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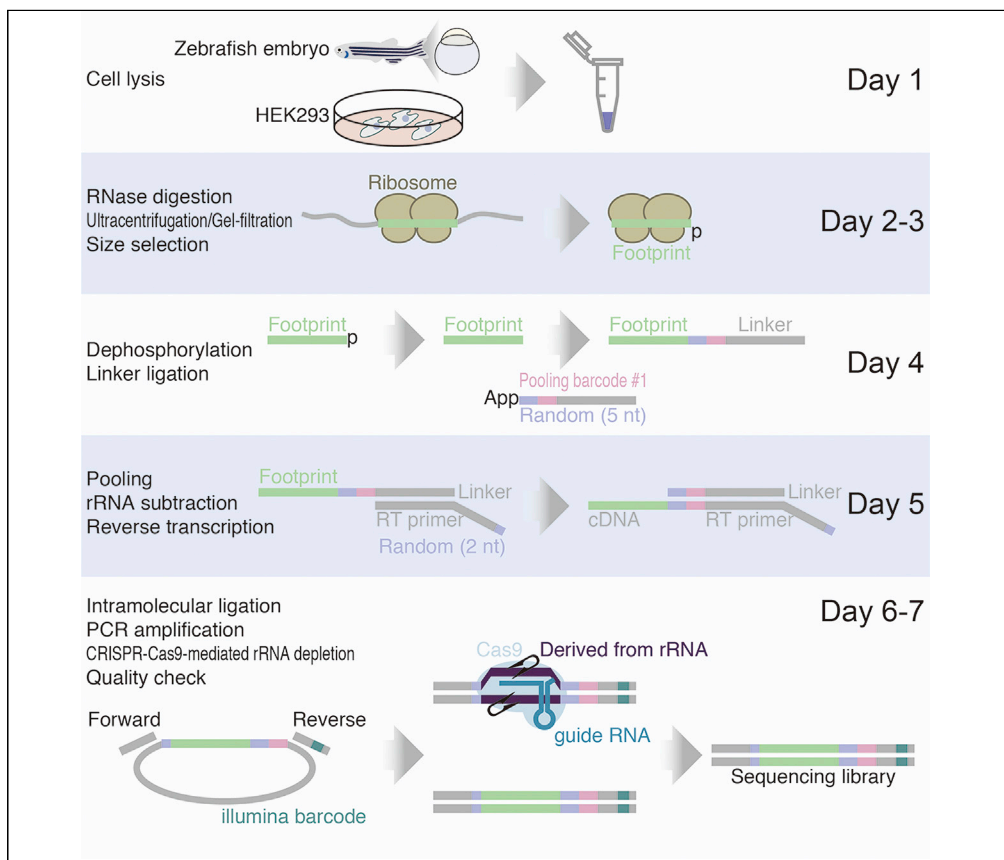
Protocol for Disome Profiling to Survey Ribosome Collision in Humans and Zebrafish



Ribosomes often encounter obstacles during translation elongation and thus collide with each other. Disome profiling, an optimized ribosome profiling method, specifically sequences the long ribosome footprints generated from collided ribosomes produced by the ribosome pause and thus allows the survey of sites in a genome-wide manner. This protocol details the procedure from lysate preparation of human tissue cultures and zebrafish embryos to sequencing library construction.

Mari Mito, Yuichiro Mishima, Shintaro Iwasaki

shintaro.iwasaki@riken.jp

HIGHLIGHTS

Isolation of disome footprints from humans and zebrafish for deep sequencing

Sample multiplexing in the middle of library preparation with custom barcoded linkers

Increased sequencing space by CRISPR-Cas9-mediated rRNA depletion

Mito et al., STAR Protocols 1, 100168
December 18, 2020 © 2020 The Author(s).
https://doi.org/10.1016/j.xpro.2020.100168

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Protocol for Disome Profiling to Survey Ribosome Collision in Humans and Zebrafish

Mari Mito,[1] Yuichiro Mishima,[2,4] and Shintaro Iwasaki[1,3,4,5,*]

[1]RNA Systems Biochemistry Laboratory, RIKEN Cluster for Pioneering Research, Wako, Saitama 351-0198, Japan

[2]Faculty of Life Sciences, Kyoto Sangyo University, Kita-ku, Kyoto 603-8555, Japan

[3]Department of Computational Biology and Medical Sciences, Graduate School of Frontier Sciences, The University of Tokyo, Kashiwa, Chiba 277-8561, Japan

[4]Technical Contact

[5]Lead Contact

*Correspondence: shintaro.iwasaki@riken.jp
https://doi.org/10.1016/j.xpro.2020.100168

SUMMARY

Ribosomes often encounter obstacles during translation elongation and thus collide with each other. Disome profiling, an optimized ribosome profiling method, specifically sequences the long ribosome footprints generated from collided ribosomes produced by the ribosome pause and thus allows the survey of sites in a genome-wide manner. This protocol details the procedure from lysate preparation of human tissue cultures and zebrafish embryos to sequencing library construction.

For complete details on the use and execution of this protocol, please refer to Han et al. (2020).

BEFORE YOU BEGIN

Prepare 25% PEG AMPure Beads, gRNAs for CRISPR-Cas9, and preadenylated linkers before starting library preparation. Preadenylated linkers will be used for ligation to dephosphorylated disome footprints (see "Dephosphorylation and Linker Ligation"). Twenty-five percent PEG AMPure Beads will be used for purification of the PCR product for gRNA transcription (see "Preparation of gRNAs for CRISPR-Cas9-Mediated rRNA Fragment Removal" in this section). gRNAs will be assembled with Cas9 to digest a fraction of the disome library with contaminated rRNA sequences (see "PCR Amplification and CRISPR-Mediated rRNA Fragment Depletion"). See also Figure 1 for an overview of the procedures in this protocol.

⚠ CRITICAL: Wipe all the surfaces of the bench and pipette with 70% ethanol to avoid contamination by RNase.

Note: Use RNase-free and low-adhesion tubes (such as those from Eppendorf) and filtered tips (such as those from Greiner Bio-One) in all the steps.

Note: The centrifuge should be prechilled at 4°C before use.

Preparation of 25% PEG AMPure Bead Solution

⏱ Timing: 10 min

This step describes the preparation of 25% PEG AMPure bead solution.

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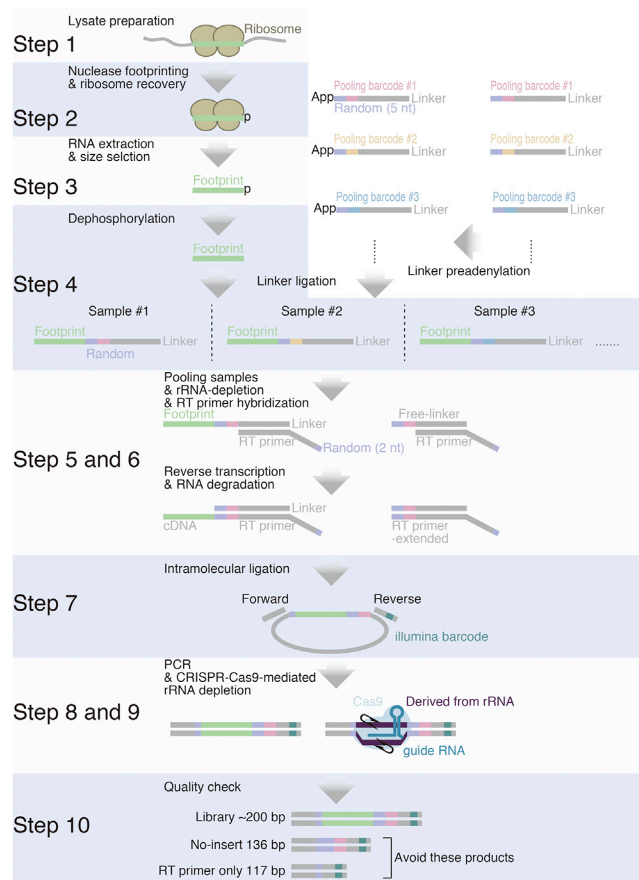

Figure 1. Overview of Disome Profiling Library Preparation

Ribosome profiling is a deep-sequencing-based technique to monitor the position of ribosomes along mRNAs, harnessing the RNase protection of mRNA fragments engulfed by ribosomes (i.e., ribosome footprints) (Ingolia et al., 2009). Whereas typical ribosome profiling isolates footprints the size of a single ribosome (~30 nucleotides [nt] long), the current protocol focuses on 40–65 nt footprints corresponding to the length of tightly packed ribosome pairs or disomes (Wolin and Walter, 1988). This protocol was modified from the standard ribosome profiling protocol (McGlincy and Ingolia, 2017). The modification included (1) long-footprint isolation at the size selection step and (2) CRISPR-mediated depletion of contaminated rRNA fragments. The unique sample index sequence in linkers allows one to pool samples into a single sample in the middle of the procedure, reducing the variability among samples during downstream reactions. Each step shown in the figure corresponds to a major step in this protocol: step 1, cell lysis; step 2, RNase digestion and ribosome purification; step 3, size selection of ribosome footprints; step 4, dephosphorylation and linker ligation; step 5, ribosome RNA depletion; step 6, reverse transcription; step 7, intramolecular ligation; step 8, optimization of PCR; step 9, PCR amplification and CRISPR-mediated rRNA fragment depletion; and step 10, quality assessment.

Note: We use AMPure XP beads and T4 RNA Ligase 2, truncated KQ with the modifications noted below.

1. Prepare 500 μL of 25% PEG solution with 250 μL of 50% PEG-8000 (an accessory of T4 RNA Ligase 2, truncated KQ) and 250 μL of 5 M NaCl at 23°C–26°C.

   Note: Use wide-bore tips to take up PEG-8000. Turn the tube upside down several times and ensure that the solution is well mixed.

2. Place 500 μL of AMPure XP beads in a 1.5-mL tube and centrifuge at 3,000 × *g* at 23°C–26°C for 5 min.

3. Place the tube on a magnetic stand for 1.5-mL tubes (such as Magna Stand for 1.5-mL tubes) and wait for 5 min for the supernatant to become clear.
4. Discard the supernatant by pipetting and add 500 µL of 25% PEG solution. Vortex vigorously before use.

Preparation of gRNAs for CRISPR-Cas9-Mediated rRNA Fragment Removal

Timing: 7 h

This step describes the preparation of gRNAs for CRISPR-Cas9 by in vitro transcription.

Note: We use T7-Scribe standard RNA IVT Kit with the modifications noted below.

5. Prepare the following PCR mix in 0.2-mL tubes.

Reagent	Final Concentration	Volume
T7-promoter-Forward (10 µM)	0.2 µM	1 µL
T7-gRNA-#1, #2, or #3-Forward (10 µM)	0.2 µM	1 µL
Scaffold-Reverse-1 (10 µM)	0.2 µM	1 µL
Scaffold-Reverse-2 (10 µM)	0.2 µM	1 µL
2× PrimeSTAR Max Mix	1×	25 µL
RNase-free water	n/a	21 µL
Total	**n/a**	**50 µL**

6. Run PCR reactions in a thermal cycler (with the lid at 105°C).

Steps	Temperature	Time	Cycles
Initial denaturation	98°C	10 s	1
Denaturation	98°C	10 s	30 cycles
Extension	68°C	10 s	
Final extension	68°C	30 s	1
Hold	4°C (or 12°C)	Forever	

7. Run 3 µL of PCR product on a 2% agarose gel to confirm that the amplified DNA is the right size (136 bp).
8. Add 40 µL of 25% PEG AMPure XP bead suspension to the remaining PCR solution, vortex, and incubate at 23°C–26°C for 15 min.
9. Place the tube on a magnetic stand for 0.2-mL tubes (such as Magna Stand for 0.2-mL tubes) and wait for 1 min for the supernatant to become clear.
10. Discard the supernatant, add 200 µL of 80% ethanol, and wait for 30 s. Repeat this step (for a total of two washes).
11. Discard the supernatant, spin down, place the tube on a magnetic stand for 0.2-mL tubes (such as Magna Stand for 0.2-mL tubes), and remove the supernatant thoroughly by pipetting. Open the lid and dry beads at 23°C–26°C for 1 min.

⚠ CRITICAL: Avoid over-drying beads, which reduces RNA elution efficiency. Over-drying can be detected by cracks in the beads collected on the tube wall.

12. Add 10 μL of RNase-free water, vortex, and incubate at 23°C–26°C for 5 min.

⚠ CRITICAL: Avoid contamination by ethanol, which inhibits downstream reactions.

13. Place the tube on a magnetic stand for 0.2-mL tubes (such as Magna Stand for 0.2-mL tubes) and wait for 5 min for the supernatant to become clear.
14. Collect the supernatant by pipetting into a new 1.5-mL tube.

Note: We recommended using the Magna Stand for magnetic bead handling since this allows flexible angles and positions of the magnet to tubes held on the stand with a strong magnetic field. As a result, beads could be trapped on the upper side of the tube wall more strongly than on other typical magnetic stands. Thus, whole solutions could easily be collected by pipetting with minimal bead contamination.

15. Measure the DNA concentration by using a micro spectrophotometer (DeNovix) with 260 nm absorbance with a conversion factor of 50.

Note: Other micro spectrometers such as Nanodrop could be used.

Note: The solution typically contains 50–75 ng/μL DNA.

Pause Point: The DNA may be stored at −20°C.

16. Prepare the following *in vitro* transcription reactions in 0.2-mL tubes and incubate at 37°C for 2–3 h in a thermal cycler (with the lid at 57°C) to transcribe gRNAs.

Reagent	Final Concentration	Volume
DNA template (0.2–0.5 μg)	n/a	X μL
RNase-free water	n/a	7.5 - X μL
10× T7-Scribe Transcription Buffer[a]	1×	2 μL
ATP (100 mM)[a]	7.5 mM	1.5 μL
GTP (100 mM)[a]	7.5 mM	1.5 μL
CTP (100 mM)[a]	7.5 mM	1.5 μL
UTP (100 mM)[a]	7.5 mM	1.5 μL
DTT (100 mM)[a]	10 mM	2 μL
ScriptGuard RNase Inhibitor 40 U/μL[a]	1 U/μL	0.5 μL
T7-Scribe Enzyme Solution[a]	n/a	2 μL
Total	n/a	**20 μL**

[a]Reagents of the T7-Scribe standard RNA IVT Kit

17. Add 1 μL of RNase-Free DNase I (1 U/μL) (an accessory of the T7-Scribe standard RNA IVT Kit) and incubate at 37°C for 20 min in a thermal cycler (with the lid at 57°C).
18. Add 38 μL of RNAClean XP, vortex, and incubate at 23°C–26°C for 15 min.
19. Place the tube on a magnetic stand for 0.2-mL tubes (such as 0.2-mL Magna Stand) and wait for 5 min for the supernatant to become clear.
20. Discard the supernatant by pipetting, add 200 μL of 70% ethanol, and wait for 30 s. Repeat this step (for a total of two washes).

21. Discard the supernatant by pipetting, spin down, place the tube on a magnetic stand for 0.2-mL tubes (such as Magna Stand for 0.2-mL tubes), and thoroughly remove the supernatant by pipetting. Open the lid and dry beads at 23°C–26°C for 1 min.
22. Add 15–30 μL of RNase-free water, vortex, and incubate at 23°C–26°C for 5 min.

⚠ CRITICAL: Avoid over-drying the beads, which reduces DNA elution efficiency.

⚠ CRITICAL: Avoid contamination by ethanol, which inhibits downstream reactions.

23. Place the tube on a magnetic stand for 0.2-mL tubes (such as 0.2-mL Magna Stand) and wait for 5 min for the supernatant to become clear.
24. Collect the supernatant by pipetting into a new 1.5-mL tube.
25. Measure the RNA concentration by using a micro spectrophotometer (DeNovix) with 260 nm absorbance with a conversion factor of 40.

Note: Other micro spectrometers such as Nanodrop could be used.

Note: The solution typically contains 2–7 μg/μL RNA.

Pause Point: The gRNA may be stored at −20°C or −80°C. For long-term storage, −80°C is preferable.

Preparation of Preadenylated Linkers

Timing: 3 h

This step describes the preparation of pre-adenylated linkers for ribosome footprint ligation.

Note: We use DNA Adenylation Kit and Oligo Clean & Concentrator Kit with the modifications noted below.

26. Prepare the following linker preadenylation reactionsin 0.2-mL tubes, incubate at 65°C for 1 h and then inactivate at 85°C for 5 min in a thermal cycler (with the lid at 105°C). Prepare 1–8 reactions (according to the number of samples designed to pool) for each linker (#1-#8).

Linker Preadenylation Reaction

Reagent	Final Concentration	Volume
Linker (100 μM) (one of #1-#8)	6 μM	1.2 μL
10× 5′ DNA Adenylation Reaction Buffer[a]	1×	2 μL
ATP (1 mM)[a]	0.1 mM	2 μL
Mth RNA Ligase (50 μM)[a]	5 μM	2 μL
RNase-free water	n/a	12.8 μL
Total	n/a	**20 μL**

[a]Reagents from the DNA Adenylation Kit

27. Add 30 μL of RNase-free water and purify the preadenylated linker by the Oligo Clean & Concentrator Kit according to the manufacturer’s instructions. The linker was eluted into 6 μL of RNase-free water to make a 20 μM stock.

⚠ CRITICAL: Use a new 2-mL reservoir tube for every step during the column purification to avoid contamination.

⏸ **Pause Point:** The preadenylated linker may be stored at −20°C for several months.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, Peptides, and Recombinant Proteins		
AMPure XP	Beckman Coulter	Cat# A63881
RNAClean XP	Beckman Coulter	Cat# A63987
PrimeSTAR Max DNA Polymerase	Takara Bio	Cat# R045A
T7-Scribe standard RNA IVT Kit	Cell Script	Cat# C-AS2607
10× T7-Scribe Transcription Buffer (Accessories of C-AS2607)	Cell Script	N/A
ATP (100 mM) (Accessories of C-AS2607)	Cell Script	N/A
GTP (100 mM) (Accessories of C-AS2607)	Cell Script	N/A
CTP (100 mM) (Accessories of C-AS2607)	Cell Script	N/A
UTP (100 mM) (Accessories of C-AS2607)	Cell Script	N/A
DTT (100 mM) (Accessories of C-AS2607)	Cell Script	N/A
ScriptGuard RNase Inhibitor (Accessories of C-AS2607)	Cell Script	N/A
T7-Scribe Enzyme Solution (Accessories of C-AS2607)	Cell Script	N/A
EnGen Cas9 NLS	New England Biolabs	Cat# M0646T
10× Cas9 Nuclease Reaction Buffer (Accessories of M0646T)	New England Biolabs	N/A
Proteinase K	Merck	Cat# 3115887001
Agarose S	NIPPON GENE	Cat# 312-01193
DNA Adenylation Kit	New England Biolabs	Cat# E2610L
10× 5′ DNA Adenylation Reaction Buffer (Accessories of E2610L)	New England Biolabs	N/A
1 mM ATP (Accessories of E2610L)	New England Biolabs	N/A
Mth RNA Ligase (Accessories of E2610L)	New England Biolabs	N/A
UltraPure DNase/RNase-Free Distilled Water	Thermo Fisher Scientific	Cat# 10977023
UltraPure SDS Solution, 10%	Thermo Fisher Scientific	Cat# 15553027
Ethanol molecular biology grade (99.5%)	FUJIFILM Wako Pure Chemical	Cat# 054-07225
3 M NaOAc pH 5.2 molecular biology grade	nacalai tesque	Cat# 06893-24
5 M Sodium Chloride Solution	nacalai tesque	Cat# 06900-14
Triton X-100 molecular biology grade	nacalai tesque	Cat# 12967-32
Isopropanol molecular biology grade	FUJIFILM Wako Pure Chemical	Cat# 168-21675
1 M Magnesium Chloride Solution	nacalai tesque	Cat# 20942-34
0.5 M EDTA molecular biology grade	FUJIFILM Wako Pure Chemical	Cat# 311-90075

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
1 M Tris-HCl (pH 7.5)	FUJIFILM Wako Pure Chemical	Cat# 318-90225
USB Dithiothreitol (DTT), 0.1 M Solution	Thermo Fisher Scientific	Cat# 707265ML
DMEM, high glucose, GlutaMAX Supplement	Thermo Fisher Scientific	Cat# 10566024
TRIzol	ThermoFisher Scientific	Cat# 15596018
D-PBS(-) without Ca and Mg, liquid	nacalai tesque	Cat# 14249-24
Sucrose molecular biology grade	FUJIFILM Wako Pure Chemical	Cat# 198-13525
Turbo DNase 2 U/μL	ThermoFisher Scientific	Cat# AM2238
SUPERase·In RNase Inhibitor (20 U/μL)	Thermo Fisher Scientific	Cat# AM2696
Cycloheximide solution, Ready-Made Solution, microbial, 100	Merck	Cat# C4859-1ML
RNase I 10 U/μL	Lucigen	Cat# N6901K
2× RNA Loading Buffer without Ethidium Bromide	FUJIFILM Wako Pure Chemical	Cat# 182-02571
SuperSep RNA 15% 17-well	FUJIFILM Wako Pure Chemical	Cat# 194-15881
Tris-Borate-EDTA Buffer(10×)	nacalai tesque	Cat# 35440-31
GlycoBlue Coprecipitant	Thermo Fisher Scientific	Cat# AM9516
Low Range ssRNA Ladder	New England Biolabs	Cat# N0364S
10,000× SYBR Gold	ThermoFisher Scientific	Cat# S11494
T4 polynucleotide kinase	New England Biolabs	Cat# M0201S
T4 RNA Ligase 2, truncated KQ	New England Biolabs	Cat# M0373L
T4 RNA Ligase Reaction Buffer (10×) (Accessories of M0373L)	New England Biolabs	N/A
50% PEG 8000 (Accessories of M0373L)	New England Biolabs	N/A
1 M Sodium hydroxide	nacalai tesque	Cat# 37421-05
CircLigaseII ssDNA ligase	Lucigen	Cat# CL9025K
10× CircLigaseII buffer (Accessories of CL9025K)	Lucigen	N/A
5 M Betaine (Accessories of CL9025K)	Lucigen	N/A
50 mM $MnCl_2$ (Accessories of CL9025K)	Lucigen	N/A
ProtoScript II Reverse Transcriptase	New England Biolabs	Cat# M0368L
5× Protoscript II buffer (Accessories of M0368L)	New England Biolabs	N/A
DTT (100 mM) (Accessories of M0368L)	New England Biolabs	N/A
10 mM dNTP mix	New England Biolabs	Cat# N0447L
Ribo-Zero Gold rRNA Removal Kit Human/Mouse/Rat	Illumina	Cat# MRZG12324
Magnetic beads (Accessories of MRZG12324)	Illumina	N/A
Magnetic Bead Resuspension Solution (Accessories of MRZG12324)	Illumina	N/A
Ribo-Zero reaction buffer (Accessories of MRZG12324)	Illumina	N/A

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
rRNA Removal solution (Accessories of MRZG12324)	Illumina	N/A
GelStar Nucleic Acid Gel Stain 10,000×	LONZA	Cat# 50535
SuperSep DNA 15% 17-well	FUJIFILM Wako Pure Chemical	Cat# 190-15481
100-bp DNA Ladder	TAKARA BIO	Cat# 3407A
Gel Loading Dye Purple 6×	New England Biolabs	Cat# B7024S
Phusion High-Fidelity DNA Polymerase	New England Biolabs	Cat# M0530L
Pronase	Merck	Cat# 10165921001
Critical Commercial Assays		
Oligo Clean & Concentrator	Zymo Research	Cat# D4060
Qubit RNA BR Assay Kit	ThermoFisher Scientific	Cat# Q10210
Direct-zol RNA MicroPrep	Zymo Research	Cat# R2062
NucleoSpin Gel and PCR Clean-up	Takara Bio	Cat# 740609.250
DNA-1000 Kit	SHIMADZU	Cat# 292-27911-91
Deposited Data		
Original images used for the figures	This study	Mendeley Data: doi: 10.17632/gjrkppp3fc.1
Experimental Models: Cell Lines		
HEK293	American Type Culture Collection (ATCC)	Cat# CRL-1573
Experimental Models: Organisms/Strains		
Zebrafish AB strain	Zebrafish International Resource Center (ZRC)	ZDB-GENO-960809-7
Oligonucleotides		
See Tables S1 and S2 for DNA/RNA oligonucleotides sequences.	N/A	N/A
Other		
NGS MagnaStand 8Ch × 1.5-mL tube	FastGene	Cat# FG-SSMAG1.5
NGS MagnaStand (YS-Model) 8Ch × 0.2-mL PCR tube	FastGene	Cat# FG-SSMAG2
10-cm dish	ThermoFisher Scientific	Cat# 150466
13 × 56 mm polycarbonate ultracentrifuge tube	Beckman Coulter	Cat# 362305
MicroSpin S-400 HR Columns	GE Healthcare	Cat# 27514001
Spin-X centrifuge tube filter 0.22 μm	CORNING	Cat# 8160
PETRI DISH, SQUARE 120×120×17 mm	Greiner Bio-One	Cat# 688161
Disposable homogenizer pestle R-1.5	ASONE	Cat# 1-2955-01
Gel loading 20 μL filter tips	ThermoFisher Scientific	Cat# 2155P
Razors	Feather	Cat# No.11
SAPPHIRE FILTER TIP, 1,250 μL XL, LOW RETENTION, NATURAL, STERILE, 96 PCS./RACK	Greiner Bio-One	Cat# 750265

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
SAPPHIRE FILTER TIP, 300 µL XL, LOW RETENTION, NATURAL, STERILE, 96 PCS./RACK	Greiner Bio-One	Cat# 738265
SAPPHIRE FILTER TIP, 20 µL XL, LOW RETENTION, NATURAL, STERILE, 96 PCS./RACK	Greiner Bio-One	Cat# 773265
Long 10 µL filter tips	WATSON	Cat# 1252P-207CS
Barrier Specialty Pipette Tips, 200, wide-bore	ART	Cat# 2069GPK
Nunc 15 mL Conical Sterile Polypropylene Centrifuge Tubes	Thermo Fisher Scientific	Cat# 339650
Nunc 50 mL Conical Sterile Polypropylene Centrifuge Tubes	ThermoFisher Scientific	Cat# 339652
DNA LoBind Tube 1.5 mL	eppendorf	Cat# 22431021
8-strip PCR 0.2-mL tube with lid	BIO-BIK	Cat# 3247-00
2-mL reservoir tube	Quality Scientific Plastics	Cat# NC-508-GRD-Q
Qubit 2.0 Fluorometer	ThermoFisher Scientific	N/A
EasySeparator	FUJIFILM Wako Pure Chemical	Cat# 058-07681
Thermal cycler	BIO-RAD	Cat# T100
TLA110 rotor	Beckman Coulter	Cat# 366735
Optima MAX-TL Ultracentrifuge	Beckman Coulter	Cat# A95761
Seesaw shaker	Biocraft	Cat# BC-700
Centrifugal evaporator	EYELA	Cat# CVE-2200
Electrophoresis power supply	Amercham Biosciences	Cat# EPS301
Dry block heater	Major science	Cat# MC-0203
Rotary mixer	Nissin	Cat# MRC-200
Refrigerated microcentrifuge	TOMY	Cat# MX-307
LED Transilluminator	Gellex International ltd.	Cat# LB-16
Microchip Electrophoresis System for DNA/RNA Analysis MultiNA	SHIMADZU	N/A
Micro spectrophotometer	DeNovix	Cat# DS-11
Pasteur pipette	Iwaki	Cat# IK-PAS-9P

MATERIALS AND EQUIPMENT

Lysis Buffer

Reagent	Final Concentration	Volume
Tris-HCl, pH 7.5 (1 M)	20 mM	100 µL
NaCl (5 M)	150 mM	150 µL
$MgCl_2$ (1 M)	5 mM	25 µL
DTT (0.1 M)	1 mM	50 µL
Triton X-100 (10%)	1%	500 µL
RNase-free water	n/a	4,170 µL
Cycloheximide (100 mg/mL)	100 µg/mL	5 µL
Total	n/a	5,000 µL

Note: Prechill on ice.

Lysis Buffer w/o Triton X-100

Reagent	Final Concentration	Volume
Tris-HCl, pH 7.5 (1 M)	20 mM	100 μL
NaCl (5 M)	150 mM	150 μL
$MgCl_2$ (1 M)	5 mM	25 μL
DTT (0.1 M)	1 mM	50 μL
RNase-free water	n/a	4,670 μL
Cycloheximide (100 mg/mL)	100 μg/mL	5 μL
Total	n/a	**5,000 μL**

Note: Prechill on ice.

Sucrose Cushion Solution

Reagent	Final Concentration	Volume
Sucrose	1 M	1.7 g
Tris-HCl, pH 7.5 (1 M)	20 mM	100 μL
NaCl (5 M)	150 mM	150 μL
$MgCl_2$ (1 M)	5 mM	25 μL
DTT (0.1 M)	1 mM	50 μL
Cycloheximide (100 mg/mL)	100 μg/mL	5 μL
SUPERase·In RNase Inhibitor (20 U/μL)	20 U/mL	5 μL
RNase-free water	n/a	3,565 μL
Total	n/a	**5,000 μL**

Note: Prechill on ice.

RNA Gel Extraction Buffer

Reagent	Final Concentration	Volume
NaOAc, pH 5.2 (3 M)	300 mM	500 μL
EDTA (0.5 M)	1 mM	10 μL
SDS (10%)	0.25%	125 μL
RNase-free water	n/a	4,365 μL
Total	n/a	**5,000 μL**

Note: Store at 23°C–26°C.

DNA Gel Extraction Buffer

Reagent	Final Concentration	Volume
NaCl (5 M)	300 mM	300 μL
Tris-HCl, pH 7.5 (1 M)	10 mM	50 μL
EDTA (0.5 M)	1 mM	10 μL
RNase-free water	n/a	4,640 μL
Total	n/a	**5,000 μL**

Note: Store at 23°C–26°C.

SYBR Gold Staining Buffer

Reagent	Final Concentration	Volume
10,000× SYBR Gold	1×	4 μL
10× TBE	1×	4 mL
RNase-free water	n/a	36 mL
Total	n/a	40 mL

Note: Store at 23°C–26°C.

⚠ CRITICAL: Wear personal protective equipment such as gloves and safety glasses during experiments.

⚠ CRITICAL: DTT, Triton X-100, SDS, and pronase are irritants.

⚠ CRITICAL: Cycloheximide is toxic and harmful.

⚠ CRITICAL: TRIzol is toxic, corrosive, and volatile.

⚠ CRITICAL: Ethanol and isopropanol are flammable and volatile.

⚠ CRITICAL: 2× RNA Loading Buffer (FUJIFILM Wako Pure Chemical) contains formamide, which is a reproductive toxin.

⚠ CRITICAL: Typically, DNA/RNA staining reagents (including SYBR Gold) are mutagenic. Handle the waste according to the institutional regulations.

⚠ CRITICAL: NaOH is corrosive.

⚠ CRITICAL: Polyacrylamide gels may still contain acrylamide monomers, which are neurotoxic.

Alternatives: TRIzol could be substituted with other equivalent reagents as far as they are compatible with the Direct-zol Kit (Zymo Research). See details for the manufacturer's instructions.

Alternatives: Instead of a micro spectrometer from DeNovix, other micro spectrometers such as Nanodrop could be used.

Alternatives: Instead of S-400 columns (GE Healthcare), equivalent spin columns, such as MobiSpin S-400 (MoBiTec), could be used.

Alternatives: Instead of 15% SuperSep RNA gels and 15% SuperSep DNA gels (FUJIFILM Wako Pure Chemical), equivalent precast denaturing and nondenaturing polyacrylamide gels (such as Novex 15% TBE-Urea Gels and Novex 8% TBE Gels, Thermo Fisher Scientific) with appropriate gel tanks could be used.

Alternatives: This protocol uses the fragment analyzer Microchip Electrophoresis System for DNA/RNA Analysis MultiNA for quality assessment of the sequencing library. Alternatively, Bioanalyzer or Tapestation (Agilent) can be used.

STEP-BY-STEP METHOD DETAILS

Cell Lysis of HEK293 Cells

Timing: 2 days

This step describes the preparation and lysis of HEK293 cells.

Day 1

Timing: 1 h

1. Seed 10 mL of HEK293 cells at 7–9 × 10^5 cells/mL into a 10-cm dish and incubate the cells for 16–24 h at 37°C in a CO_2 incubator.

Day 2

Timing: 3 h

Note: We use Qubit RNA BR Assay Kit with the modifications noted below.

2. Aspirate the medium from the dish. Place the dish on ice, rinse the dish with 10 mL of PBS (pre-chilled on ice), and then aspirate the PBS.
3. Add 600 μL of lysis buffer, pipette several times to cover the entire dish surface and transfer the cell lysate to a 1.5-mL tube by pipetting.

   Note: Leaning the dish and pipetting lysis buffer pooled at the lower corner of the dish toward the top of dish several times allows the entire detachment of cells from the dish.

4. Add 7.5 μL of 2 U/μL Turbo DNase I and incubate on ice for 10 min.
5. Centrifuge the sample at 20,000 × *g* at 4°C for 10 min.
6. Collect the supernatant by pipetting into a new 1.5-mL tube and mix well by pipetting.
7. Measure the RNA concentration in lysate by a Qubit Fluorometer and Qubit RNA BR Assay Kit according to the manufacturer's instructions.
8. Flash-freeze the sample by liquid nitrogen and store at −80°C. Proceed to step 20.

Pause Point: The lysate may be stored at −80°C.

Note: The lysate typically contains 200–300 ng/μL RNA.

Cell Lysis from Zebrafish Embryos

Timing: 2 days

This step describes the preparation and lysis of zebrafish embryos.

Day 1

Timing: 30 min

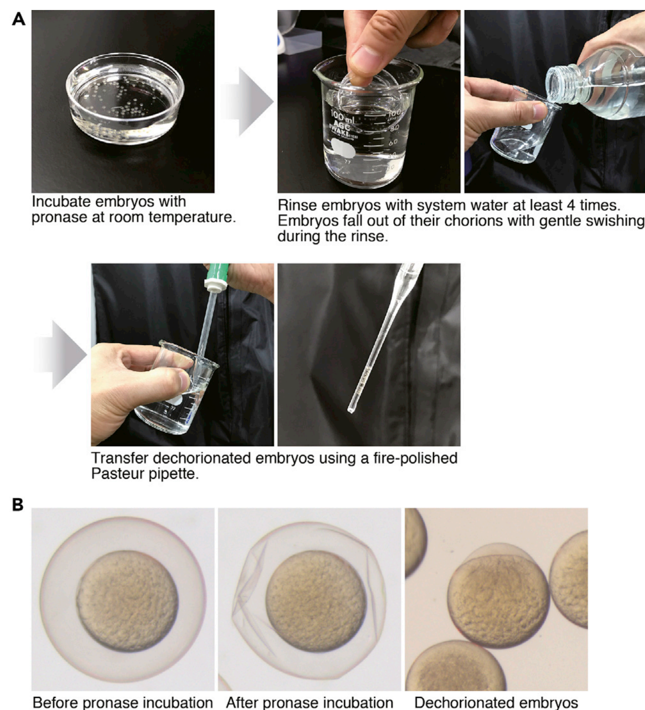

Figure 2. Dechorionation of Zebrafish Embryos

(A) Handling of embryos during dechorionation.

(B) Representative images of zebrafish embryos before (left) and after (middle) pronase treatment and those that were dechorinated (right).

9. In the evening, a day before collecting fertilized eggs, transfer adult zebrafish at a ratio of 1 male to 2 females in a mating tank and incubate them for 14–16 h.

Day 2

Timing: Variable

Note: We use Qubit RNA BR Assay Kit with the modifications noted below.

10. At the beginning of the next light cycle, allow males and females to mate naturally.
11. Collect fertilized eggs and let them develop in system water at 28.5°C.
12. Add pronase at a concentration of 1 mg/mL and incubate embryos at 26°C–28.5°C until their chorions lose elasticity. See Figure 2A for the suggestion of embryo handling and Figure 2B for representative pictures of dechorionated embryos.

⚠ CRITICAL: Wear personal protective equipment such as gloves and safety glasses when handling pronase.

13. Rinse embryos with system water at least four times to remove pronase. Embryos fall out of their chorions with gentle swishing during the rinse (see Figures 2A and 2B).
14. Transfer 50–60 dechorionated embryos to a 1.5-mL tube using a fire-polished Pasteur pipette (see Figures 2A and 2B).
15. Gently aspirate water from a tube but leave a minimum amount of water so that embryos are covered with water.

⚠ CRITICAL: Dechorionated embryos are fragile and sticky. Handle gently in water and use glass dishes and pipettes.

⏸ **Pause Point:** The embryos may be flash-frozen by liquid nitrogen and stored at −80°C.

16. Add 800 μL of lysis buffer and mix well by vortexing for 30 s.

    Note: Homogenizing pestles may be used to lyse later-stage embryos (after the gastrulation stage).

17. Centrifuge the sample at 20,000 × *g* at 4°C for 10 min.
18. Collect the supernatant in a new 1.5-mL tube and mix well.
19. Measure the RNA concentration as described in step 7. Proceed to step 29.

    ⏸ **Pause Point:** The lysate may be stored at −80°C.

    Note: The lysate typically contains 40–50 ng/μL RNA.

RNase Digestion and Ribosome Purification

Note: Here, two different methods for ribosome isolation (ultracentrifugation and gel filtration spin column) are described. Both options should work well in either HEK293 or zebrafish embryo lysates. The difference in digestion conditions is due to the reaction volume that the downstream ribosome isolation method can tolerate.

Isolation from HEK293 Cell Lysate

⏲ Timing: 3.5 h

This step describes the RNase treatment to generate ribosome footprints and its isolation from HEK293 cell lysate.

Note: We use Direct-zol RNA MicroPrep Kit with the modifications noted below.

20. Prepare the following RNase digestion reactions in 1.5-mL tubes on ice.

RNase Digestion Reaction: HEK293 Cells

Reagent	Final Concentration	Volume
Lysate (containing 10 μg of total RNA)	n/a	X μL
Lysis buffer	n/a	298 - X μL
RNase I (10 U/μL)	0.067 U/μL	2 μL
Total	n/a	300 μL

⚠ CRITICAL: Keep the reaction on ice to avoid over-digestion.

⚠ CRITICAL: The ratio of total RNA to RNase I can be optimized for certain conditions such as different amounts of RNA (large or small) in the lysate.

21. Incubate the reaction at 25°C for 45 min in a heat block.
22. Place the tube on ice and then add 10 μL of SUPERase·In RNase Inhibitor (20 U/μL).

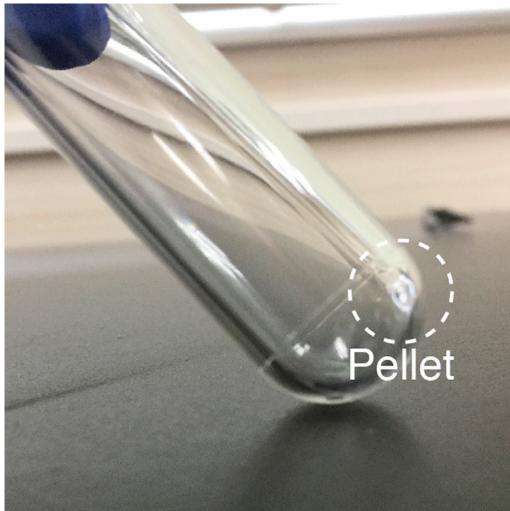

Figure 3. A Representative Image of the Ribosome Pellet Generated by Sucrose Cushion
The circle indicated by a dashed white line is the ribosome pellet at the bottom of an ultracentrifuge tube that was used for downstream processes.

23. Transfer the reaction at the bottom of an Open-Top Thickwall Polycarbonate Tube.
24. Underlay 900 μL of sucrose cushion solution at the bottom of the tube. See Methods Video S1 for details.

Note: The clear interface of the solutions should be visible.

25. Pellet ribosomes by ultracentrifugation with a TLA110 rotor at 100,000 rpm at 4°C for 1 h.
26. Discard the supernatant by pipetting. See Figure 3 as a representative picture of the ribosome pellet. Resuspend the pellet in 300 μL of TRIzol reagent by pipetting. Leave the resuspension for 10 min to ensure complete dissolution. Transfer the solution into a new 1.5-mL tube.

Pause Point: The RNA in TRIzol Reagent may be stored at −80°C.

27. Add 300 μL of 99.5% ethanol and purify RNA with the Direct-zol RNA MicroPrep Kit according to the manufacturer's instructions. Elute the RNA into 7 μL of RNase-free water.

⚠ CRITICAL: Use a new 2-mL reservoir tube every step during the column purification to avoid contamination.

28. Add 7 μL of 2× RNA Loading Buffer (FUJIFILM Wako Pure Chemical). Proceed to step 41.

Pause Point: The purified RNA may be stored at −80°C.

Isolation from Zebrafish Embryo Lysate

Timing: 3 h

This step describes RNase treatment to generate ribosome footprints and its isolation from zebrafish embryo lysate.

Note: We use Direct-zol RNA MicroPrep Kit with the modifications noted below.

29. Prepare the following RNase digestion reactions in 1.5-mL tubes on ice.

RNase Digestion Reaction: Zebrafish Embryos

Reagent	Final Concentration	Volume
Lysate (containing 15 μg of total RNA)	n/a	X μL
Lysis buffer	n/a	397.75 - X μL
RNase I (10 U/μL)	0.005 U/μL	2.25 μL
Total	n/a	400 μL

⚠ CRITICAL: Keep the reaction on ice to avoid over-digestion.

⚠ CRITICAL: The ratio of total RNA to RNase I can be optimized for certain conditions, such as different amounts of RNA (large or small) in the lysate.

30. Incubate the reaction at 25°C for 45 min in a heat block.

Note: During the incubation, prepare S-400 columns as described in step 32.

31. Place the tube on ice and then add 10 μL of SUPERase·In RNase Inhibitor (20 U/μL).

32. Prepare 4 S-400 columns as follows:
    a. Mix S-400 columns well, remove the tips of the columns, and place them into 2-mL reservoir tubes.
    b. Spin at 740 × *g* at 4°C for 1 min.
    c. Load 700 μL of lysis buffer w/o Triton X-100 into a column and spin at 740 × *g* at 4°C for 1 min. Place the columns into new 2-mL reservoir tubes.
    d. Repeat step c two times (for a total of three washes).

Note: The last spin should be done immediately before the sample loading to avoid resin over-drying.

    e. Place the S-400 columns into new clean 2-mL tubes.

33. Load 100 μL of the RNase digestion reaction on each column.
34. Centrifuge columns at 740 × *g* at 4°C for 2 min.
35. Load an additional 100 μL of lysis buffer w/o Triton X-100 to each column.
36. Centrifuge columns at 740 × *g* at 4°C for 2 min.

Note: The eluted reaction is ∼220 μL.

37. Add 660 μL of TRIzol reagent to the flow-through in each tube and mix well.
38. Add 880 μL of 99.5% ethanol to each tube. Pool the solution from four tubes and purify RNA with the Direct-zol RNA MicroPrep Kit according to the manufacturer's instructions. One Direct-zol column was used.

Note: As the sample volume exceeds the volume of the MicroPrep column, divide samples into a smaller volume (700–800 μL).

⚠ CRITICAL: Use a new 2-mL reservoir tube every step during the column purification to avoid contamination.

39. Elute the RNA from the column into 7 μL of RNase-free water.
40. Add 7 μL of 2× RNA Loading Buffer (FUJIFILM Wako Pure Chemical). Proceed to step 41.

Pause Point: The purified RNA may be stored at −80°C.

Size Selection of Ribosome Footprints

Timing: 2 h, 12–20 h, 2 h

This step describes the gel extraction of ribosome footprints.

41. Prepare RNA size marker mixes according to the following tables.

Size Marker: Mix 1

Reagent	Final Concentration	Volume
17 nt RNA (SI-029) (10 μM)	0.42 μM	0.5 μL
26 nt RNA (NI-800) (10 μM)	0.42 μM	0.5 μL
34 nt RNA (NI-801) (10 μM)	0.42 μM	0.5 μL
RNase-free water	n/a	5.5 μL
2× RNA Loading Buffer (FUJIFILM Wako Pure Chemical)	1×	7 μL
Total	n/a	14 μL

Size Marker: Mix 2

Reagent	Final Concentration	Volume
Low range ssRNA ladder, including 50 and 80 nt RNAs	n/a	3 μL
RNase-free water	n/a	4 μL
2× RNA Loading Buffer (FUJIFILM Wako Pure Chemical)	1×	7 μL
Total	n/a	14 μL

42. Denature samples and size marker mixes for 3 min at 95°C and then cool on ice for 2 min.
43. Set up denaturing gel (SuperSep RNA, 15%, FUJIFILM Wako Pure Chemical) on EasySeparator.
44. Load the entire sample on the gel.

Note: Prepare a blank lane between samples to avoid cross contamination.

45. Run electrophoresis at 10 mA constant for 50 min.
46. Prepare the following SYBR Gold staining buffer, pour into rectangular plastic tray, and then transfer the gel into it. Stain the gel for 3 min on a gentle shaker.
47. Discard the staining buffer, visualize the gel by an LED transilluminator, and excise the region corresponding from 50 to 80 nt, demarcated by size markers from each footprinting sample, with a blade. Excise size markers as a control and proceed to downstream steps with the same procedure as the footprint samples. See Figure 4 as a representative result.

Optional: The footprints ranging from 17 to 34 nt could be excised for monosome profiling (or standard ribosome profiling).

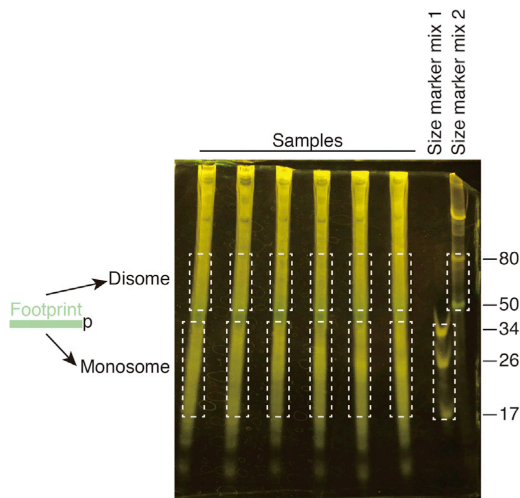

Figure 4. A Representative Gel Image for Footprinting Fragment Purification

The areas indicated by dashed white lines are gel-excised and used for downstream processes.

48. Place the gel pieces into a 1.5-mL tube and manually squeeze them with a disposable homogenizer pestle.

    Note: Use the buffer to rinse and collect the gel pieces from the pestle.

49. Add 400 μL of RNA gel extraction buffer to gel pieces and then flash-freeze by liquid nitrogen.

    Pause Point: The gel pieces may be stored at −80°C.

50. Leave the sample for 12–20 h at 23°C–26°C on a rotary mixer (Nissin).
51. Place a 0.22-μm filter (accompanied by Spin-X centrifuge tube) into a 1.5-mL tube and transfer RNA gel extraction buffer and broken gel with wide-bore 200-μL tips. Centrifuge at 10,000 × *g* at 4°C for 1 min. Ensure that no buffer remains on the filter.

    Note: The original tube supplied with Spin-X is fragile. Do not use for downstream steps.

52. Add 3 μL of GlycoBlue and 500 μL of isopropanol, mix well by vortexing, incubate in a −20°C freezer for 1 h, and then precipitate RNA at 20,000 × *g* at 4°C for 30 min.

    Pause Point: Precipitates may be stored at −20°C for several months.

    Note: Remove 800 μL of supernatant first by pipetting and then centrifuge at 20,000 × *g* at 4°C for 2 min to collect the remaining supernatant on the tube wall. Then, carefully remove all supernatant from the bottom of the tube with a 200-μL pipette.

53. Discard supernatant thoroughly by pipetting. Add 1 mL of 70% ethanol to rinse and centrifuge at 20,000 × *g* at 4°C for 3 min.

    Note: Remove the 900 μL of supernatant first by pipetting and then centrifuge at 20,000 × *g* at 4°C for 2 min to collect the remaining supernatant on the tube wall. Then, carefully remove all liquid from the bottom of the tube by a 200-μL pipette.

54. Discard the supernatant thoroughly by pipetting and dry up the pellet by centrifugal evaporation for 2–5 min.

⏸ **Pause Point:** RNA may be stored at −20°C or −80°C.

Dephosphorylation and Linker Ligation

⏱ **Timing: 12 h**

This step describes the dephosphorylation and linker ligation of ribosome footprints.

Note: We use T4 polynucleotide kinase, T4 RNA Ligase 2, truncated KQ, and Oligo Clean & Concentrator Kit with the modifications noted below.

55. Resuspend the precipitated RNA in 7 μL of 10 mM Tris pH 7.5 (or RNase-free water) and transfer to a 0.2-mL PCR tube.
56. Denature RNA at 95°C for 2 min in a thermal cycler (with the lid at 105°C) and then place on ice for 3 min.
57. Prepare the dephosphorylation reaction and incubate at 37°C for 1 h in a thermal cycler (with the lid at 57°C).

Dephosphorylation Reaction

Reagent	Final Concentration	Volume
RNA sample	n/a	7 μL
10× T4 PNK Buffer[a]	1×	1 μL
T4 PNK (10 U/μL)[a]	1 U/μL	1 μL
SUPERase·In RNase Inhibitor (20 U/μL)	2 U/μL	1 μL
Total	n/a	**10 μL**

[a]Reagents of the T4 polynucleotide kinase

⏸ **Pause Point:** Samples may be stored at −20°C or −80°C.

58. Prepare the linker ligation reaction in the same tube by adding components as described below. Incubate at 22°C for 3 h in a thermal cycler (with the lid at 45°C). Use a preadenylated linker (one of #1-#8) for each sample for pooling.

Linker Ligation Reaction

Reagent	Final Concentration	Volume
Dephosphorylation reaction	n/a	10 μL
PEG-8000 (50%)[a]	17.5%	7 μL
10× T4 RNA ligase buffer[a]	0.5×	1 μL
Preadenylated linker (20 μM) (one of #1-#8)	1 μM	1 μL
T4 Rnl2 truncated KQ (200 U/μL)[a]	10 U/μL	1 μL
Total	n/a	**20 μL**

[a]Reagents of the T4 RNA Ligase 2, truncated KQ

Note: Use wide-bore tips to take up PEG-8000. Turn the tube upside down several times and ensure that the reaction is well mixed.

59. Add 30 μL of RNase-free water to the reaction and purify linker-ligated RNA by the Oligo Clean & Concentrator Kit according to the manufacturer's instructions. Elute the linker into 7 μL of RNase-free water.

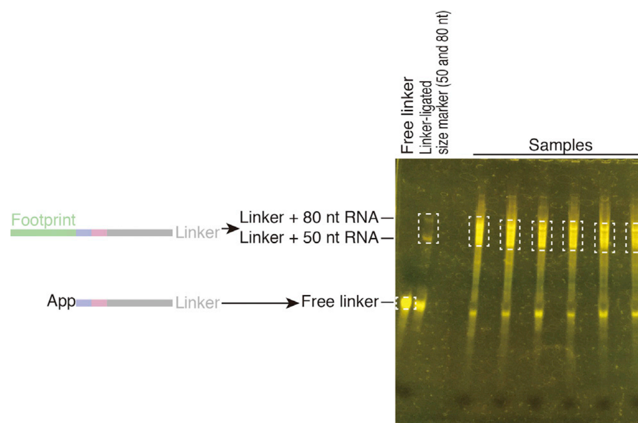

Figure 5. A Representative Gel Image for Linker-Ligated Footprints
The areas indicated by dashed white lines are gel-excised and used for downstream processes.

⚠ CRITICAL: Use a new 2-mL reservoir tube every step during the column purification to avoid contamination.

60. Add 7 μL of 2× RNA Loading Buffer (FUJIFILM Wako Pure Chemical).

Pause Point: Samples may be stored at −20°C or at −80°C.

61. Gel-purify the linker-ligated RNA as described in steps 42–54. Add the following free linker control in the gel-purification step. See Figure 5 for a representative result.

Linker Control Solution

Reagent	Final Concentration	Volume
Preadenylated linker (20 μM)	1.43 μM	1 μL
RNase-free water	n/a	6 μL
2× RNA Loading Buffer (FUJIFILM Wako Pure Chemical)	1×	7 μL
Total	n/a	14 μL

62. Resuspend the linker-ligated RNA with 10 mM Tris pH 7.5. Pool samples, scaling up to 26 μL by 10 mM Tris pH 7.5.

Note: Do not pool marker/free linker controls into samples.

Note: For marker/free linker controls, resuspend with 10 μL of 10 mM Tris pH 7.5.

Ribosome RNA Depletion

Timing: 30 min

This step describes the rRNA depletion with Ribo-Zero kit from linker-ligated RNAs.

Note: We use Ribo-Zero Gold rRNA Removal Kit Human/Mouse/Rat and Oligo Clean & Concentrator Kit with the modifications noted below.

Note: Skip this step for marker/free linker controls.

63. Prepare the beads as follows:
    a. Slowly pipette 225 μL of beads into a 1.5-mL tube.
    b. Place the tube onto a magnetic stand for 1.5-mL tubes (such as Magna Stand for 1.5-mL tubes) and wait for 1 min for the supernatant to become clear.
    c. Discard the supernatant by pipetting, add 225 μL of RNase-free water, and vortex vigorously.
    d. Repeat steps b-c (for a total of two washes). Discard RNase-free water by pipetting and re-suspend beads in 65 μL of magnetic bead resuspension solution. Vortex well and keep on ThermoMixer preset at 25°C.
64. Perform rRNA depletion as follows:
    a. Prepare the following rRNA depletion solution in a 0.2-mL PCR tube as described below.

rRNA Depletion Solution

Reagent	Final Concentration	Volume
Linker-ligated RNA	n/a	26 μL
10× Ribo-Zero reaction buffer[a]	1×	4 μL
Ribo-Zero rRNA removal solution[a]	n/a	10 μL
Total	n/a	40 μL

[a]Reagents of the Ribo-Zero Gold rRNA Removal Kit Human/Mouse/Rat

    b. Incubate at 68°C for 10 min and then at 25°C for 5 min in a thermal cycler (with the lid at 88°C).
    c. Add the reaction to 65 μL of beads in 1.5-mL tube and immediately mix by pipetting ten times, followed by vortexing for 10 s.
    d. Incubate at 25°C for 5 min on a ThermoMixer and then vortex for 10 s.
    e. Spin down the tube and place the tube onto a magnetic stand for 1.5-mL tubes (such as Magna Stand for 1.5-mL tubes) and wait for 1 min for the supernatant to become clear.
    f. Collect the supernatant by pipetting into a new 1.5-mL tube and place on ice.
    g. Purify the linker-ligated RNA using the Oligo Clean & Concentrator Kit according to the manufacturer's instructions. Elute the RNA into 10 μL of RNase-free water.

⚠ CRITICAL: Use a new 2-mL reservoir tube every step during the column purification to avoid contamination.

Note: The typical ~100 μL of supernatant obtained requires 200 μL of Oligo Binding Buffer and 800 μL of 99.5% ethanol before being spun onto the column.

Note: Human Ribo-Zero works for zebrafish samples.

Note: Ribo-Zero Kit accompanied by TruSeq Stranded Total RNA Library Prep Gold (Illumina, cat. no. 20020598) can be used.

Pause Point: Samples may be stored at −20°C or −80°C for several months.

Reverse Transcription

Timing: 8 h

This step describes the generation of cDNA by reverse transcription.

Note: We use ProtoScript II Reverse Transcriptase and Oligo Clean & Concentrator Kit with the modifications noted below.

65. Prepare the following mixture to hybridize the reverse transcription (RT) primer (NI-802). Prepare free reverse transcription primer control.

Reverse Transcription Primer Hybridization

Reagent	Final Concentration	Volume
Linker-ligated pooled samples, linker-ligated size markers, free linker control, or RNase-free water	n/a	10 μL
RT primer (NI-802) (1.25 μM)	0.21 μM	2 μL
Total	n/a	12 μL

66. Denature at 65°C for 5 min in a thermal cycler (with the lid at 105°C) and then place on ice.
67. Prepare the following reverse transcription reaction, add to 12 μL of the RT primer hybridized linker-RNA, and incubate at 50°C for 30 min in a thermal cycler (with the lid at 70°C).

Reverse Transcription Reaction

Reagent	Final Concentration	Volume
5× Protoscript II buffer[a]	1×	4 μL
dNTPs (10 mM each)	0.5 mM each	1 μL
DTT (100 mM)[a]	5 mM	1 μL
SUPERase·In RNase Inhibitor (20 U/μL)	1 U/μL	1 μL
Protoscript II (200 U/μL)[a]	10 U/μL	1 μL
Total	n/a	8 μL

[a]Reagents of ProtoScript II Reverse Transcriptase

68. Add 2.2 μL of 1 M NaOH and incubate at 70°C for 20 min in a thermal cycler (with the lid at 105°C) to degrade RNA.
69. Add 28 μL of RNase-free water and purify cDNA by the Oligo Clean & Concentrator Kit according to the manufacturer's instructions. Elute linker into 7 μL of RNase-free water.

⚠ CRITICAL: Use a new 2-mL reservoir tube every step during the column purification to avoid contamination.

70. Add 7 μL of 2× RNA Loading Buffer (FUJIFILM Wako Pure Chemical).
71. Gel-purify the cDNA as described in steps 42–54. Use DNA extraction buffer instead of RNA extraction buffer. See Figure 6 for a representative result.

⏸ **Pause Point:** Precipitates may be stored at −20°C.

Intramolecular Ligation

⏲ Timing: 2 h

This step describes the circularization of cDNA by intramolecular ligase.

Note: We use CircLigase II ssDNA ligase with the modifications noted below.

72. Resuspend the precipitated cDNA in 12 μL of 10 mM Tris pH 7.5 and transfer to a 0.2-mL PCR tube.

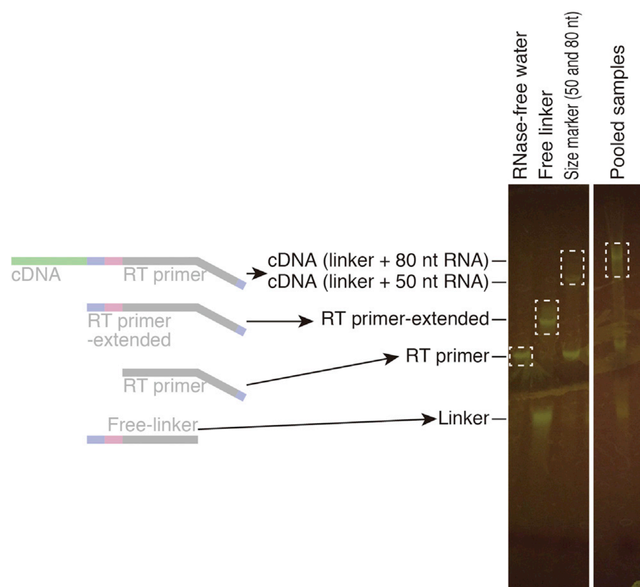

Figure 6. A Representative Gel Image for Reverse Transcribed Products

The areas indicated by dashed white lines are gel-excised and used for downstream processes.

Pause Point: cDNA may be stored at −20°C.

73. Prepare the following intramolecular ligation reaction, incubate at 60°C for 1 h, and then heat-inactivate for 10 min at 80°C in a thermal cycler (with the lid at 105°C).

Intramolecular Ligation Reaction

Reagent	Final Concentration	Volume
cDNA	n/a	12 μL
10× CircLigase II buffer[a]	1×	2 μL
Betaine (5 M)[a]	1 M	4 μL
$MnCl_2$ (50 mM)[a]	2.5 mM	1 μL
CircLigase II (100 U/μL)[a]	5 U/μL	1 μL
Total	n/a	20 μL

[a]Reagents of CircLigase II ssDNA ligase

Pause Point: Circularized DNA may be stored at −20°C.

Optimization of PCR

Timing: 3 h

This step describes the optimization of library amplification by PCR.

Note: We use Phusion High-Fidelity DNA Polymerase with the modifications noted below.

74. Prepare the following PCR reaction.

PCR: Small Scale

Reagent	Final Concentration	Volume
5× Phusion HF buffer[a]	1×	10 μL
dNTPs (10 mM each)	0.2 mM each	1 μL
Forward library PCR primer (NI-798) (100 μM)	500 nM	0.25 μL
Reverse library PCR primer (NI-822) (100 μM)	500 nM	0.25 μL
Circularized cDNA template	n/a	2.5 μL
RNase-free water	n/a	35.5 μL
Phusion polymerase (2 U/μL)[a]	0.02 U/μL	0.5 μL
Total	n/a	**50 μL**

[a]Reagents of Phusion High-Fidelity DNA Polymerase

75. Set up 3 8-strip PCR tubes for three different PCR (8, 10, 12×) cycles and transfer a 16.5-μL aliquot of the PCR mixture into one tube in each 8-strip tube.
76. Perform the following PCR amplification with three different numbers of cycles (8, 10, and 12 cycles) in a thermal cycler (with the lid at 105°C) with the program below.

PCR Conditions

Steps	Temperature	Time	Cycle
Initial denaturation	98°C	30 s	1×
Denaturation	98°C	10 s	8, 10, 12×
Annealing	65°C	10 s	
Extension	72°C	5 s	
Hold	4°C (or 12°C)	Forever	1×

77. At the end of each cycle of PCR, place an 8-strip PCR tube from the thermal cycler on ice.

Note: Increase or decrease cycle numbers, if necessary.

78. Add 3.3 μL of Gel Loading Dye Purple 6× (NEB) to each sample.
79. Set a SuperSep DNA nondenaturing gel on an EasySeparator.
80. Load the entire volume of the PCR reaction on the gel.

Note: Load the same samples with different PCR cycles in adjacent wells for direct comparison.

81. Separate by electrophoresis at 20 mA for 80 min.
82. Stain and visualize the gel with SYBR Gold according to steps 46 and 47. See Figure 7 for a representative result.
83. Select the best cycle with a prominent product band but with minimum reannealed partial duplex library products.

Optional: Excise the PCR product by marker control and free linker control as well for the quality assessment step.

Note: For monosome profiling, directly proceed to step 92.

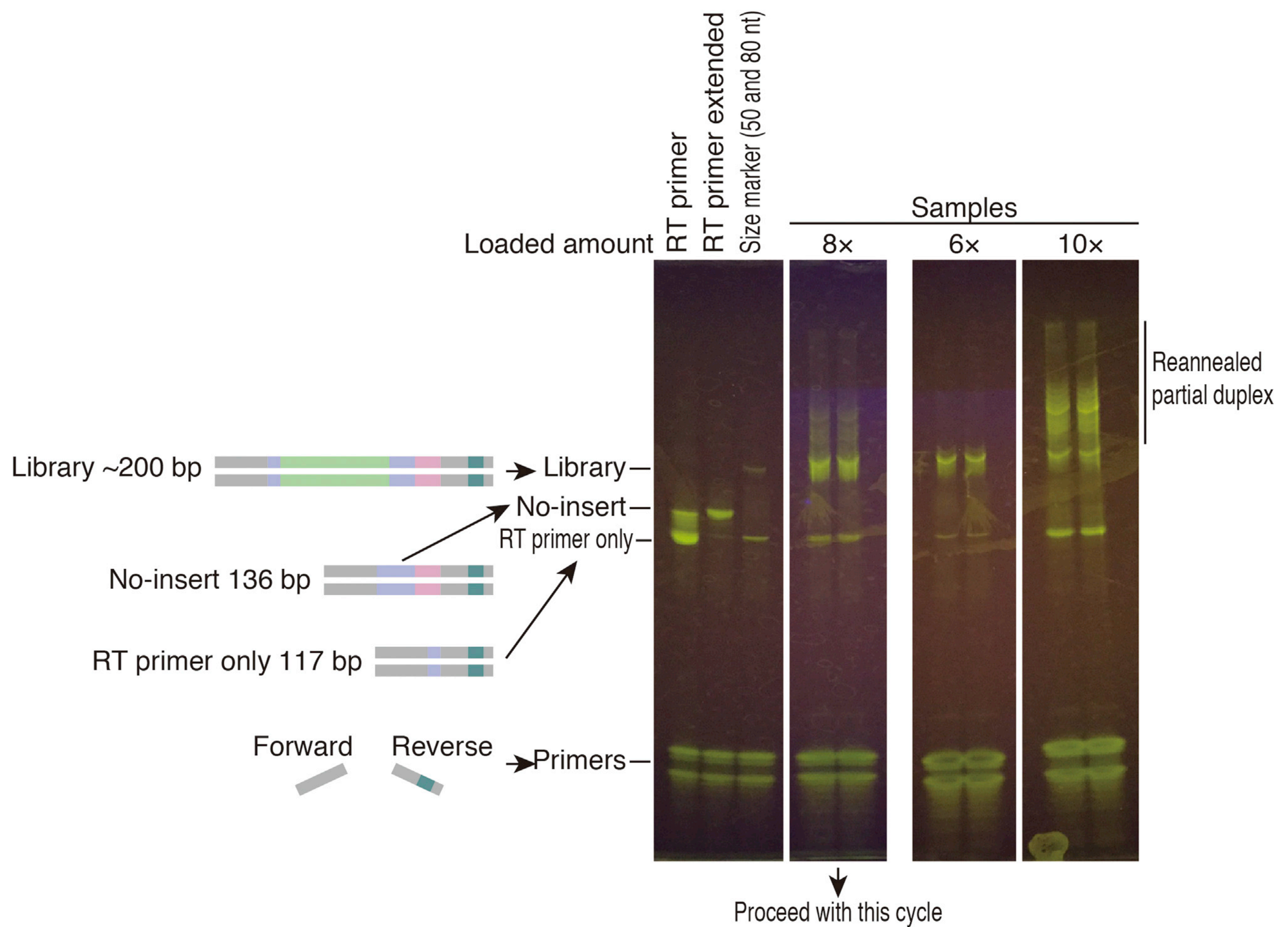

Figure 7. A Representative Gel Image for the PCR Product to Optimize the PCR Cycles
Optimal PCR conditions (eight cycles in this analysis) were used for downstream processes.

Note: For zebrafish disome profiling, directly proceed to step 92.

PCR Amplification and CRISPR-Mediated rRNA Fragment Depletion

Timing: 5 h, 12–20 h, 2 h

This step describes the depletion of a fraction of DNA with rRNA sequence by CRISPR-Cas9.

Note: We use Phusion High-Fidelity DNA Polymerase, EnGen Cas9 NLS, and NucleoSpin Gel and PCR Clean-up with the modifications noted below.

84. Prepare the following PCR in a 0.2-mL PCR tube as follows.

Note: Use a different reverse primer with other Illumina index sequences for pooling.

85. Mix 10 μg each of gRNA #1, #2, and #3 in 20 μL of RNase-water to make the gRNA mixture (1.5 μg/μL or 34.5 μM).

PCR: Large Scale

Reagent	Final Concentration	Volume
5× Phusion HF buffer[a]	1×	20 μL
dNTPs (10 mM each)	0.2 mM each	2 μL
Forward Primer (100 μM)	500 nM	0.5 μL
Reverse Primer (100 μM)	500 nM	0.5 μL
Circularized cDNA template	n/a	5 μL
RNase-free water	n/a	71 μL
Phusion polymerase (2 U/μL)[a]	0.02 U/μL	1 μL
Total	**n/a**	**100 μL**

[a]Reagents of Phusion High-Fidelity DNA Polymerase

86. Prepare the following CRISPR-Cas9 solution in a PCR tube as follows and incubate at 25°C for 10 min in a thermal cycler (with the lid at 45°C).

CRISPR-Cas9 Solution

Reagent	Final Concentration	Volume
Sample		
gRNA mixture (34.5 μM)	3.45 μM	3 μL
10× Cas9 Reaction Buffer[a]	4×	12 μL
Cas9 (20 μM)*	1 μM	1.5 μL
RNase-free water	n/a	13.5 μL
Total	**n/a**	**30 μL**
Control		
gRNA mixture (34.5 μM)	3.45 μM	0.5 μL
10× Cas9 Reaction Buffer[a]	4×	2 μL
RNase-free water	n/a	2.5 μL
Total	**n/a**	**5 μL**

[a]Reagents of EnGen Cas9 NLS

87. Add 90 μL of PCR to 30 μL of CRISPR-Cas9 solution (hereafter "sample"), split into two 0.2-mL PCR tubes, and incubate at 37°C for 30 min in a thermal cycler (with the lid at 57°C). For the negative control, add 5 μL of PCR and 10 μL of RNase-free water to 5 μL of CRISPR-Cas9 control solution (hereafter "control").
88. Add 1.5 μL of RNase I (10 U/μL) to each tube of sample solution and 0.5 μL to the control solution. Incubate at 37°C for 10 min in a thermal cycler (with the lid at 57°C).
89. Add 1.5 μL of proteinase K (20 mg/mL) to each tube of sample solution and 0.5 μL to the control solution. Incubate at 37°C for 10 min in a thermal cycler (with the lid at 57°C).
90. Purify DNA by the Oligo Clean & Concentrator Kit according to the manufacturer's instructions. Use two columns for samples and one for CRISPR-Cas9 control. Elute samples into 36 μL of RNase-free water and pool into one tube. Elute control into 12 μL of RNase-free water.

⚠ CRITICAL: Use a new 2-mL reservoir tube for every step during the column purification to avoid contamination.

91. Prepare the following reactions and load the entire volume on a nondenaturing gel (SuperSep DNA, 15%, FUJIFILM Wako Pure Chemical) and visualize DNAs as described in steps 79 and 82. Load on multiple wells if the volume in a well exceeds 20 μL. See Figure 8 for a representative result.

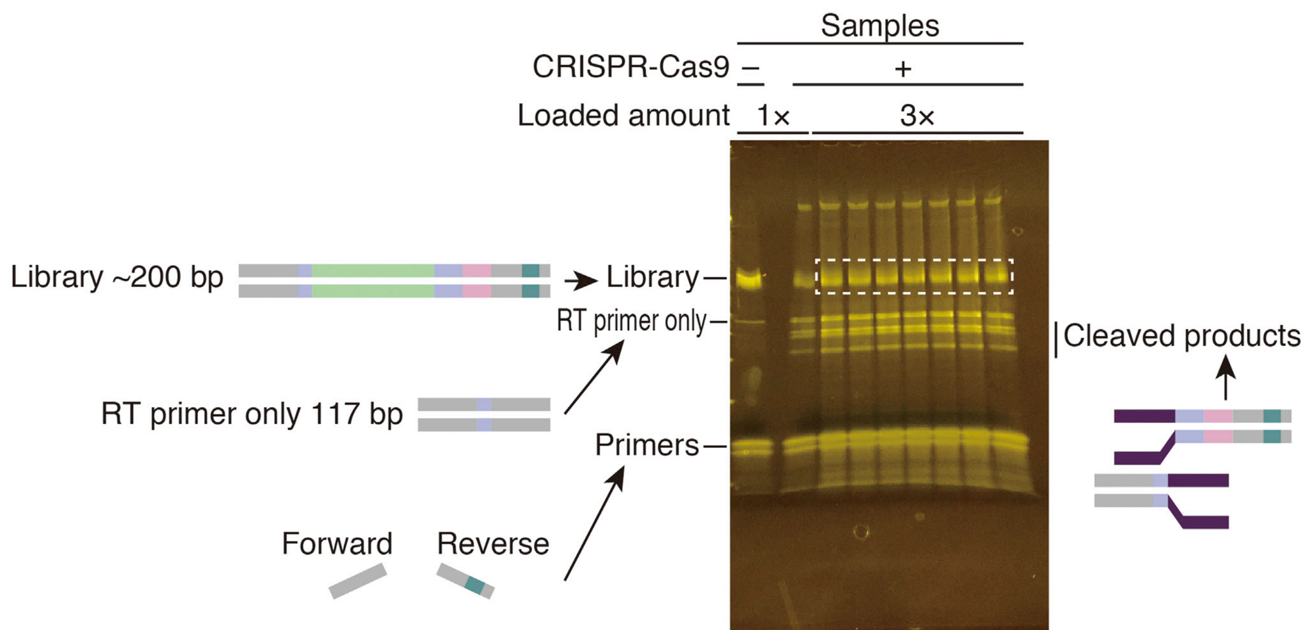

Figure 8. A representative Gel Image for CRISPR-Cas9-Mediated rRNA Depletion
The areas indicated by dashed white lines are gel-excised and used for downstream processes.

Library Gel Purification: Sample, Low Amount

Reagent	Final Concentration	Volume
Sample	n/a	4 μL
RNase-free water	n/a	6 μL
Gel Loading Dye Purple 6×	1.7×	4 μL
Total	n/a	14 μL

Library Gel Purification: Control

Reagent	Final Concentration	Volume
Control	n/a	10 μL
Gel Loading Dye Purple 6×	1.7×	4 μL
Total	n/a	14 μL

Library Gel Purification: Sample, High Amount

Reagent	Final Concentration	Volume
Sample	n/a	64 μL
Gel Loading Dye Purple 6×	1.5×	22 μL
Total	n/a	86 μL

Note: Keep gel loading buffer at 1.5× to 3× to avoid flotation of reactions in TBE buffer.

Note: Compare the sample (low amount) to the control to estimate the reaction efficiency.

Optional: Use the remaining 1 μL of sample for the quality assessment step.

Pause Point: Double-stranded DNA may be stored at −20°C.

92. Excise and break the gels as described in steps 48–51. Use 130 μL of DNA extraction buffer per lane of the gels.
93. Purify the DNA with NucleoSpin Gel and PCR Clean-up according to the manufacturer's instructions. Elute DNA into 15 μL of NE buffer (accompanied with the kit, 5 mM Tris-HCl pH 8.5).

Pause Point: DNA may be stored at −20°C.

Quality Assessment

Timing: 1 h

This step describes the quality assessment of the library by fragment analyzer.

Note: We use DNA-1000 Kit with the modifications noted below.

94. Bring separation buffer (a reagent of the DNA-1000 Kit), marker solution (a reagent of the DNA-1000 Kit), 25 times diluted GelStar (stock solution diluted 25 times with TE), and 50 times diluted 100-bp DNA ladder (stock solution diluted 50 times with TE) to 23°C–26°C.
95. Prepare the required amount of buffer solution for MultiNA (table below shows a typical example) into a separation buffer tube.

Library Gel Purification

Reagent	Final Concentration	Volume
1/25 diluted GelStar	n/a	2 μL
Separation buffer (a reagent of the DNA-1000 Kit)	n/a	398 μL
Total	n/a	400 μL

96. Dilute the marker solution (a reagent of the DNA-1000 Kit) 5 times with RNase-free water. Required amount: (No. of chips + No. of samples) × 6 μL.
97. Prepare the following reaction in a PCR plate, place into the MultiNA, and start the run.

MultiNA Run: Sample

Reagent	Final Concentration	Volume
Sample	n/a	2 μL
Diluted marker solution (a reagent of the DNA-1000 Kit)	n/a	4 μL
Total	n/a	6 μL

MultiNA Run: Ladder

Reagent	Final Concentration	Volume
1/50 diluted 100 bp ladder	n/a	3 μL
Diluted marker solution (a reagent of the DNA-1000 Kit)	n/a	6 μL
Total	n/a	9 μL

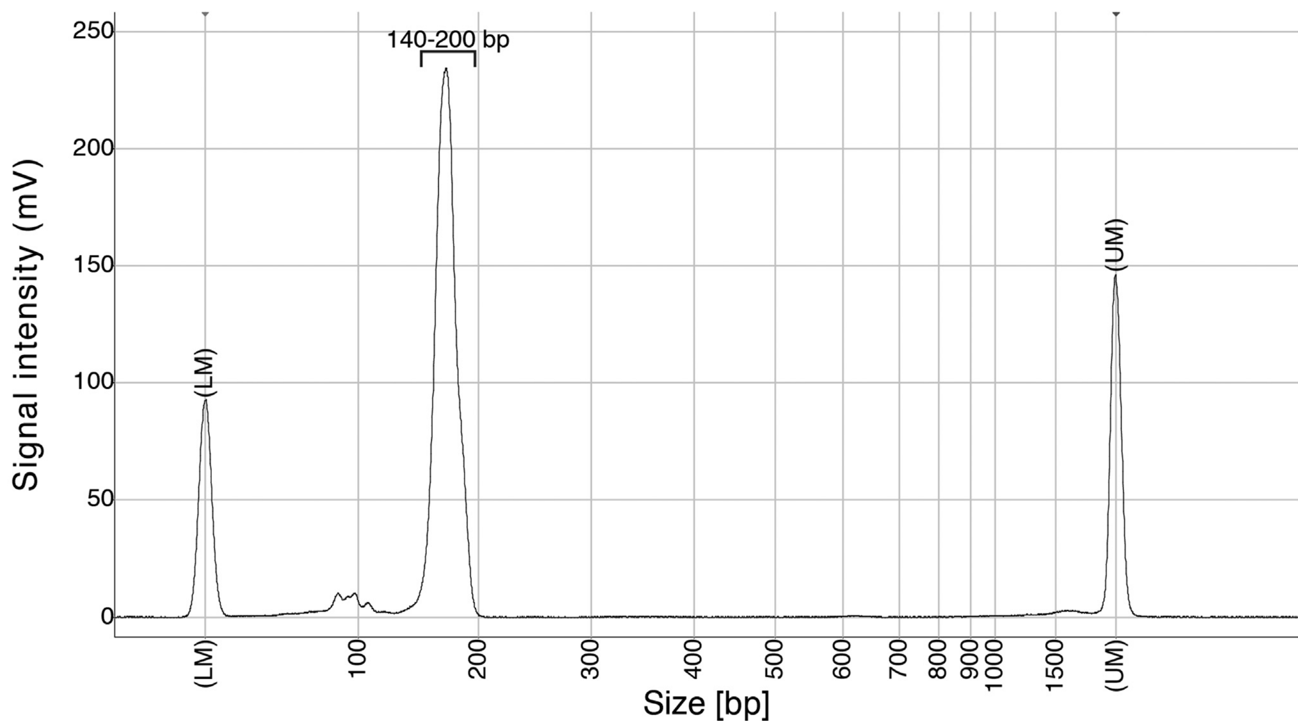

Figure 9. A Representative Result of the Fragment Analyzer for Sequencing the DNA Library
LM, lower marker; UM, upper marker.

Note: Run MultiNA with a high-sensitivity mode. The high-sensitivity mode requires a specific setup of software that was not preinstalled in the MultiNA but was available from Shimazu.

98. Use the concentrations shown in the MultiNA results for downstream purposes.

Note: Libraries can be either single-end or pair-end sequenced.

EXPECTED OUTCOMES

The final DNA libraries for disome (or monosome) profiling are ready to be sequenced by an Illumina sequencer such as HiSeq4000. Typical disome profiling libraries are ~25 nM (~3 ng/μL) with a peak at 140–200 bp (Figure 9) (the size could be smaller than expected for our equipment setup, probably because of the limitation of MultiNA for size estimation for small DNA fragments). Successful libraries have little accumulation of reannealed partial duplex products.

LIMITATIONS

Disome profiling is a powerful method to probe the position of collided ribosomes across the transcriptome in cells. Since such queued ribosomes are relatively rare compared to regularly translating monosomes, the experiment might require more initial cells. As this could vary among conditions, optimization would be necessary.

By bioinformatic analysis, the peak position with a disome footprint will be analyzed. Since biased PCR amplification may occur, caution will be necessary. The random sequence placed in linkers (5 nt) and reverse transcription primer (2 nt) can be used as a unique molecular index (UMI) to suppress such a PCR duplication.

TROUBLESHOOTING

Problem

Inefficient reverse transcription (visible at step 72 and Figure 6).

Potential Solution

The contamination by ethanol in the wash buffer from the Oligo Clean & Concentrator Kit may reduce the efficacy of reverse transcription. Dry the column of the Oligo Clean & Concentrator Kit well by centrifugation. Use a new 2-mL reservoir tube at every step during the column purification to avoid ethanol contamination.

Problem

Ethanol remains after drying the pellet by centrifugal evaporator (at step 54).

Potential Solution

The 70% ethanol should be removed from tubes thoroughly. To achieve this, we remove 900 μL of supernatant first by pipetting and then centrifuge at 20,000 × *g* at 4°C for 2 min to collect the remaining supernatant on the tube wall. Then, we carefully remove all liquid from the bottom of the tube using a 200-μL pipette (see also Note at step 53).

RESOURCE AVAILABILITY

Lead Contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Shintaro Iwasaki (shintaro.iwasaki@riken.jp).

Materials Availability

This study did not generate new unique materials.

Data and Code Availability

Original images used for the figures are deposited in the Mendeley database (https://doi.org/10.17632/gjrkppp3fc.1).

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.xpro.2020.100168.

ACKNOWLEDGMENTS

We thank all the members of the Iwasaki laboratory for constructive discussion, technical help, and critical reading of the manuscript. S.I. was supported by the Ministry of Education, Culture, Sports, Science and Technology (MEXT) (a Grant-in-Aid for Scientific Research on Innovative Areas “nascent chain biology” JP17H05679), the Japan Society for the Promotion of Science (JSPS) (a Grant-in-Aid for Young Scientists [A] JP17H04998, a Challenging Research [Exploratory] JP19K22406, and a Grant-in-Aid for Transformative Research Areas [B] 20H05784), RIKEN (Pioneering Projects "Cellular Evolution" and Ageing Project), and the Takeda Science Foundation. Y.M. was supported by a Grant-in-Aid for Scientific Research on Innovative Areas “nascent chain biology” JP17H05662 from MEXT and a Grant-in-Aid for Scientific Research (B) JP18H02370 from JSPS.

AUTHOR CONTRIBUTIONS

M.M. performed all the experiments with the help of Y.M.; S.I. supervised the project; M.M., Y.M., and S.I. wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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