrogen and resuspend in 600 μL lysis buffer by vortex.

⚠ CRITICAL: Do not use paper tissue to remove excess media on the roots, which would compromise the integrity of the roots and lower the amount and quality of the recovered ribosomes.

⚠ CRITICAL: A successful lysate preparation depends on the quality of grinding.

3. Incubate the samples 20 min on ice and vortex twice during the incubation for 5 s.
4. Centrifuge the samples at 16,000 × *g* for 15 min at 4°C.
5. Recover 500 μL of the soluble supernatant and transfer to a fresh Eppendorf tube.

⚠ CRITICAL: The lysates should be kept on ice until the next step.

RNA digestion

Timing: 45 min

Nuclease digestion is a critical step during the preparation of ribosome footprints since both the stability of ribosomal RNA and the complete conversion of polysomes to monosomes are very important for ribosome profiling. The nuclease treatment step generates both ribosomal RNA fragments and rRNA contaminating the samples, making the choice of an appropriate RNase enzyme and control of the time for sample digestion crucial. Improper digestion potentially compromises ribosomal integrity, which may cause experimental bias and loss of information. Here, we chose RNase I, which degrades single-stranded RNA to cyclic nucleotide monophosphates, leaving a 5′-OH and 2′-, 3′-cyclic monophosphate. While RNase I is able to digest mRNA outside the ribosomes, the integrity of the ribosomes is largely preserved.

6. Add 12.5 μL of RNase I (100 U/μL) to the recovered 500 μL of lysate and incubate for 45 min at room temperature with gentle mixing on a nutator.
7. After the digestion, take 500 μL of lysate and add 16.66 μL of SUPERase*In RNase Inhibitor to stop the nuclease digestion.

Sucrose density gradient fractionation

Timing: 4 h

This step describes the separation of ribosomes from other cellular components by sucrose density gradient ultracentrifugation.

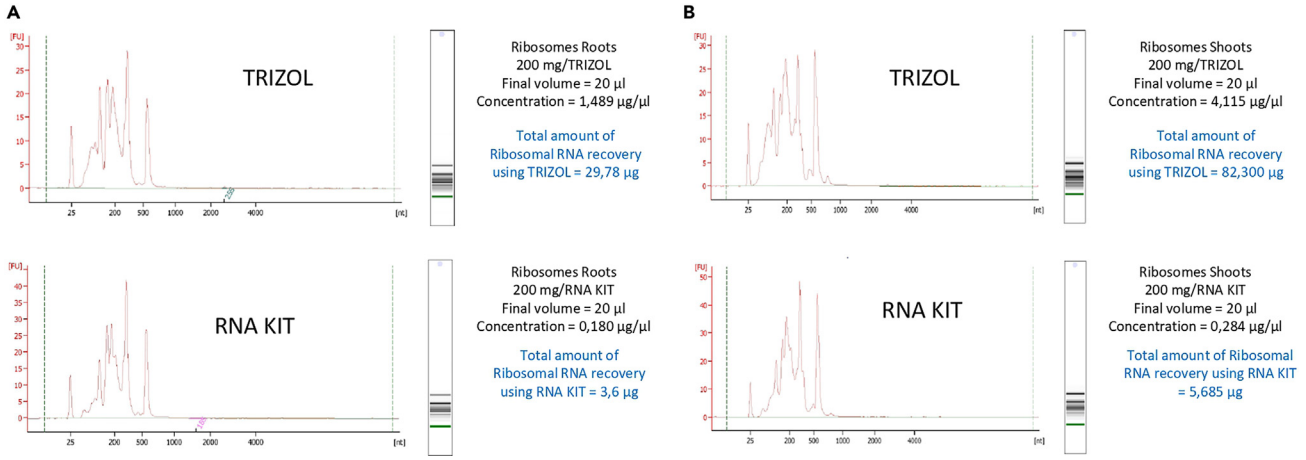


Figure 1. BioAnalyzer image of size selection of ribosome footprint fragments from root and shoot samples

(A and B) Images show RNA purification profiles derived from RNA extraction with Trizol (upper panel) or an RNA extraction kit (lower panel). (A) Root samples; (B) Shoot samples. One µL of a 1/10 dilution was used in each case.

8. Transfer the digested lysate into a 13 × 56 mm polycarbonate ultracentrifuge tube.
9. Slowly add 1.5 mL of 1 M sucrose cushion buffer.

⚠ CRITICAL: During the addition of the sucrose cushion buffer, it is recommended to position the pipette tip against the wall of the ultracentrifuge tube, carefully dropping the sucrose solution to get two distinct layers in order to achieve consistency between the replicates (the lysate will be located on top of the sucrose).

10. Centrifuge the samples in a TLA 100.4 rotor at 548,800 × *g* at 4°C for 4 h to recover the ribosomes.

⚠ CRITICAL: Precool the TLA 100.4 rotor at 4°C in advance to prevent RNA degradation.

RNA footprint extraction

Timing: 1 h 30 min

This step describes the extraction of RNA footprints.

11. Remove the supernatant carefully. Locate the translucent ribosomal pellet, but avoid to touch the bottom of the tube.

Note: Since the ribosomal pellet is translucent and may be hard to detect, you can mark the location of the pellet on the ultracentrifuge tube right after removing the tube from the rotor.

12. Resuspend the ribosomal pellet in 1 mL of TRIzol reagent in a 13 × 56 mm polycarbonate ultracentrifuge tube.

Note: For RNA isolation, we followed the TRIzol protocol from Invitrogen. We compared the yield and quality of the isolated RNA using RNA extraction kits and TRIzol reagent and obtained the best results with TRIzol (Figures 1 and 2).

13. Transfer the resuspended pellet to a fresh 1.5 mL Eppendorf tube, add 0.2 mL of chloroform, shake the tube vigorously by hand for 15 s, and incubate for 3 min at room temperature.

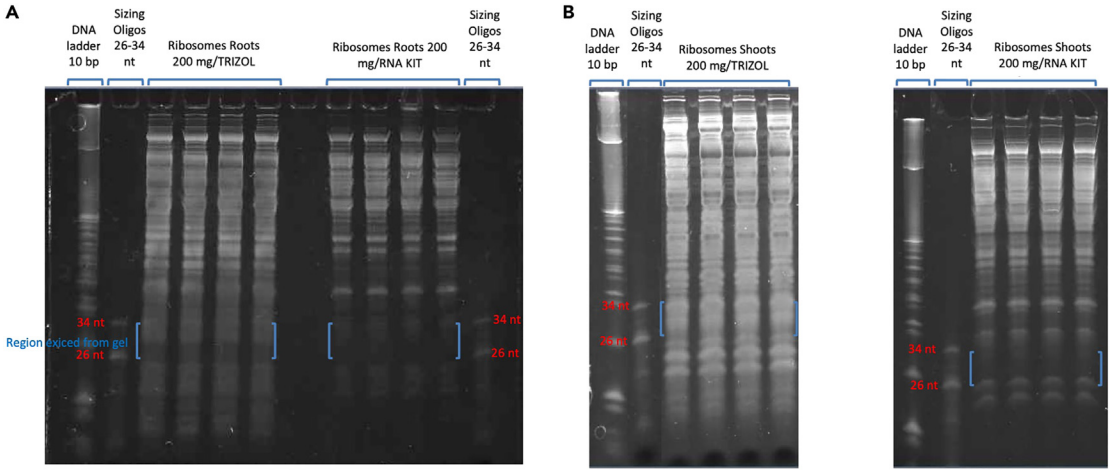


Figure 2. Gel containing ribosome footprints from root and shoot samples using TRIzol or a kit for RNA extraction

(A and B) ribosome footprints from roots (A) and shoots (B). Blue brackets indicate the gel region that should be excised. Upper (34 nt) and lower (26 nt) markers are indicated in the gels.

14. Centrifuge the sample at 12,000 × *g* for 15 min at 4°C.
15. Remove the aqueous phase of the sample very carefully. Avoid contamination of the interphase or organic layer, and pipet the solution into a fresh 1.5 mL tube.
16. Add 0.5 mL of 100% isopropanol to the aqueous phase, vortex for 5 s, incubate at −20°C for 30 min, and centrifuge at 12,000 × *g* for 10 min at 4°C.
17. Remove the supernatant, leaving only the RNA pellet in the tube.
18. Add 1 mL of 75% ethanol to the samples to wash the pellet.

⚠ CRITICAL: Prepare fresh ethanol with RNase-free reagents. The percentage of ethanol should be 75%, a lower percentage can lead to DNA contamination.

19. Centrifuge the samples at 7,500 × *g* for 5 min at 4°C and discard the ethanol.

Note: Remove the ethanol very well using a pipet. Do not disturb the pellet.

20. Air dry the RNA pellet for 10 min at room temperature.
21. Resuspend the RNA pellet in 20 μL of 10 mM Tris-HCl (pH 8.0).

Pause point: RNA can be stored overnight at −20°C or for several months at −80°C.

Footprint fragment extraction

Timing: 3 h 15 min – overnight

This step describes the footprint fragment extraction, where the RNA fragments of 30–34 nt length—corresponding to the ribosome-protected fragments (RPFs)—were excised from a polyacrylamide gel.

22. Pre-run a 15% polyacrylamide TBE-urea gel at 200 V for 15 min using 1 X TBE running buffer.

⚠ CRITICAL: It is advised to keep an electrophoresis tank in the lab free of RNase contamination. If the tank is used for other purposes, is necessary decontaminate the tank by soaking it in 0.1 N sodium hydroxide (NaOH) and 0.1% EDTA in nuclease-free water overnight, and then extensively rinse with nuclease-free water before use. Alternatively, you can also use commercially available products such as RNase ZAP (Ambion).

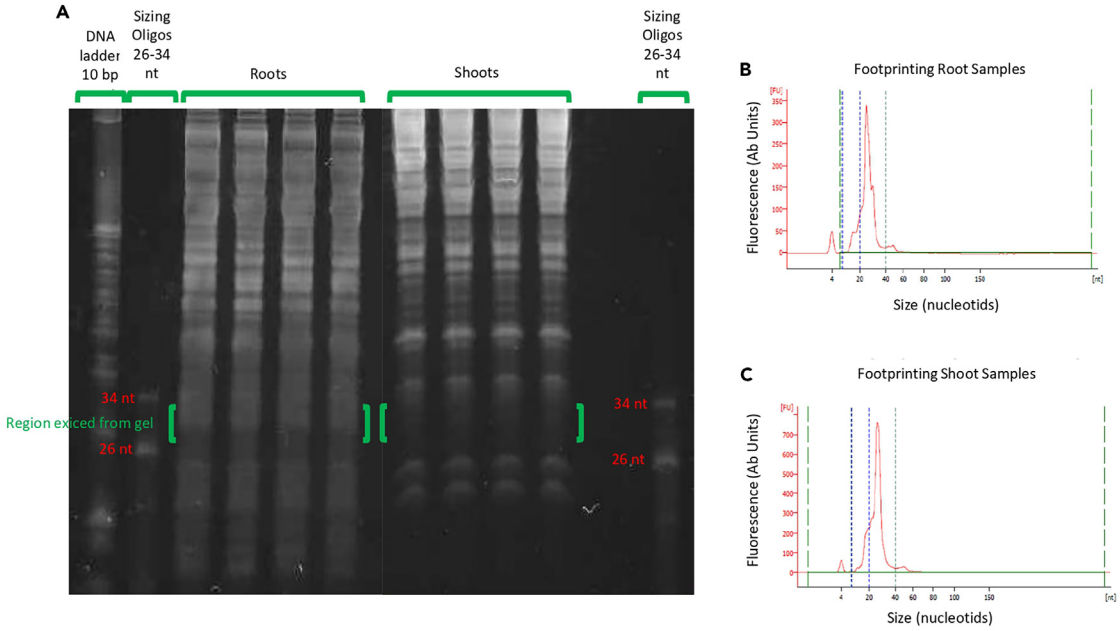


Figure 3. Polyacrylamide gel containing Arabidopsis footprint samples from roots and shoots

(A) Size selection of ribosome footprint samples. Samples derived from lysates of 15 μg of input RNA. Green brackets indicate the gel region that should be excised.

(B) BioAnalyzer profile of footprint samples from roots after gel purification of the excised region.

(C) BioAnalyzer profile of footprint samples from shoots after gel purification of the excised region.

Molecular biology grade water should be used to prepare the running buffer due to the large volume of nuclease-free water needed for this purpose. We suggest to prepare DEPC-treated water as follows: add 1 mL of DEPC (diethylpyrocarbonate) to 1 L of MilliQ water, incubate for 12 h at 37°C, and autoclave the DEPC-water for 15 min to degrade the DEPC. It is advised to wear protective gloves, eye protection, and face protection when manipulating DEPC.

23. Just prior to the electrophoresis, add 10 μL of 2× denaturing sample buffer to each RNA sample, and prepare the upper and lower markers by adding 1.0 μL of upper and lower markers (stock 10 μM), 8.0 μL of 10 mM Tris-HCl (pH 8.0), and 10.0 μL of 2× denaturing sample buffer in a fresh 1.5 mL tube.
24. Prepare a ladder sample with 0.5 μL of 10 bp ladder (1 μg/μL), 4.5 μL of 10 mM Tris-HCl (pH 8.0), and 5.0 μL of 2× denaturing samples buffer.
25. Denature the samples for 90 s at 80°C.
26. Load the samples on the polyacrylamide gel with control oligo sample (mixed upper and lower markers) at both sides of the RNA samples.

⚠ CRITICAL: It is very important to clean the wells before loading the samples on the gel since urea may compromise proper running of the samples.

Note: We suggest to run only 6 μL of each RNA sample in the gel (6 lines in total per sample).

27. Run the gel for 65 min at 200 V.
28. Stain the gel for 3 min with 1× SYBR Gold in 1× TBE running buffer with gentle shaking.
29. Illuminate the stained gel using ultraviolet transillumination and excise the 30 nt – 34 nt region between the NI-NI-19 and NI-NI-20 oligo markers from each footprint sample.

⚠ CRITICAL: RPFs are usually ~30 nt in length. We recommended to cut the middle region between 30 nt – 34 nt to avoid contamination from rRNAs since the major contamination with rRNA was found between ~28 nt and 35 nt (Figure 3A).

30. Transfer each excised gel slice in a 2 mL non-stick RNase-free microfuge tube.

Note: You can also process the oligo marker bands as an internal control for the experiment.

31. Add 400 μL of RNA gel extraction buffer to the polyacrylamide gel slices and freeze the samples for 1 h at −80°C.
32. Incubate the samples overnight on a nutator at room temperature.

Note: Overnight incubation increases the reproducibly of the experiment.

Pause point: At this point, the protocol could be continued the next day.

Purification of footprint fragments

Timing: 30 min - overnight

This step describes the purification of footprint fragments.

33. Centrifuge the samples containing the gel slides at 16,100 × *g* for 15 min at 4°C and transfer the supernatant (400 μL) into a 1.5 mL non-stick RNase-free microfuge tube.
34. Add 1.5 μg of GlycoBlue to each sample and vortex for 5 s.

Note: It is suggested to use GlycoBlue to increase the visibility of the pellet and to avoid losing the recovered RNA footprints.

35. Add 500 μL of isopropanol to the samples and vortex for 5 s. To precipitate the RNA footprints, keep the samples overnight at −80°C.

Pause point: The samples can be stored for one week at −80°C.

RNA footprint recovery

Timing: 1 h

This step describes the RNA footprint recovery.

36. Centrifuge the samples at 16,100 × *g* for 35 min at 4°C.
37. Remove the supernatant and dry the pellet for 10 min at room temperature.
38. Resuspend the RNA footprint pellets in 12 μL of 10 mM Tris-HCl (pH 8.0).

Note: In total, 6 different RNA footprint pellets belonging to a unique sample should be re-suspended together in 12 μL of 10 mM Tris-HCl (pH 8.0) as follows: resuspend the first tube in 12 μL of 10 mM Tris-HCl (pH 8.0). Transfer the resuspended RNA footprints from the first to the second tube, and repeat this procedure until the sixth tube.

Note: It is advised to use 2 μL for the BioAnalyzer to check the quality of the RNA footprints before generating the libraries for sequencing.

Note: The BioAnalyzer profiles after purification of the excised gel regions from roots and shoots are shown in Figures 3B and 3C.

Pause point: The samples can be stored indefinitely at −80°C.

Library construction

Timing: 5 days

The construction of the library is one of the key points of the protocol. Linker ligation and rRNA depletion are the most important steps for up to standard results.

Dephosphorylation of the RNA footprint samples

Timing: 1 h 30 min - overnight

This step describes the dephosphorylation of RNA footprint samples.

39. Add 33 µL of nuclease-free water to each 10 µL of RNA footprints (from step 38) and denaturate for 2 min at 75°C.
40. Equilibrate the samples at 37°C, add the dephosphorylation reaction -outlined below, and incubate for 1 h at 37°C.

Dephosphorylation reaction mix	
Reagent	Amount per sample
RNA sample	25 µL
T4 PNK buffer (10×)	5.0 µL
SUPERase*In (20 U/µL)	0.625 µL
T4 PNK (10 U/µL)	2.5 µL
Nuclease-free water	16.875 µL

41. Incubate the samples for 10 min at 70°C to inactivate the enzyme.
42. Add 450 µL of nuclease-free water, 2.0 µL of 5 mg/mL linear acrylamide, and 50.0 µL of 3 M NaOAc to the samples and mix thoroughly.
43. Add 500 µL of isopropanol, vortex for 5 s, and keep the samples overnight at −80°C.

Pause point: The samples can be stored overnight at −80°C.

Linker ligation of the samples

Timing: 7 h 20 min – overnight

This step describes the linker ligation of ribosome footprints.

44. Centrifuge the samples at 16,100 × *g* for 45 min at 4°C.
45. Add ice-cold 80% ethanol and centrifuge the samples at 16,100 × *g* for 15 min at 4°C.
46. Remove the supernatant and dry the pellet for 5 min at room temperature.
47. Resuspend the samples in 8.5 µL of 10 mM Tris-HCl (pH 7.0).

Pause point: The samples can be stored indefinitely at −80°C.

48. Add 0.5 μL of preadenylated linker (0.25 μg/μL) to the samples.
49. Incubate the samples for 2 min at 75°C and cool them on ice for 15 min.
50. Prepare the ligation reaction and incubate for 4 h at room temperature.

Ligation reaction mix	
Reagent	Amount per sample
RNA and linker	9.0 μL
100% DMSO	2.0 μL
T4 Rnl2 buffer (10×)	2.0 μL
PEG 8000 (50% w/v)	5.0 μL
SUPERase*In (20 U/μL)	1.0 μL
T4 Rnl2(tr) (200 U/μL)	1.0 μL

51. Run the samples in a polyacrylamide gel electrophoresis as follows:
    a. Add 20 μL of 2× NOVEX TBE-urea sample buffer to the linker ligation products.
    b. Prepare an RNA ladder: 2 μL of low range ssRNA ladder, 0.2 μL of 20 μL M lower/upper marker (optional), 5.8 μL of nuclease-free water, and 8 μL of 2× NOVEX sample buffer.
    c. Denature the samples for 5 min at 90°C and place them on ice. Spin-down before use.
    d. Take a 10% TBE-urea gel, wash the wells, and load the samples with a p20 pipette.
    e. Run the gels containing the linker ligation samples for 10 min at 100 V and then for 45 min at 200 V to resolve the fragments well.

    Alternatives: You can adjust the running conditions according to your tank if is different from the brand that we provide in this protocol.

    f. Stain the gel in 30 mL of nuclease-free water and 3 μL of SYBR Gold at 30 rpm for 10 min in the dark.
    g. Visualize the gel with an ultra-slim-LED illuminator.
52. Cut the region near the 43–51 nt (+17 nt) from the gel, and transfer the linker ligation product bands to a 1.5 mL non-stick RNase-free microfuge tube (Figure 4).
53. Grind the gel with a pestle in a 1.5 mL tube.
54. Add 500 μL of RNA gel extraction buffer to the polyacrylamide gel slices.
55. Heat the samples for 10 min at 72°C.
56. Transfer the samples to a Spin-X column (Coring 8160) and centrifuge at 16,100 × *g* for 10 min at 4°C.
57. Transfer 500 μL of the supernatant into a non-stick RNase-free 1.5 mL microfuge tube.
58. Add 50.0 μL of 3 M NaOAc (pH 5.2) and 2.2 μL of 5 mg/mL linear acrylamide to each sample and mix thoroughly.
59. Add 550 μL of isopropanol to each sample and vortex to resuspend well.

Pause point: The samples can be stored overnight at −80°C.

Reverse transcription

Timing: 3 h 45 min – overnight

This step describes the generation of cDNA via reverse transcription.

60. Centrifuge the samples at 16,100 × *g* for 45 min at 4°C.
61. Add ice-cold 80% ethanol and centrifuge the samples at 16,100 × *g* for 15 min at 4°C.
62. Remove the supernatant and dry the pellet for 5 min at room temperature.
63. Resuspend the samples in 10.5 μL of 10 mM Tris-HCl (pH 7.0).

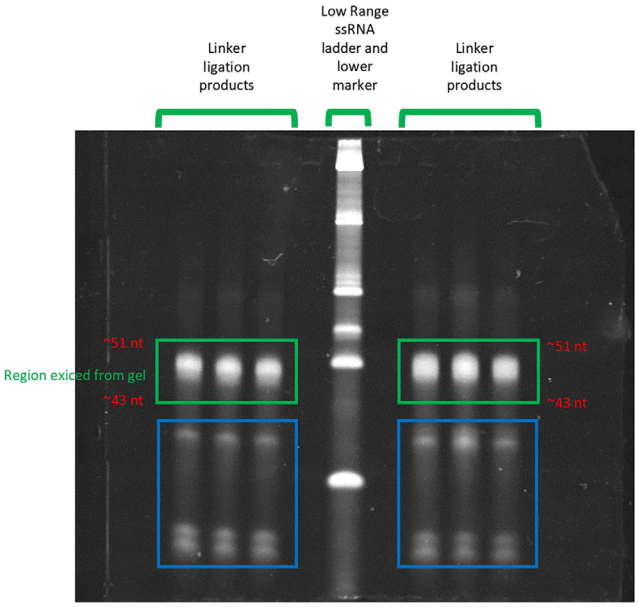


Figure 4. Visualization of the linker ligation fragments after gel electrophoresis

Green boxes indicate the pre-adenylated linker band; non-ligated RPF fragments are marked by blue boxes.

Pause point: The samples can be stored indefinitely at −80°C.

64. Add 0.5 μL of 25 μM reverse transcription primer to the samples.
65. Denature the samples for 5 min at 65°C in the thermal cycler and keep the samples on ice.
66. Prepare and incubate the reverse transcription reaction for 30 min at 48°C in the thermal cycler.

Reverse transcription reaction mix	
Reagent	Amount
Ligation and primer	11.0 μL
First-strand buffer (5×)	3.6 μL
dNTPs (10 mM)	1.0 μL
DTT (0.1M)	0.9 μL
SUPERase*In (20 U/μL)	0.5 μL
SuperScript III (200 U/μL) 1.0	1.0 μL
Nuclease-free water	0 μL

67. Add 2.0 μL of 1 N NaOH to the samples and incubate at 98°C for 20 min.
68. Run the samples in a polyacrylamide gel electrophoresis as previously described in step 51, but switch voltage to 200 V and run the gel for 65 min or use the conditions suggested for your electrophoresis tank.
69. Cut and transfer the reverse-transcription product bands from the gel to a 1.5 mL non-stick DNase-free microfuge tube (Figure 5).
70. Grind the gel with a pestle in a 1.5 mL tube.
71. Add 500 μL of DNA gel extraction buffer to the polyacrylamide gel slices.
72. Heat the samples for 10 min at 72°C.
73. Transfer the samples to a Spin-X column (Coring 8160) and centrifuge at 16,100 × *g* for 10 min at 4°C.
74. Transfer 500 μL of the supernatant into a non-stick DNase-free 1.5 mL microfuge tube.
75. Add 50.0 μL of 3 M NaOAc (pH 5.2) and 2.2 μL of 5 mg/mL linear acrylamide to each sample and mix thoroughly.

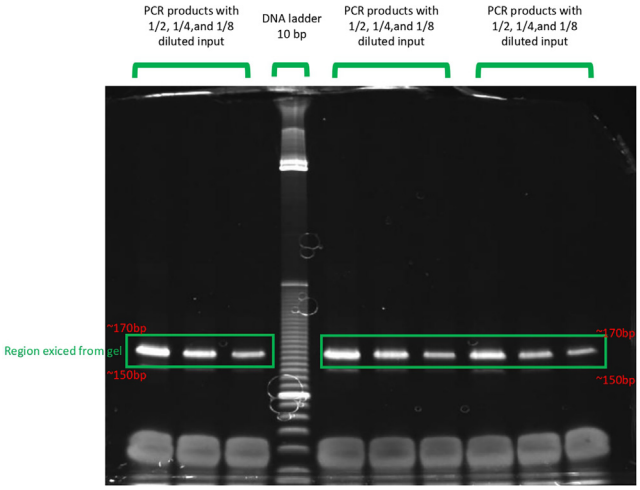


Figure 5. Reverse transcription of ligated footprints
Excised bands are shown in green boxes.

76. Add 550 μL of isopropanol to each sample and vortex to resuspend well.

Note: Instead of marker oligo samples, prepare 1 μL of 25 ng reverse transcription primer, 7 μL of nuclease-free water, and 8 μL of 2× NOVEX sample buffer.

Pause point: The samples can be stored overnight at −80°C.

Circularization and rRNA depletion

Timing: 3 h – overnight

This step describes the circularization and rRNA depletion, a vital step for the generation of high-quality Ribo-seq data.

77. Centrifuge the samples at 16,100 × *g* for 45 min at 4°C.
78. Add ice-cold 80% ethanol and centrifuge the samples at 16,100 × *g* for 15 min at 4°C.
79. Remove the supernatant and dry the pellet for 5 min at room temperature.
80. Resuspend the samples in 15.0 μL of 10 mM Tris-HCl (pH 8.0).

Pause point: The samples can be stored indefinitely at −80°C.

81. Incubate the circularization reaction for 1 h at 60°C and incubate the samples for 10 min at 80°C to inactivate the enzyme.

Circularization reaction mix	
Reagent	Amount
First-strand cDNA	15.0 μL
CircLigase buffer (10×)	2.0 μL
ATP (1 mM)	1.0 μL
$MnCl_2$ (50 mM)	1.0 μL
CircLigase (100 U/μL)	1.0 μL

Pause point: The samples can be stored indefinitely at −20°C.

82. Add 1.0 μL of 200 μM subtraction oligo pool, 1.0 μL of 20× SSC, and 3.0 μL of nuclease-free water to 5.0 μL of circularization reaction.

Subtraction oligo pool mix		
Oligo 200 μM	Pool ratio	Pool volume
Oligo 1	7.5%	2.25 μL
Oligo 2	60.0%	18 μL
Oligo 3	25.0%	7.5 μL
Oligo 4	7.5%	2.25 μL

Note: For designing the subtraction oligos used for removing rRNA from the libraries, it is recommended to conduct a pilot Ribo-seq experiment to identify abundant contaminant sequences. Contamination from rRNA can be similar across biological replicate samples, but can also vary greatly across developmental stages or tissues as well as among samples from different species. Thus, sequencing with fewer reads to evaluate the percentage of rRNA is highly recommended. The question as to whether this step is recommended is determined by the rRNA carry-over degree and rRNA removal efficiency; if the cost of subtraction is lower than the cost of sequencing ribosome RNA, rRNA depletion should be considered.

⚠ CRITICAL: For the RNA depletion steps, it is necessary to use 2 reactions per sample to collect sufficient DNA.

83. Denature the samples for 90 s at 100°C and anneal at 0.1°C/s to 37°C.
84. Incubate the samples for 15 min at 37°C.
85. Prepare MyOne Streptavidin C1 DynaBeads (10 mg/mL) as follows:
    a. Dilute the binding and washing (B&W) buffer (2×) to 1× as a working solution.
    b. Resuspend the Dynabeads in the vial (i.e., vortex for >30 s).
    c. Transfer 25 μL of Dynabeads to a 1.5 mL DNase-free non-stick tube, and place the tubes on a magnetic rack for 1 min.
    d. Discard the supernatant, remove the tube from the rack, and resuspend the beads in 25 μL of 1× B&W buffer.
    e. Wash the beads two more times.
    f. Resuspend the Dynabeads in 10 μL of 2× B&W buffer, and equilibrate the beads at 37°C.
86. Add 10.0 μL of subtraction reaction to the beads, mix well, and incubate for 15 min at 37°C in the ThermoMixer.
87. Place the tubes in the magnetic rack for 1 min.
88. Pipet 17.5 μL of the supernatant into a 1.5 mL DNase-free non-stick tube. Pool the eluate from the same sample to a final volume of 35 μL.
89. Add 15.0 μL of bind/wash buffer, 1.0 μL of 5 mg/mL linear acrylamide, and 5.0 μL of 5 M NaCl to the samples and mix thoroughly.
90. Add 234 μL of isopropanol to each sample, vortex for 5 s, and store the samples overnight at −80°C.

Pause point: The samples can be stored overnight at −80°C.

PCR amplification and barcoding

Timing: 3 h – overnight

This step describes PCR amplification and barcoding.

91. Centrifuge the samples at 16,100 × *g* for 45 min at 4°C.

92. Add ice-cold 80% ethanol and centrifuge the samples at 16,100 × *g* for 15 min at 4°C.
93. Remove the supernatant and dry the pellet for 5 min at room temperature.
94. Resuspend the depleted DNA samples in 10.0 μL of 10 mM Tris-HCl (pH 8.0).

Pause point: The samples can be stored indefinitely at −80°C.

95. Prepare a two-fold serial dilution of depletion products (1/2, 1/4, 1/8).
96. Set up a PCR mixture for 3 reactions per sample as follows:

PCR reaction master mix	
Reagent	Amount
Depleted DNA	1.0 μL
5× Phusion HF buffer	3.0 μL
10 mM dNTP mix	0.3 μL
Multiplex PCR primer F (10 μM)	0.75 μL
Multiplex PCR primer R (10 μM)	0.75 μL
Phusion Pol. 2 U/μL	0.15 μL
Nuclease-free water	9.05 μL

97. Perform the PCR amplification using the conditions given below.

PCR cycling conditions			
Steps	Temperature	Time	Cycles
Initial Denaturation	98°C	30 s	1
Denaturation	98°C	10 s	8–12 cycles
Annealing	60°C	10 s	
Extension	72°C	5 s	
Final extension	72°C	5 min	1
Hold	4°C	Indefinite	

98. Remove the PCR tube strips containing the reactions and add 3.0 μL of 6× non-denaturing loading dye to each PCR tube.

Note: As a control, mix 1.0 μL of Ultra-Low Range DNA Ladder (Invitrogen), 15.7 μL of 10 mM Tris-HCl (pH 8.0), and 3.3 μL of 6× non-denaturing loading dye.

99. Run the amplification reactions in an 8% polyacrylamide non-denaturing gel by electrophoresis for 10 min at 100 V and then for 50 min at 200 V.

Note: Load amplification reactions for the same sample in adjacent wells to allow for direct comparison.

100. Stain the gel for 20 min with 2 μL of 1× SYBR Gold in 40 mL of nuclease-free water at 30 rpm for 15–20 min in the dark.
101. Determine the optimal PCR cycle number and template input volume based on the PCR results. Select conditions featuring a prominent product band but little accumulation of re-annealed partial duplex library products.
102. Prepare the DNA template and mix with index primer, 4 reactions per sample.
103. Setup the PCR reaction again as described in steps 96–100.
104. Illuminate the stained gel using an ultraviolet transillumination and excise and transfer the PCR product bands from the gel to a 1.5 mL non-stick DNase-free microfuge tube (Figure 6).

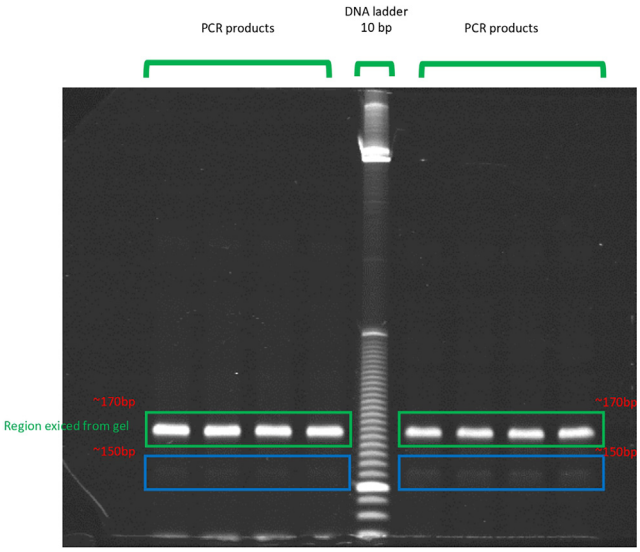


Figure 6. Indexed PCR product bands
Green boxes indicate excised bands. Blue boxes denote unextended reverse transcription primer that should be avoided.

⚠ CRITICAL: Avoid to cut any lower product band derived from unextended reverse transcription primers (Figure 6).

105. Grind the gel with a pestle in a 1.5 mL tube.
106. Add 500 µL of DNA gel extraction buffer to the polyacrylamide gel slices.
107. Heat the samples for 10 min at 72°C.
108. Transfer the samples to a Spin-X column (Coring 8160) and centrifuge at 16,100 × *g* for 10 min at 4°C.
109. Transfer 500 µL of the supernatant into a non-stick DNase-free 1.5 mL microfuge tube.
110. Add 50.0 µL of 3 M NaOAc (pH 5.2) and 2.2 µL of 5 mg/mL linear acrylamide to each sample and mix thoroughly.
111. Add 550 µL of isopropanol to each sample and vortex to resuspend well.
112. Centrifuge the samples at 16,100 × *g* for 45 min at 4°C.
113. Add ice-cold 80% ethanol and centrifuge the samples at 16,100 × *g* for 15 min at 4°C.
114. Remove the supernatant and dry the pellet for 5 min at room temperature.

Pause point: The samples can be stored overnight at −80°C.

115. Resuspend every DNA library in 20.0 µL of 10 mM Tris-HCl (pH 8.0).

Pause point: The samples can be stored indefinitely at −80°C.

Library sequencing

Timing: 5 days

This step describes the quantification and sequencing of the libraries.

116. Quantify and check the library quality by loading 1.5 µL of library with 6.0 µL of nuclease-free water in a high-sensitivity DNA chip on the Agilent BioAnalyzer following the manufacturer's protocol (https://www.agilent.com/cs/library/usermanuals/Public/G2938-90321_SensitivityDNA_KG_EN.pdf).

117. The libraries should be sequenced for 50 bases using an Illumina Hiseq 2500 SR50 system.

Note: Approximately 5% of the reads map to coding gene regions without any ambiguity. Thus, 100 M reads should yield circa 5 M unambiguous reads.

EXPECTED OUTCOMES

While RNA-seq determines the total amount of mRNA to survey transcriptional regulation, Ribo-seq can be used to measure mRNAs undergoing translation, providing a more accurate view of protein expression. Since the Ribo-seq technique allows detailed and accurate *in vivo* analysis of protein production, this approach can be used to estimate translation efficiency (TE), to identify open reading frames (ORFs) and upstream (uORFs), to determinate the approximate translation elongation rate, the positions and densities of ribosomes on individual mRNAs through the mapping of the sequenced RPFs, and to predict protein abundance.

QUANTIFICATION AND STATISTICAL ANALYSIS

Data analysis

Timing: 5 days

Using this protocol, at least 22 million Ribo-seq reads will be generated for each biological replicate. Of these, typically between 5–15 million Ribo-seq reads will be mapped to protein-coding regions of the Arabidopsis genome.

The analysis of the sequencing data is similar to the standard RNA-seq data analysis and depends on the aims of the experiment.

Mapping Ribo-seq reads

In order to specifically deal with Ribo-seq reads of Arabidopsis samples, we wrapped our procedure into three scripts (filtering, mapping, and counting), plus one environment checking script. Together with prebuild bowtie2 index files and gene coordinate files, these scripts are available via file riboFMC.tar.gz at https://maccu.project.sinica.edu.tw/20230330/.

Hardware requirements: The wrapper scripts have been tested on an Ubuntu 20.04 virtual machine with 8GB RAM and 4 vCPUs.

Software requirements: Running these wrapper scripts requires a standard Linux/Unix environment with Java (version 7 or later), B-owtie2, and SAMtools available in the command line. The Perl module Bio::DB::Fasta is also required, which would be available by installing the B-ioPerl package in Linux distributions like Debian and Ubuntu, or by using CPAN install.

1. Process the Ribo-seq data by Cutadapt for adapter removal. Hereafter, we assume the cleaned file is a FASTQ gzip file named root-ctr-0h-r1.fastq.gz.
2. Download the package, extract it, and run the setup script setup.pl. The setup script will check the existences of Java, B-owtie2, SAMtools, and the Perl module Bio::DB::Fasta. It will also verify if the Arabidopsis TAIR10 genome assembly is in directory riboFMC/db. If not, entering "yes" will let the script download files from the TAIR FTP archive.

```
> wget https://maccu.project.sinica.edu.tw/20230330/riboFMC.tar.gz

> tar -xzvf riboFMC.tar.gz

> riboFMC/setup.pl
```

3. Run the filtering script riboFMC/filter.pl. This script filters the data by mapping reads to Araport11 tRNAs, miRNAs, snoRNAs, snRNAs, and rRNAs. The mapping is performed by employing B-owtie2 against a prebuild B-owtie2 index in riboFMC/db/filterBase. This script requires two parameters: (i) input FASTQ file (can be compressed by gzip) and (ii) output FASTA file (must be compressed). The output FASTA file stores reads that cannot be mapped to the filter base. The command line option -p should be applied if the computer is capable of concurrently running more than 3 threads (default setting). In the output text, the line starting with "Executing command" shows the command being executed.

```
> riboFMC/filter.pl root-ctr-0h-r1.fastq.gz root-ctr-0h-r1.pass1.fasta.gz
```

⚠ CRITICAL: lncRNAs were included for further analysis.

4. Run the mapping script riboFMC/mapping.pl. Taking the output file from the last step as input, this script maps the remaining reads to Araport11 transcriptome using Bowtie2 and keep alignments with at least 95% identity. Alignment results can be saved in the file assigned by the second parameter in BAM format. Similarly, option -p should be considered if the computer can run more than three threads. Some short explanations on the executed command: the Bowtie2 part is to map reads to Araport11 transcripts and alignments are in transcript coordinates. The J-ava part (rackj.jar) is responsible for keeping only alignments with an identity at least 95% and for transferring transcript coordinates into genome coordinates. Perl script psl2sam.pl and SAMtools are for transforming results into a BAM file.

```
> riboFMC/mapping.pl root-ctr-0h-r1.pass1.fasta.gz root-ctr-0h-r1.transcriptome.translated.
bam
```

5. Run the counting script riboFMC/counting.pl. This wrapper script calls a Java program inside riboFMC/jar/rackj.jar for per-gene read counting given the BAM file is assigned by the first parameter. The default coordinate file of genes to be counted are stored in riboFMC/db/target.cgff for Araport11 mRNAs, lnc_RNAs, and ncRNAs. The second parameter functions as a prefix string of a number of output files. The counting result file to be used in this protocol is suffixed with ".geneRPKM". This file is in tab-delimited text format and can be opened with Excel. The five columns of the table denote: (i) gene ID, (ii) sum of exon lengths (in Kbps), (iii) sum of unique-reads and multi-reads, (iv) RPKM, and (v) ratio of multi-reads. Note that read counting of unique-reads and multi-reads as well as computation of RPKMs follow the description described in Mortazavi et al.[24]

```
> riboFMC/counting.pl root-ctr-0h-r1.transcriptome.translated.bam root-ctr-0h-r1
```

Computing differentially expressed genes

6. To identify differentially expressed genes, Z-tests were performed as described in Lan et al.,[25] and genes with a P value of ≤ 0.05 were defined as being differentially expressed. For Riboseq, genes with a log2 ≥ 1.2 of the ratio treatment/control are selected as upregulated genes and genes with a log2 ≤ -1.2 of the ratio treatment/control are defined as down-regulated. To facilitate the computation, a template Excel file CompareRPKMs.xlsx is provided in https://maccu.project.sinica.edu.tw/20230330/. Some manual operations are needed to use the Excel file: (i) adjust the numbers of control columns and treatment columns by inserting or deleting columns, and verify that the Excel formulas in columns Pt and Pc are covering treatment and control columns, respectively, (ii) ascertain that all ".geneRPKM" files from the last step are in the same

order, (iii) copy and fill columns with gene information and RPKM values, and (iv) use the Excel autofill function to duplicate formulas in row 2 under columns Pt, Pc, …, logFC to all rows. The column "Z-TEST" contains Z-test P values, the column "logFC" contains log2-fold-change.

LIMITATIONS

One of the limitations of the Ribo-seq technique is the limited detection of lowly expressed genes.

The way the samples are harvested is very critical; keeping the integrity of the roots yields more ribosomes.

Another problem is associated with RNase digestion which is highly stochastic, causing high variability in ribosome-protected fragment lengths.

A further drawback of this method is related to changes in the ribosome profiles. Since the protocol includes the use of cycloheximide (a drug that blocks translation elongation to prevent ribosome run-off), which can cause excessive ribosome accumulation at the initiation site, the distinction between direct and indirect effects on translation is rendered difficult.

TROUBLESHOOTING

Problem 1

Degradation of RNA (steps 1–5).

Potential solution

- Use RNase-free water.
- Wear disposable gloves to prevent contamination by ribonucleases present on your hands.
- Use reagents of the highest quality available.
- Filter-sterilize all buffers for long-term storage.
- Pre-chill rotors and centrifuges before use.

Problem 2

Poor enrichment of polysomes after lysis and sucrose gradient fractionation (steps 1–10).

Potential solution

- Sustain tissue integrity when collecting the samples.
- During the lysis step, it is important to use salts to stabilize the ribosomes.
- Try an alternative polysome and/or lysis buffer.

Problem 3

Low amount of recovered RNA after size selection (steps 11–21).

Potential solution

- Use TRIzol instead of a kit for RNA purification.

Problem 4

Low number of reads and concomitant high amount of rRNA contamination (steps 82–90).

Potential solution

- Cut the region between 28 nt - 30 nt and avoid to cut the region (marked in blue) that is contaminated with rRNA (Figure 7).

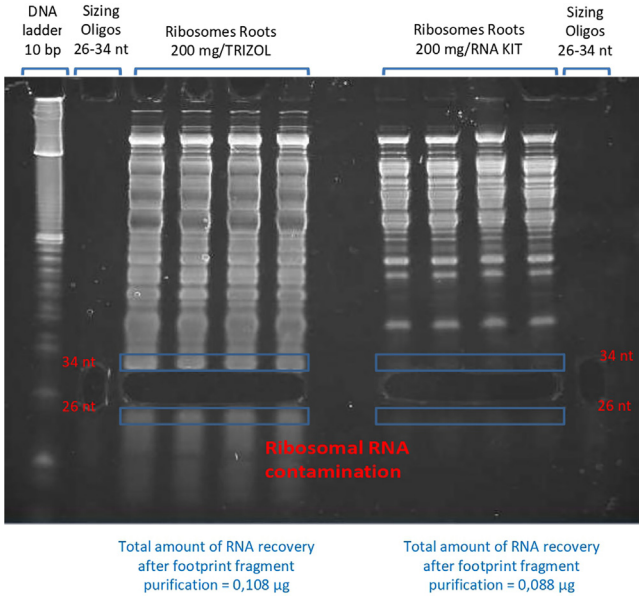


Figure 7. Gel picture of root and shoot samples after excision of the bands

The excised region is between 30–34 nt in length. Regions shown in blue brackets (26 nt and 35 nt, respectively) contain rRNA contamination and should not be used.

- Fewer RPFs obtained by sequencing, resulting in less mapped reads, can be the result of the presence of rRNA in the libraries, which is the most predominant RNA. Most contaminating rRNAs are generated from ribonuclease digestion of the ribosomes (Figure 8). One way of depleting rRNA is to use subtractive hybridization of biotinylated oligos customized to library preparations. If the contamination for rRNA is still high, you may clean up the libraries again with the Ribo-Zero Removal Kit (Plant Leaf, Illumina, MRZPL1224- or Plant Seed/Root, Illumina, MRZSR116) as complementary strategy.

Problem 5

- Multiple site mappings, ambiguous reads, and underestimation of the differential translational regulation in the coding sequences by the length of footprints (~30 nts) (steps 118–122).

Potential solution

- Use appropiate and specific bioinformatic tools to filter the alignments.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Wolfgang Schmidt (wosh@gate.sinica.edu.tw).

Materials availability

This study did not generate new unique reagents.

Data and code availability

The methodology for computational analysis is available at https://maccu.project.sinica.edu.tw/20230330/. Any additional information required is available from the lead contact upon request.

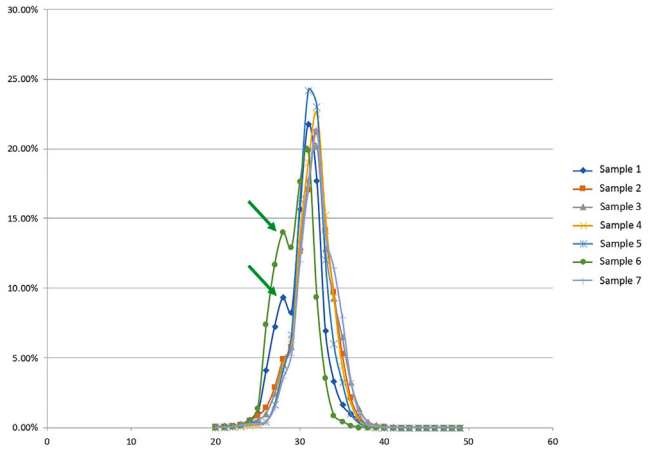


Figure 8. Quantitative competitive plot of the RPF sequence results

Two samples (indicated by green arrows) are showing different patterns (small insertion size, between 14%–18%). After sequencing, these two samples show different results compared to samples without any small RNA insertion.

ACKNOWLEDGMENTS

This work was supported by grants from Academia Sinica (AS-TP-109-L01) and the National Science and Technology Council (111-2313-B-001-01). The graphical abstract was created with BioRender.com.

AUTHOR CONTRIBUTIONS

W.S. and I.C.V.-B. designed and conceived this study. I.C.V.-B., S.-J.C., and A.-P.C. performed the experiments. S.-J.C. and A.-P.C. prepared the libraries and designed the subtraction oligos. W.-D.L. developed the methodology for computational analysis. W.S., I.C.V.-B., S.-J.C., A.-P.C., and W.-D.L. analyzed the results. I.C.V.-B. wrote the manuscript. W.S. supervised the study and edited the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

1. Ingolia, N.T., Ghaemmaghami, S., Newman, J.R.S., and Weissman, J.S. (2009). Genome-wide analysis in vivo of translation with nucleotide resolution using ribosome profiling. Science *324*, 218–223. https://doi.org/10.1126/science.1168978.

2. Brar, G.A., and Weissman, J.S. (2015). Ribosome profiling reveals the what, when, where and how of protein synthesis. Nat. Rev. Mol. Cell Biol. *16*, 651–664. https://doi.org/10.1038/nrm4069.

3. Ingolia, N.T., Brar, G.A., Rouskin, S., McGeachy, A.M., and Weissman, J.S. (2012). The ribosome profiling strategy for monitoring translation in vivo by deep sequencing of ribosome-protected mRNA fragments. Nat. Protoc. *7*, 1534–1550. https://doi.org/10.1038/nprot.2012.086.

4. Juntawong, P., Hummel, M., Bazin, J., and Bailey-Serres, J. (2015). Ribosome profiling: A tool for quantitative evaluation of dynamics in mRNA translation. Methods Mol. Biol. *1284*, 139–173. https://doi.org/10.1007/978-1-4939-2444-8_7.

5. Merchante, C., Hu, Q., Heber, S., Alonso, J., and Stepanova, A. (2016). A ribosome footprinting protocol for plants. Bio. Protoc. *6*, e1985. https://doi.org/10.21769/BioProtoc.1985.

6. Hsu, P.Y., Calviello, L., Wu, H.-Y.L., Li, F.-W., Rothfels, C.J., Ohler, U., and Benfey, P.N. (2016). Super-resolution ribosome profiling reveals unannotated translation events in Arabidopsis. Proc. Natl. Acad. Sci. USA *113*, E7126–E7135. https://doi.org/10.1073/pnas.1614788113.

7. Bazin, J., Baerenfaller, K., Gosai, S.J., Gregory, B.D., Crespi, M., and Bailey-Serres, J. (2017). Global analysis of ribosome-associated noncoding RNAs unveils new modes of translational regulation. Proc. Natl. Acad. Sci. USA *114*, E10018–E10027. https://doi.org/10.1073/pnas.1708433114.

8. Chen, H., Alonso, J.M., and Stepanova, A.N. (2022). A Ribo-seq method to study genome-wide translational regulation in plants. In Environmental Responses in Plants, *2494*, P. Duque and D. Szakonyi, eds (Humana). https://doi.org/10.1007/978-1-0716-2297-1_6.

9. Wu, H.Y.L., and Hsu, P.Y. (2022). A custom library construction method for super-resolution ribosome profiling in Arabidopsis. Plant Methods *18*, 115. https://doi.org/10.1186/s13007-022-00947-2.

10. Liu, M.J., Wu, S.H., Wu, J.F., Lin, W.D., Wu, Y.C., Tsai, T.Y., Tsai, H.L., and Wu, S.H. (2013). Translational landscape of photomorphogenic Arabidopsis. Plant Cell *25*, 3699–3710. https://doi.org/10.1105/tpc.113.114769.

11. Kurihara, Y., Makita, Y., Kawashima, M., Fujita, T., Iwasaki, S., and Matsui, M. (2018).

Transcripts from downstream alternative transcription start sites evade uORF-mediated inhibition of gene expression in *Arabidopsis*. Proc. Natl. Acad. Sci. USA *115*, 7831–7836. https://doi.org/10.1073/pnas.1804971115.

12. Vélez-Bermúdez, I.C., Lin, W.D., Chou, S.J., Chen, A.P., and Schmidt, W. (2023). Transcriptome and translatome comparison of tissues from *Arabidopsis thaliana*. Preprint at bioRxiv. https://doi.org/10.1101/2023.07.22.550136.

13. Xu, G., Greene, G.H., Yoo, H., Liu, L., Marqués, J., Motley, J., and Dong, X. (2017). Global translational reprogramming is a fundamental layer of immune regulation in plants. Nature *545*, 487–490. https://doi.org/10.1038/nature22371.

14. Yoo, H., Greene, G.H., Yuan, M., Xu, G., Burton, D., Liu, L., Marqués, J., and Dong, X. (2020). Translational regulation of metabolic dynamics during effector-triggered immunity. Mol. Plant *13*, 88–98. https://doi.org/10.1016/j.molp.2019.09.009.

15. Lukoszek, R., Feist, P., and Ignatova, Z. (2016). Insights into the adaptive response of *Arabidopsis thaliana* to prolonged thermal stress by ribosomal profiling and RNA-Seq. BMC Plant Biol. *16*, 221. https://doi.org/10.1186/s12870-016-0915-0.

16. Juntawong, P., Girke, T., Bazin, J., and Bailey-Serres, J. (2014). Translational dynamics revealed by genome-wide profiling of ribosome footprints in Arabidopsis. Proc. Natl. Acad. Sci. USA *111*, E203–E212. https://doi.org/10.1073/pnas.1317811111.

17. Merchante, C., Brumos, J., Yun, J., Hu, Q., Spencer, K.R., Enríquez, P., Binder, B.M., Heber, S., Stepanova, A.N., and Alonso, J.M. (2015). Gene-specific translation regulation mediated by the hormone-signaling molecule EIN2. Cell *163*, 684–697. https://doi.org/10.1016/j.cell.2015.09.036.

18. Planchard, N., Bertin, P., Quadrado, M., Dargel-Graffin, C., Hatin, I., Namy, O., and Mireau, H. (2018). The translational landscape of *Arabidopsis* mitochondria. Nucleic Acids Res. *46*, 6218–6228. https://doi.org/10.1093/nar/gky489.

19. Willems, P., Ndah, E., Jonckheere, V., Stael, S., Sticker, A., Martens, L., Van Breusegem, F., Gevaert, K., and Van Damme, P. (2017). N-terminal proteomics assisted profiling of the unexplored translation initiation landscape in *Arabidopsis thaliana*. Mol. Cell. Proteomics *16*, 1064–1080. https://doi.org/10.1074/mcp.M116.066662.

20. Martin, M. (2011). Cutadapt removes adapter sequences from high-throughput sequencing reads. EMBnet. J. *17*, 10–12. https://doi.org/10.14806/ej.17.1.200.

21. Langmead, B., and Salzberg, S.L. (2012). Fast gapped-read alignment with Bowtie 2. Nat. Methods *9*, 357–359. https://doi.org/10.1038/nmeth.1923.

22. Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., Marth, G., Abecasis, G., and Durbin, R.; 1000 Genome Project Data Processing Subgroup (2009). The Sequence Alignment/Map format and SAMtools. Bioinformatics *25*, 2078–2079. https://doi.org/10.1093/bioinformatics/btp352.

23. Cheng, C.-Y., Krishnakumar, V., Chan, A.P., Thibaud-Nissen, F., Schobel, S., and Town, C.D. (2017). Araport11: a complete reannotation of the *Arabidopsis thaliana* reference genome. Plant J. *89*, 789–804. https://doi.org/10.1111/tpj.13415.

24. Mortazavi, A., Williams, B.A., McCue, K., Schaeffer, L., and Wold, B. (2008). Mapping and quantifying mammalian transcriptomes by RNA-Seq. Nat. Methods *5*, 621–628. https://doi.org/10.1038/nmeth.1226.

25. Lan, P., Li, W., Lin, W.D., Santi, S., and Schmidt, W. (2013). Mapping gene activity of *Arabidopsis* root hairs. Genome Biol. *14*, R67. https://doi.org/10.1186/gb-2013-14-6-r67.