

Mitochondrial Ribosome (Mitoribosome) Profiling for Monitoring Mitochondrial Translation In Vivo

UNIT 4.28

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Translation in the mitochondria is regulated by mechanisms distinct from those acting in the cytosol and in bacteria, yet precise methods for investigating it have lagged behind. This unit describes an approach, mitochondrial ribosome (mitoribosome) profiling, to quantitatively monitor mitochondrial translation with high temporal and spatial resolution in *Saccharomyces cerevisiae*. Mitoribosomes are immunoprecipitated from whole-cell lysate and the protected mRNA fragments are isolated. These fragments are then converted to sequencing libraries or analyzed by northern blot hybridization to reveal the distribution of mitoribosomes across the mitochondrial transcriptome. As information about RNA abundance is required to resolve translational from RNA effects, we also present an RNA sequencing approach that can be performed in parallel. Accurately capturing the biologically relevant distribution of mitoribosome positions depends on several critical parameters that are discussed. Application of mitoribosome profiling can reveal mechanisms of mitochondrial translational control that were not previously possible to uncover. © 2017 by John Wiley & Sons, Inc.

Keywords: mitochondria • ribosome profiling • ribosome immunoprecipitation • RNA-seq • northern blot • translation

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INTRODUCTION

Mitochondria have their own genome that is expressed by a dedicated mitochondrial RNA polymerase and mitochondrial ribosome (mitoribosome; Despande & Patel, 2012; Kehrein, Bonnefoy, & Ott, 2013). This unit presents a set of protocols for monitoring mitochondrial gene expression in *Saccharomyces cerevisiae* with high temporal resolution and in any condition (Fig. 4.28.1). Basic Protocols 1 and 2 describe mitoribosome profiling: A method to monitor mitoribosome positions with codon resolution across the mitochondrial transcriptome. The method is adapted from the ribosome profiling technique developed to monitor cytosolic ribosome (cytoribosome) positions by deep sequencing ribosome-protected fragments (footprints; Ingolia, 2010; Ingolia, Ghaemmaghami, Newman, & Weissman, 2009). Here, to ensure enrichment of mitoribosomes (74 S) from the similar-sized but more abundant cytoribosomes (80 S), they are immunoprecipitated via a 3X-FLAG epitope tag added endogenously to a mitoribosomal protein. Two critical modifications required to capture mitoribosome footprints are (1) buffer conditions that do not induce subunit dissociation and (2) size selection that accounts for the larger footprint of mitoribosomes (Couvillion, Soto, Shipkovenska, & Churchman, 2016). Once isolated, mitoribosome footprints are used to generate a library to be sequenced on the Illumina platform.

Preparation and
Analysis of RNA

4.28.1



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Supplement 119

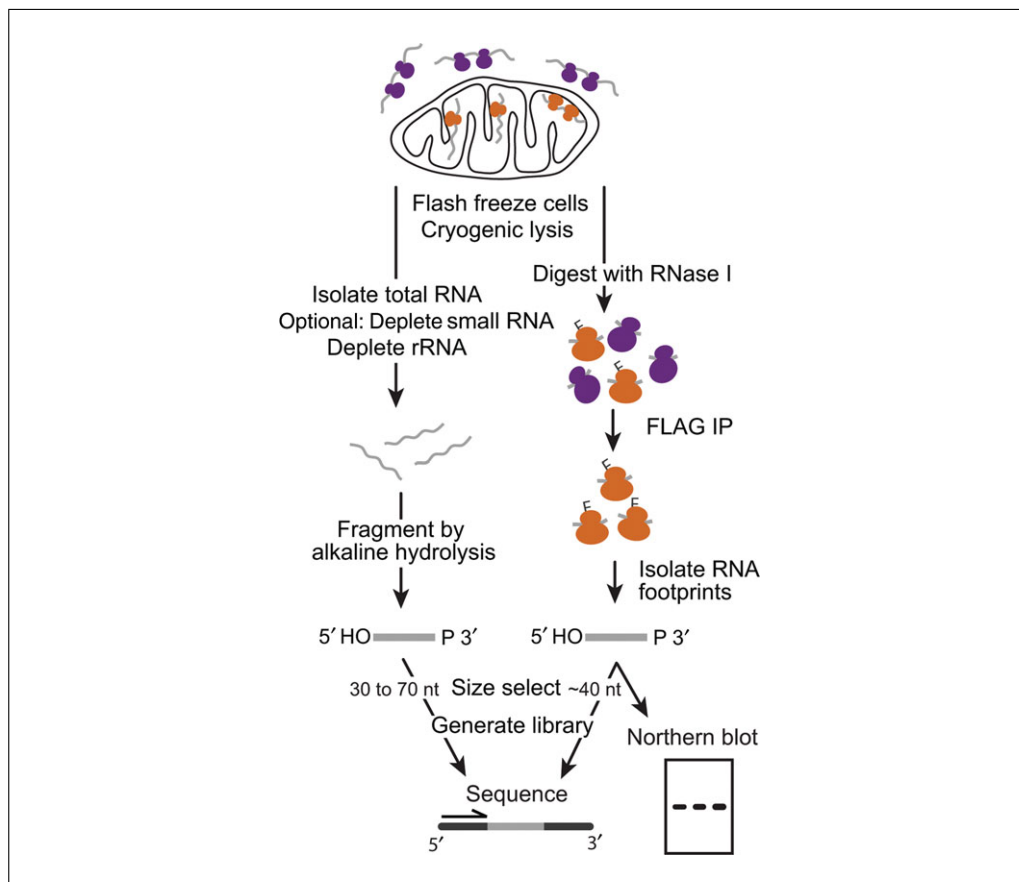


Figure 4.28.1 Flowchart of the protocols presented in this unit. mRNA isolation and fragmentation (left, Support Protocol), and mitribosome footprint isolation (right, Basic Protocol 1) result in chemically similar RNA species that are then size selected and converted to libraries for sequencing (Basic Protocol 2). Alternatively, mitribosome footprints can be analyzed by northern blot hybridization (Alternate Protocol). IP, immunoprecipitation.

Two additional protocols expand the potential of mitribosome profiling (Support Protocol and Alternate Protocol). One, RNA sequencing (RNA-seq), performed in parallel allows the deconvolution of RNA-level and translational effects. Two, detection of mitribosome footprints by northern blot hybridization, can be used instead of or in addition to library generation and sequencing. It provides a rapid way to verify the success of the experiment and the size range of footprints. It can also be used to compare ribosome occupancy on a transcript across conditions or strains.

Here, steps to isolate mitribosome footprints from the yeast *S. cerevisiae* are presented (Basic Protocol 1), as well as how to isolate total mRNA fragments in parallel for RNA sequencing (Support Protocol). Mitribosome footprints (and total mRNA) can then be converted into libraries for deep sequencing (Basic Protocol 2), or footprints can be analyzed by northern blotting (Alternate Protocol).

BASIC PROTOCOL 1

Mitochondrial Ribosome (Mitribosome) Profiling

4.28.2

ISOLATE MITRIBOSOME-PROTECTED FRAGMENTS BY IMMUNOPRECIPITATION

This protocol describes the isolation of mitochondrial ribosome footprints. A yeast strain with 3X-FLAG tagged mitribosomes is rapidly harvested and lysed under cryogenic conditions to preserve ribosome positions. Endogenously tagged mitribosome small subunit protein MrpS17 (MrpS17-FLAG) is used here, but tagged large subunit proteins Mrp20 (Mrp20-FLAG) and Img1 (Img1-FLAG) yield similar results. The lysate is thawed and treated with RNase I to digest unprotected RNA. Next, FLAG antibody-conjugated

agarose beads are used for batch purification, the beads are washed, and mitoribosomes are eluted by competition with 3X-FLAG peptide. The RNA is then extracted from the immunoprecipitate using phenol (Ni, Xu, Sabanayagam, & Gallagher, 2016).

Materials

Yeast strain expressing a C-terminal epitope tagged (3X-FLAG) MrpS17 grown in medium of choice
RNase-free water
Lysis buffer (including protease inhibitors and lauryl maltoside; see recipe)
50 U/ μ l RNase I (RNase I_f; New England Biolabs, cat. no. M0243)
5 $\times$ SDS-PAGE sample buffer (see recipe)
20 U/ μ l SUPERase-In
Agarose slurry conjugated with FLAG antibody (Sigma-Aldrich)
Wash buffer (including Triton X-100; see recipe)
5 mg/ml 3X-FLAG peptide in tris-buffered saline (TBS; aliquot and store at -80°C , avoid freeze-thaw; Sigma-Aldrich, cat. no. F4799)
125:24:1 (v/v/v) acid-phenol/chloroform/isoamyl alcohol, pH 4.5
3 M sodium acetate, pH 5.5 (RNase-free; *APPENDIX 2*)
25 mg/ml linear polyacrylamide (LPA)
Isopropanol
70% (v/v) ethanol
10 mM Tris-Cl, pH 7.0 (RNase-free; *APPENDIX 2*)
Anti-FLAG M2 antibody (Sigma-Aldrich, cat. no. F3165)
Liquid nitrogen
Microfiltration assembly (90-mm, ULTRA-WARE)
 4-liter side-arm flask with a fritted glass support base
 Glass funnel
 Anodized aluminum clamp
 No. 8 silicone stopper
Nitrocellulose membrane (0.45- μ m, 90-mm diameter membranes; Whatman)
Mixer mill, 50-ml chambers and 25-mm stainless steel ball (Retsch)
100 $^{\circ}\text{C}$ heat block
Floor centrifuge with rotor able to reach 20,000 $\times g$ (e.g., Sorvall with SS-34 rotor and Oak Ridge tubes)
End-over-end rotator
Costar Spin-X centrifuge tube filter (0.45- μ m cellulose acetate in 2-ml tube; Corning)
Spectrophotometer
Vacuum source
Styrofoam container
Forceps
15- and 50-ml conical tubes
20-G needles
Plastic spatula (Cell Lifter, 18 cm; GeneMate, cat. no. T-2443-4)
Metal spatulas with curved ends
Tongs
Cryo-gloves
Kimwipes
 -80°C freezer
Refrigerated centrifuge
Vortex

CAUTION: Phenol/chloroform is harmful if swallowed or comes in contact with skin, causes severe skin burns and eye damage, is fatal if inhaled, and is potentially carcino-

genic. It should be used with appropriate safety measures such as protective gloves, glasses, clothing, and sufficient ventilation. All waste should be handled according to hazardous waste regulations.

Culture yeast cells and harvest by filtration

1. Culture cells (Sherman, 2002) as desired for experiment. For analysis by northern blotting, collect ~60 OD₆₀₀ equivalents (small scale, ~100 ml). For analysis by sequencing, collect ~400 OD₆₀₀ equivalents (large scale, ~700 ml).

Keep cell number equivalent across samples to be compared. See Treco and Winston (2008) for a discussion on relating optical density and cell number.

2. Set up filtration apparatus and prepare 50-ml conical tubes submerged in and filled with liquid nitrogen. Additionally, use a needle to make a hole in tube caps for removal of liquid nitrogen later.

Prepare all materials prior to retrieving culture from shaker in order to minimize time between removing cells from condition of interest and freezing.

3. Measure culture volume for desired cell number and harvest cells by filtration.
4. Using a flat, plastic spatula (Cell Lifter), scrape all cells from filter and transfer to liquid nitrogen-filled tube.

To remove cell paste from spatula after freezing, use a second, prechilled spatula to scrape it off.

5. Cap tubes and remove liquid nitrogen through hole in cap.

Tubes with cell pellets can be stored for 6 months at –80°C.

Prepare frozen lysis buffer

Alternatively, lysis buffer can be prepared and frozen first and cells collected into the same tube.

6. Prepare 2.6 ml (small scale) or 4 ml (large scale) of 1× lysis buffer per sample. Make 2.6 or 4 ml aliquots, respectively, and chill on ice 10 min.
7. Prepare 50-ml conical tubes submerged in and filled with liquid nitrogen, with holes in caps as in step 2.

Additional liquid nitrogen may have to be poured on top as it boils off during the subsequent step.

8. Drip lysis buffer 150 µl at a time into liquid nitrogen-filled tubes.

Wait several seconds after each drop until the buffer is completely frozen before adding more. If many samples are being prepared at once, move on to the next tubes, cycling through to reduce waiting time.

9. Cap tubes and remove liquid nitrogen through hole in cap.
10. Transfer one tube frozen buffer into each tube of frozen cells, cap, and shake to combine.

Tap tubes to remove material from cap before proceeding or storing. Samples can be stored for 6 months at –80°C.

Cryogenically lyse by mixer mill

11. Prechill mixer mill chamber, disassembled except for the plastic O-ring, and stainless steel ball in liquid nitrogen.

Wait for vigorous boiling to stop; this may take several minutes.

12. Remove chamber and place frozen yeast/buffer mix, then ball, inside. Screw chamber together and place into liquid nitrogen.
13. Load chambers and run mixer mill 3 min at 15 Hz. Place chambers back into liquid nitrogen until vigorous boiling stops. Repeat for a total of 6 rounds.
14. Prepare 50-ml conical tubes submerged in liquid nitrogen, but do not fill.
15. Prechill rounded side of metal spatula.
16. Remove chamber from liquid nitrogen, open, and scrape grindate out; transfer to prepared 50-ml tube.

Remove as much of grindate as possible, transferring the steel ball a couple times and scraping the walls of both sides of the chamber. Samples can be stored for 6 months at -80°C .

Digest RNA for ribosome footprinting

17. For large scale preparation only, freshly prepare additional $1\times$ lysis buffer to dilute thawed sample (see below for amount needed).
18. Thaw grindate in a bath of room temperature water.

As grindate thaws, gently swirl to maintain more homogenous temperatures throughout the sample. As soon as ice is melted, move immediately to the next step.

19. For large scale preparation only, add $1\times$ lysis buffer to dilute lysate to a concentration of 25 OD₆₀₀/ml. Starting volume is ~ 4 ml, e.g., if you collected 400 OD₆₀₀ cell equivalents, add 12 ml to bring volume to 16 ml. Mix gently by inverting several times.

20. Remove aliquot for RNA sequencing.

Yield is at least 100 ng total RNA/ μl lysate. Typically 200 μl is sufficient for downstream applications. For best quality, proceed directly to isolate RNA (see Support Protocol) or alternatively, flash freeze and store for up to 6 months at -80°C .

21. Add RNase I to 500 U/ml lysate, swirl thoroughly to mix, incubate 30 min in room temperature (25°C) water bath. Swirl to mix again after 15 min.

Optional: Save 40 μl of this input sample for analysis by immunoblotting: Combine with 10 μl $5\times$ SDS-PAGE sample buffer, vortex 5 sec, boil 5 min, and store -20°C .

22. To stop digestion add Suprase-In to 100 U/ml. Mix by swirling, and place on ice.

23. Centrifuge $20,000 \times g$ at 4°C , 15 min.

For small scale preparation, lysate can be transferred to microcentrifuge tubes for centrifugation in a microcentrifuge. For large scale preparation, transfer to Oak Ridge centrifuge tube and use Sorvall floor centrifuge with SS-34 rotor or equivalent.

Immunoprecipitate mitoribosomes

24. Prepare agarose slurry (12 μl per ml lysate) by washing three times in at least 40 volumes of wash buffer. To wash, invert tube several times, centrifuge 1 min at $1000 \times g$ to pellet beads, and discard supernatant. Resuspend beads by adding 4.5 volumes wash buffer.

To avoid crushing the agarose beads, do not centrifuge at high speed.

25. Transfer supernatant (cleared lysate) from step 23 to fresh tube at 4°C .

Optional: Save 40 μl of this soluble sample for analysis by immunoblotting: Combine with 10 μl $5\times$ SDS-PAGE sample buffer, vortex 5 sec, boil 5 min, and store at -20°C .

26. To cleared lysate, add 60 μ l washed, diluted agarose slurry (from step 24) per ml of lysate.

Agarose beads settle quickly; pipet up and down before removing each aliquot.

27. Incubate lysate with beads 3 hr at 4°C, rotating end over end.

28. Centrifuge 2 min at 1000 $\times$ g, 4°C, to pellet beads.

Optional: Save 40 μ l of the supernatant as unbound sample for analysis by immunoblotting: Combine with 10 μ l 5 $\times$ SDS-PAGE sample buffer, vortex 5 sec, boil 5 min, and store at -20°C .

29. Remove and discard supernatant.

30. Wash beads in 50 volumes wash buffer three times, rotating end over end, 10 min at room temperature each time. To pellet beads between washes, centrifuge 1 min at 1000 $\times$ g.

31. Elute 3X-FLAG-tagged mitoribosomes from beads: After final wash, pellet beads and resuspend in 6 bead slurry volumes wash buffer containing 200 μ g/ml 3X-FLAG peptide.

For example, if the original lysate volume was 15 ml, 180 μ l agarose slurry was used and the elution volume added should be 1080 μ l.

32. Incubate 40 min rotating end over end at room temperature.

33. To remove beads, apply mixture to 0.45- μ m filter column (Costar Spin-X) and centrifuge 1 min at 16,000 $\times$ g. Transfer flow-through (eluate) to a 1.5-ml microcentrifuge tube.

Optional: Save 20 μ l of the eluate for analysis by immunoblotting: Combine with 5 μ l 5 $\times$ SDS-PAGE sample buffer, vortex 5 sec, boil 5 min, and store at -20°C .

Extract RNA

34. If eluate volume is >600 μ l, split sample into two 1.5-ml tubes. Add equal volume of acid-phenol/chloroform/isoamyl alcohol (pH 4.5).

Avoid upper aqueous buffer layer when removing phenol/chloroform/isoamyl alcohol from stock bottle.

35. Shake vigorously by hand 10 sec, then vortex at top speed 10 sec. Centrifuge 5 min at 10,000 $\times$ g, room temperature.

36. Transfer aqueous (top) layer to a fresh 1.5-ml tube, being careful to leave behind any debris, interphase, or organic material.

Collect organic (bottom) phase for appropriate disposal in phenol/chloroform waste.

37. To reserved aqueous phase, add one-tenth volume 3 M sodium acetate and 0.7 μ l LPA. Vortex 10 sec.

Isopropanol precipitate RNA

38. Add 1.25 volumes room temperature isopropanol. Vortex 10 sec. Incubate 20 min at -80°C .

Alternatively, incubate 10 to 15 min in ethanol/dry ice bath, or overnight at -20°C .

RNA can be stored at this step for 6 months at -80°C .

39. Let thaw, mix, and centrifuge 15 min at 20,000 $\times$ g at 4°C to pellet RNA.

Note orientation of tube in centrifuge.

40. Remove most of supernatant, leaving 50 to 100 μ l. Wash pellet by adding 800 μ l 70% ethanol at room temperature, vortex 5 sec. Centrifuge 1 min at 20,000 $\times$ g, room temperature.
41. Remove supernatant, being careful not to disturb pellet.

To remove as much supernatant as possible, centrifuge again briefly to collect ethanol wash from the walls and aspirate liquid using a fine tip. Best results are obtained if all ethanol is removed instead of allowing it to dry off, but take great care to not disturb pellet.

42. Resuspend pellet in 11 μ l 10 mM Tris-Cl, pH 7.0.

Optional: Check quality of lysis and immunoprecipitation by immunoblot

43. Perform immunoblot (Ni et al., 2016) on input, soluble, unbound, and eluate samples using anti-FLAG antibody.

Solubility and immunoprecipitation efficiency should be at least 60%.

CONSTRUCT DNA SEQUENCING LIBRARIES FROM MITORIBOSOME FOOTPRINTS AND/OR FRAGMENTED TOTAL mRNA

**BASIC
PROTOCOL 2**

RNase I treatment to generate footprints (Basic Protocol 1) and fragmentation of total mRNA by alkaline hydrolysis (Support Protocol) result in chemically similar RNA species with 5' hydroxyl and 3' phosphate ends, facilitating identical library generation methods. To minimize bias, enzymatic steps should be as efficient as possible. Here we follow, with minor modifications, the method devised for cytosolic ribosome profiling (Ingolia, Brar, Rouskin, McGeachy, & Weissman, 2012). First the $\sim$ 40 nucleotide (nt) footprints are size selected from mitoribosome-associated RNA. For fragmented mRNA that has already been size selected, begin at step 15, in which the 3' phosphate group is removed and a pre-adenylated linker (linker-1) is ligated using a modified RNA ligase. Reverse transcription is then performed with a primer containing carbon spacers to increase flexibility. The cDNA product is then circularized to create a template with known flanking sequences for PCR. PCR is performed with primers containing unique indexing sequences (Table 4.28.1) so that samples can be pooled for sequencing and sorted in silico. The resulting product is compatible for sequencing on Illumina platforms. Typically a single MiSeq run ($\sim$ 30 million reads) produces sufficient coverage across five indexed mitoribosome profiling libraries, and a single NextSeq run ($\sim$ 400 million reads) produces sufficient coverage across five indexed RNA-seq libraries.

Materials

Mitoribosome-associated RNA (from Basic Protocol 1) and/or fragmented and size-selected mRNA (from Support Protocol)
 2 $\times$ urea RNA loading buffer (see recipe)
 1 $\times$ TBE buffer (diluted from UltraPure 10 $\times$ stock; Invitrogen, cat. no. 15581044)
 10-bp DNA ladder
 15% and 10% TBE-urea, 8% TBE polyacrylamide gels
 SYBR gold (10,000 $\times$ concentrate)
 RNase-free water
 3 M sodium acetate, pH 5.5 (RNase-free)
 25 mg/ml linear polyacrylamide (LPA)
 Isopropanol
 70% (v/v) ethanol
 10 $\times$ T4 polynucleotide kinase (PNK) buffer
 10 U/ μ l T4 polynucleotide kinase (PNK)
 Oligonucleotide linker and primers (see Table 4.28.1)

**Preparation and
Analysis of RNA**

4.28.7

Table 4.28.1 DNA Oligonucleotides

Name	Sequence
Library preparation	
Linker-1 ^a	5' /5rApp/CTGTAGGCACCATCAAT/3ddC/3'
oSMD-RT1 ^{b,c}	5' /5Phos/GATCGTCGGACTGTAGAACTCTGAACCTGTC/iSp18/CACTCA/iSp18/CAAGCAGAAGACGGCATACGAGATATTGATGGTGCCTACAG 3'
oSMD2 ^b	5' CAAGCAGAAGACGGCATACGAGATATTGATGG 3'
oSMD1 ^{b,d}	5' AATGATACGGCGACCACCGAGATCTACACGATCGGAAGAGCACACGTCTGAACTCCAGTCAC <u>ATC</u> <u>ACG</u> CACAGGTTTCAGAGTTC 3'
oMTC1 ^{b,d}	5' AATGATACGGCGACCACCGAGATCTACACGATCGGAAGAGCACACGTCTGAACTCCAGTCAC <u>TGACC</u> ACGACAGGTTTCAGAGTTC 3'
oMTC2 ^{b,d}	5' AATGATACGGCGACCACCGAGATCTACACGATCGGAAGAGCACACGTCTGAACTCCAGTCAC <u>ACATG</u> TCGACAGGTTTCAGAGTTC 3'
oMTC3 ^{b,d}	5' AATGATACGGCGACCACCGAGATCTACACGATCGGAAGAGCACACGTCTGAACTCCAGTCAC <u>GCCAAT</u> CGACAGGTTTCAGAGTTC 3'
oMTC4 ^{b,d}	5' AATGATACGGCGACCACCGAGATCTACACGATCGGAAGAGCACACGTCTGAACTCCAGTCAC <u>CAGATC</u> CGACAGGTTTCAGAGTTC 3'
oMTC5 ^{b,d}	5' AATGATACGGCGACCACCGAGATCTACACGATCGGAAGAGCACACGTCTGAACTCCAGTCAC <u>ACTTG</u> ACGACAGGTTTCAGAGTTC 3'
oNTI202 ^b	5' CGACAGGTTTCAGAGTTCTACAGTCCGACGATC 3'
DNA template generation for northern probes	
COB(+)	5' GTACCTGAATGATACTTATTACCATTCTATGC 3'
COB(-)	5' TACCGATATAGAATAAAACATTTTCAATAGTAGAG 3'
COX1(+)	5' ATGGTACAAAGATGATTATATTCAACAAATGC
COX1(-)	5' TGTAATCCTGATAAAGACTTGTAAGAAGTTTCG 3'
COX2(+)	5' ATGTTAGATTTATTAAGATTACAATTAACAAC 3'
COX2(-)	5' CAGAACTTGATTTAATCTACCAGGAG 3'
COX3(+)	5' CCATTTTCATATGGTTATGCC'TTCACCATGAC 3'
COX3(-)	5' GAAATGTAATCCTGTACCAGCATAGAATACTG 3'
ATP6(+)	5' TCACCATTAGATCAATTTGAGATTAGAC 3'
ATP6(-)	5' ACCTAATCTTAAACCTAATGAAATAGCTC 3'
ATP9(+)	5' GATTAAATATTTAATAAATAAATATTATGC 3'
ATP9(-)	5' TTATACACCGAATAATAATAAGAATGAAACC 3'

^a Available as IDT part # 11-04-03-05.^b Order as PAGE purified.^c iSp18 are 18-carbon spacers.^d Used when indexing on the Illumina platform is desired. The bold, underlined sequence is the index read during sequencing.

50% (w/v) PEG 8000 (RNase-free)

10× T4 RNA ligase reaction buffer (New England Biolabs, cat. no. B0216L)

200 U/μl T4 RNA ligase 2 (RNL2), truncated (New England Biolabs)

10 mM Tris·Cl, pH 8.0 (RNase-free; *APPENDIX 2*)

5× First-strand (FS) RT reaction buffer (Invitrogen, cat. no. 18080-993)

10 mM dNTPs

0.1 M DTT

20 U/μl SUPERase-In

200 U/μl Superscript III

1 N sodium hydroxide
 3 M sodium chloride
 10× CircLigase reaction buffer (Epicentre, cat. no. CL4111K)
 1 mM ATP
 50 mM manganese chloride
 100 U/μl CircLigase ssDNA ligase
 5× Phusion HF reaction buffer (New England Biolabs, cat. no. M0530S)
 2 U/μl Phusion HF DNA polymerase
 10× DNA loading buffer (see recipe)
 100-bp DNA ladder
 DNA gel extraction buffer (see recipe)
 10 mM Tris·Cl, pH 8.5 (*APPENDIX 2*)
 Qubit dsDNA HS assay kit

Heat blocks: 80°C, 70°C, 37°C, 25°C

Typhoon fluorescent/phosphorescent image scanner, or UV transilluminator with camera

Blue light transilluminator (or UV transilluminator) box

End-over-end rotator

Costar Spin-X centrifuge tube filter (0.45-μm cellulose acetate in 2-ml tube; Corning)

Qubit Fluorometer

Bioanalyzer DNA high sensitivity chip and reagents

Polyacrylamide electrophoresis apparatus and power source

0.5- and 1.5-ml RNase-free non-stick microcentrifuge tubes

Plastic sheet protector

20-G needle

Razor blades or scalpels

Thermocycler and tubes (PCR tubes)

Size select mitoribosome footprints

1. Combine 10 μl RNA from large scale mitoribosome purification with 10 μl 2× urea RNA loading buffer.
2. Prepare 0.5 μg 10-bp ladder in 20 μl (final volume) of 1× urea RNA loading buffer.
3. Denature samples and ladder 90 sec at 80°C, immediately followed by at least 90 sec on ice.
4. Pre-run a 15% TBE-urea polyacrylamide gel in 1× TBE 15 min at 200 V.

Clear urea from wells with a syringe and needle prior to pre-run and again immediately before loading samples.

5. Load gel and run 1 hr at 200 V.

For a 1-mm thick, 12-lane gel, load each sample in two wells. Alternatively, use a 1.5-mm thick 10-lane gel and load each sample in one well.

6. Stain gel with SYBR gold (3 μl in 30 ml 1× TBE) 3 min with shaking at room temperature. Rinse gel in water, place in a sheet protector, and image on Typhoon scanner with 520 BP emission filter, blue (488) laser, and PMT voltage setting 400 to 600, depending on the power of the laser. Alternatively, image using UV transilluminator.

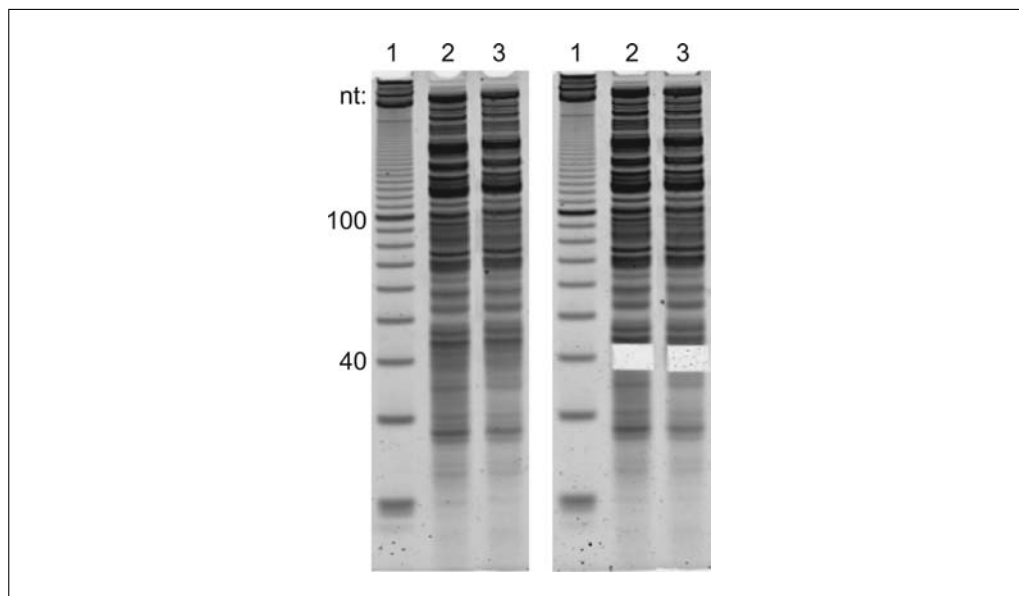


Figure 4.28.2 Representative example of gel excision to size select mitoribosome footprints. Left and right panels are before and after size selection, respectively. Lane 1 shows a denatured 10-bp DNA ladder, and lanes 2 and 3 show two different samples of RNA extracted after mitoribosome immunoprecipitation (IP). The majority of RNA is fragmented mito- and cytoribosomal RNA.

Perform rapid gel extraction

7. Pierce a 0.5-ml RNase-free, non-stick microcentrifuge tube with a 20-G needle and place it inside a 1.5-ml RNase-free, non-stick tube with the cap cut off.
8. Place gel on a blue light or UV light box and excise fragments in the range of 37 to 42 nt using the 10-bp ladder as a guide (Fig. 4.28.2). Place each gel slice into a pierced 0.5-ml RNase-free microcentrifuge tube of the nested tubes from step 7.

We recommend performing northern blotting (see Alternate Protocol) on a fraction of the sample prior to gel extraction to verify size range of footprints.

9. Centrifuge nested tubes 2 min at $20,000 \times g$, room temperature, to force the gel through the needle hole. Transfer any residual gel from the small tube into the larger tube.
10. Add 360 μ l RNase-free water to gel pieces and incubate 10 min at 70°C.
11. Vortex gel slurry 30 sec and transfer gel mixture to a Costar Spin-X column using a wide-bore tip.

To make a wide-bore tip, cut the end of a tip with a clean razor blade.

12. Centrifuge 2 min at $20,000 \times g$, room temperature to recover the elution mixture free of gel debris. Transfer flow-through to a 1.5-ml microcentrifuge tube.
13. Add 40 μ l 3 M sodium acetate and 0.7 μ l LPA. Vortex 10 sec.
14. Add 500 μ l room temperature isopropanol. Vortex 10 sec. Precipitate RNA as described above (Basic Protocol 1, steps 38 to 41).

Dephosphorylate 3' ends

15. Resuspend pellet in 5 μ l RNase-free water.
16. Denature 90 sec at 80°C then immediately place on ice for at least 90 sec.
17. Add 4.5 μ l PNK mix: 0.95 μ l 10 $\times$ T4 PNK buffer, 2.55 μ l RNase-free water, 1 μ l T4 PNK.

18. Incubate at 37°C, 1 hr.
19. Heat kill T4 PNK by incubating 70°C, 10 min.

After this step, centrifuge tube before opening to collect any condensate from walls and cap.

Ligate 3' adaptor

20. To dephosphorylated sample, add 0.5 μ l 1 μ g/ μ l Linker-1 (Table 4.28.1; 0.5 μ g, 85 pmol).

Linker-1 should be present in at least five-fold molar excess compared to substrate RNA.

21. Denature 90 sec at 80°C then immediately place on ice at least 90 sec.
22. Add 10 μ l ligation mix: 8 μ l 50% PEG 8000, 1 μ l 10 $\times$ T4 RNA ligase buffer, 1 μ l T4 RNL2, truncated.

PEG 8000 is viscous and care must be taken in preparing this reaction. Use a wide-bore tip to pipet PEG 8000, and thoroughly mix the master mix solution before aliquoting to samples.

23. Incubate at 25°C, 3 hr.
24. Add 340 μ l RNase-free water, 40 μ l 3 M sodium acetate, 0.7 μ l LPA, vortex 10 sec.
25. Add 500 μ l isopropanol, vortex 10 sec. Precipitate RNA as described above (Basic Protocol 1, steps 38 to 41).
26. Resuspend pellet in 10 μ l 1 $\times$ urea RNA load buffer.
27. Prepare 0.3 μ g 10-bp ladder in a final volume of 10 μ l 1 $\times$ urea RNA load buffer.
28. Prepare and run samples and ladder on 15% TBE-urea polyacrylamide gel, stain, and image as described above.

Each sample can now be loaded in one well of a 1-mm thick 12-lane gel.

29. Excise ligated product at ~60 nt (50 to 90 nt for RNA-seq) and perform rapid gel extraction and isopropanol precipitation.

Perform reverse transcription

30. Resuspend pellet in 10 μ l 10 mM Tris-Cl, pH 8.0, and transfer to a PCR tube; all subsequent incubation steps should be performed in a thermocycler.
31. Add 2 μ l 1.25 μ M oSMD-RT1 (Table 4.28.1) reverse transcription primer.
32. Denature 90 sec at 80°C then immediately place on ice at least 90 sec.
33. Add 8 μ l reverse transcription mix: 4 μ l 5 $\times$ FS buffer, 1 μ l 10 mM dNTPs, 1 μ l 0.1 M DTT, 1 μ l Suprase-In, 1 μ l Superscript III. Mix well.
34. Incubate 30 min at 48°C.
35. Add 2.2 μ l 1 N sodium hydroxide and incubate 20 min at 98°C to degrade RNA.
36. Transfer to a 1.5-ml microcentrifuge tube, and add 157.1 μ l water, 20 μ l 3 M sodium acetate, and 0.7 μ l LPA. Vortex 10 sec.
37. Add 300 μ l isopropanol, vortex 10 sec. Precipitate RNA as described above (Basic Protocol 1, steps 38 to 41).
38. Resuspend pellet in 10 μ l 1 $\times$ urea RNA load buffer.
39. Prepare 0.3 μ g 10-bp ladder in a final volume of 10 μ l 1 $\times$ urea RNA load buffer.

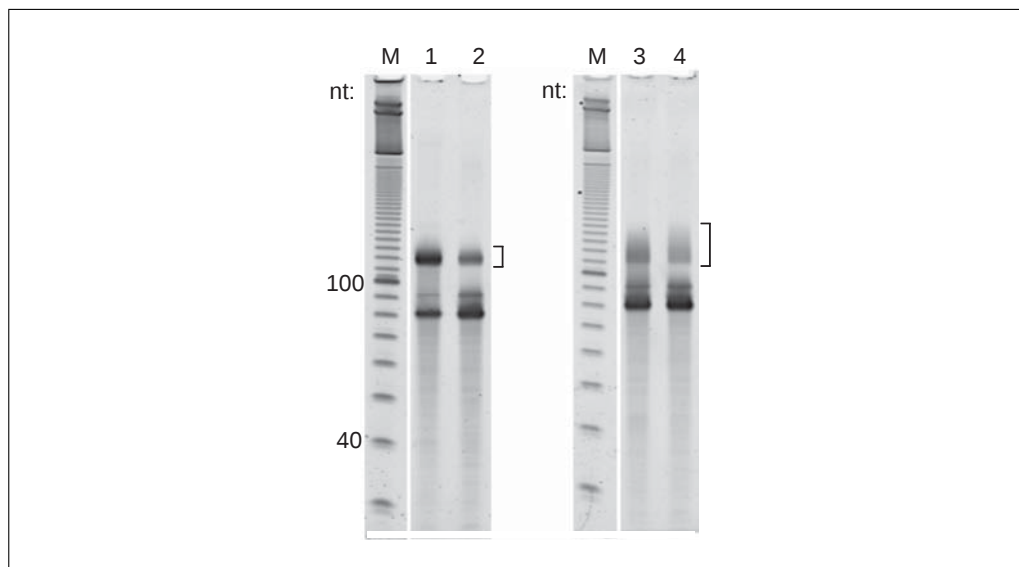


Figure 4.28.3 Representative examples of reverse transcription products. The brackets indicate the region to be excised for cDNA produced from mitoribosome footprints (lanes 1 and 2), or from fragmented RNA that has been rRNA-depleted (lane 3) or rRNA- and small RNA-depleted (lane 4). The ~80-nt species is unextended reverse transcription primer.

40. Prepare and run samples and ladder, as described above, on 10% TBE-urea polyacrylamide gel at 200 V, 65 min, stain and image as described above (step 6).

Load each sample in one well of a 1-mm thick 12-lane gel.

41. Excise cDNA product, which should be >100 nt (Fig. 4.28.3), and perform rapid gel extraction and isopropanol precipitation with 3 M sodium chloride in place of 3 M sodium acetate.

For best results, blot excess buffer from gel after staining and work rapidly. Diffusion of the unextended reverse transcription primer into excess buffer can contaminate desired product and result in PCR amplification of unextended reverse transcription primer in subsequent steps.

Circularize cDNA

42. Resuspend pellet in 5 μ l water.
43. Add 15 μ l circularization mix: 10 μ l water, 2 μ l 10 $\times$ circularization buffer, 1 μ l 1 mM ATP, 1 μ l 50 mM manganese chloride, 1 μ l Circ ligase.
44. Incubate 1 hr at 60°C.
45. Heat kill Circ ligase by incubating 10 min, 80°C.

Circles can be stored for 6 months at -20°C.

Determine cycle number to be used for PCR amplification

46. Combine 5 μ l circularization reaction with 78.5 μ l PCR mix: 56.3 μ l water, 16.7 μ l 5 $\times$ Phusion HF buffer, 1.7 μ l 10 mM dNTPs, 1.5 μ l 20 μ M oSMD2 (Table 4.28.1), 1.5 μ l 20 μ M indexed primer (e.g., oSMD1, oMTC1, oMTC2; Table 4.28.1), 0.8 μ l 2 U/ μ l Phusion polymerase.

Perform this PCR test with the index primer that will be used for each library.

47. Aliquot 16.7 μ l reaction mixture into each of five PCR tubes.
48. Program thermocycler 30 sec at 98°C and 14 cycles of: 15 sec at 98°C, 15 sec at 65°C, 7 sec at 72°C.

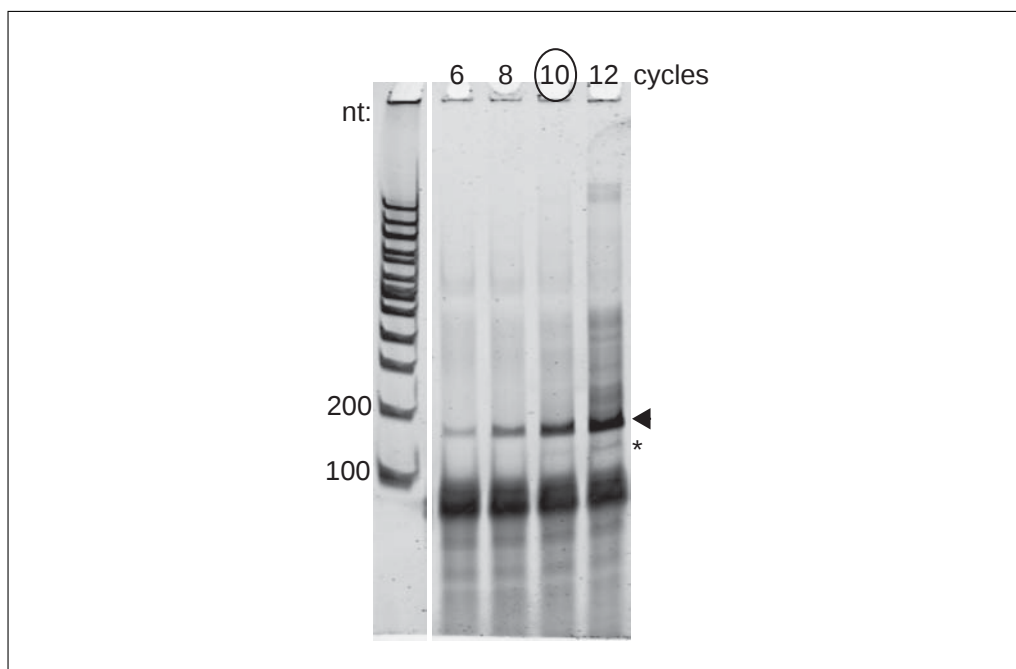


Figure 4.28.4 PCR cycle number determination. The arrowhead indicates the ~180-nt product to be purified. The asterisk indicates the product derived from unextended RT primer and should be avoided. In this example, 10 PCR cycles were chosen because the larger products that result after 12 cycles indicate the PCR amplification has approached saturation.

49. Remove samples during elongation step (72°C) after 6, 8, 10, 12, and 14 cycles.
50. Add 1.8 μ l 10 $\times$ DNA loading buffer.
51. Prepare 0.3 μ g 100 bp in a final volume of 20 μ l 1 $\times$ DNA load buffer.
52. Resolve on 8% TBE non-denaturing polyacrylamide gel 45 min at 180 V. Do not heat samples prior to loading.

Pre-running the non-denaturing gel is not necessary.

53. Stain and image gel as previously described (step 6).

Choose cycle number that produces product in the linear range of amplification and does not produce the larger products that result from reannealed partial duplexes (Fig. 4.28.4).

Perform PCR amplification

54. Prepare PCR reaction mix as described in step 46.

This reaction can be scaled down to use 3- μ l circles depending on yield.

55. Aliquot 16.7 μ l reaction mixture into PCR tubes.
56. Program thermocycler 30 sec at 98°C and N cycles of: 15 sec at 98°C, 15 sec at 65°C, 7 sec at 72°C.

N is determined by the PCR test above.

57. Resolve samples as described above.
58. Excise amplified PCR product, avoiding any lower product band resulting from unextended reverse transcription primer.
59. Perform overnight gel extraction: To gel pieces, add 600 μ l DNA gel extraction buffer. Incubate overnight at room temperature, rotating end over end.

Do not heat.

60. Remove gel pieces by applying to Costar Spin-X column as described, add 0.7 μ l LPA, and precipitate with 750 μ l isopropanol as described.
61. Resuspend pellet in 12 μ l 10 mM Tris·Cl, pH 8.5.
62. Assess library concentration and quality using Qubit Fluorometric Quantitation dsDNA kit (high sensitivity), and Bioanalyzer DNA high-sensitivity chip according to manufacturer's instructions.
63. When submitting samples for sequencing, supply custom "Read 1" sequencing primer: oNTI202 (Table 4.28.1).

ALTERNATE PROTOCOL

QUANTIFY MITORIBOSOME-PROTECTED FRAGMENTS BY NORTHERN BLOTTING

Analysis of mitoribosome footprints by northern blotting can provide valuable information when ribosome occupancy on a message needs to be directly compared across samples, codon resolution is not required, or sequencing facilities are not readily available. Because of the limited number of mitochondrial mRNAs, virtually all of them can be probed in a matter of days. As of this writing, the authors have successfully probed for *COB*, *COX1*, *COX2*, *COX3*, *ATP6*, and *ATP9*, but not *VAR1* or *ATP8* transcripts. The following northern blotting method has been optimized for detection of small RNA species. RNA is blotted to a nylon membrane after polyacrylamide gel electrophoresis. A probe is prepared from a template amplified from the region of interest by internally labeling random-primed polymers. The double-stranded probe mix is then denatured and hybridized to the RNA blot at low (room) temperature. Non-specific interactions are washed away by incubating with decreasing concentrations of salt, and specific probe hybridizations are visualized by exposing to a storage phosphor screen.

Additional materials (also see Basic Protocol 1)

Mitoribosome-associated RNA (Basic Protocol 1)
300 to 500 bp dsDNA template for probe generation (produced using standard PCR and agarose gel extraction methods)
3 μ g/ μ l random hexamers
Nuclease-free water
10 $\times$ NEB2 reaction buffer (New England Biolabs)
90 μ M dATP
0.3 mM dG, dC; 0.9 mM dT mix
10 mCi/ml α^{32} P-dATP
5 U/ μ l DNA Polymerase I Klenow fragment (3' to 5' exo-)
1 $\times$ TE buffer (see recipe)
Church's hybridization buffer with formamide (see recipe)
3 $\times$ blot wash buffer (see recipe)
2 $\times$ blot wash buffer (see recipe)

Hybond N+ nylon membrane
Biorad mini trans-blot electrophoretic transfer cell
UV crosslinker chamber
Plexiglass shielding, Plexiglass tube racks, and appropriate licensing for handling radioactive material
Geiger counter
100°C heat block
Typhoon fluorescent/phosphorescent image scanner and storage phosphor screen, or autoradiography film

Storage phosphor cassette
NucAway Spin Columns (Invitrogen, cat. no. AM10070)
Hybridization oven
Hybridization tube

CAUTION: ^{32}P poses a radiological hazard. It should be used with appropriate safety measures such as shielding and personal protective equipment, especially safety glasses. Measures should be taken to reduce exposure time and maximize distance from source. Follow institutional guidelines for handling and disposing of radioactive material.

Prepare RNA blot

1. Resolve RNA (1 μl from large scale or 5 μl from small scale mitoribosome purification) on a 15% TBE-urea polyacrylamide gel, along with 0.3 μg 10-bp ladder.

Bring sample volume to 10 μl in 1 $\times$ urea RNA loading buffer before heating and loading.

2. Stain and image gel as previously described (Basic Protocol 2, step 6).
3. Transfer to nylon membrane (Hybond N+) in 0.5 $\times$ TBE using Biorad mini trans-blot electrophoretic transfer cell, 80 V, 1 hr at room temperature.
4. Disassemble blotting sandwich with gel still on membrane and mark bottoms of wells with a pencil. Remove gel and let membrane dry 10 min.
5. Place blot in UV crosslinker chamber and apply 120,000 microjoules/ cm^2 at a wavelength of 254 nm.

After crosslinking, the blot can be stored at room temperature indefinitely.

Prepare radioactive probe

6. Combine 25 ng double stranded template DNA in 4 μl water with 5 μl 3 $\mu\text{g}/\mu\text{l}$ random hexamers.

Primer sequences for probe template amplification are provided in Table 4.28.1.

Template DNA should be 300 to 500 bp and gel purified. The ratio of DNA to random hexamers is important for probe quality.

7. Incubate 2 min at 100°C and immediately ice at least 2 min.
8. Add 16 μl extension mix:

5 μl water
2.5 μl 10 $\times$ NEB2 buffer
2.5 μl 90 μM dATP
2.5 μl 0.3 mM each dG, dC, 0.9 mM dT
2.5 μl 10 mCi/ml $\alpha^{32}\text{P}$ -dATP
1 μl 5 U/ μl Klenow fragment (3' to 5' exo-).

For dATP and dG, dC, and dT, the amounts given optimize synthesis of AT-rich probes. For templates that are not AT-rich, use 50 μM dATP, 0.5 mM each dG, dC, dT.

9. Incubate overnight (or at least 5 hr) at room temperature.
10. Remove unincorporated nucleotides with Ambion NucAway spin column according to manufacturer's instructions.

As a crude check for label incorporation efficiency, use Geiger counter to compare counts per minute of probe to counts per minute of unincorporated nucleotide in spent spin column. Ideally the probe should produce a higher signal.

11. Add 75 μl 1 $\times$ TE to probe solution.

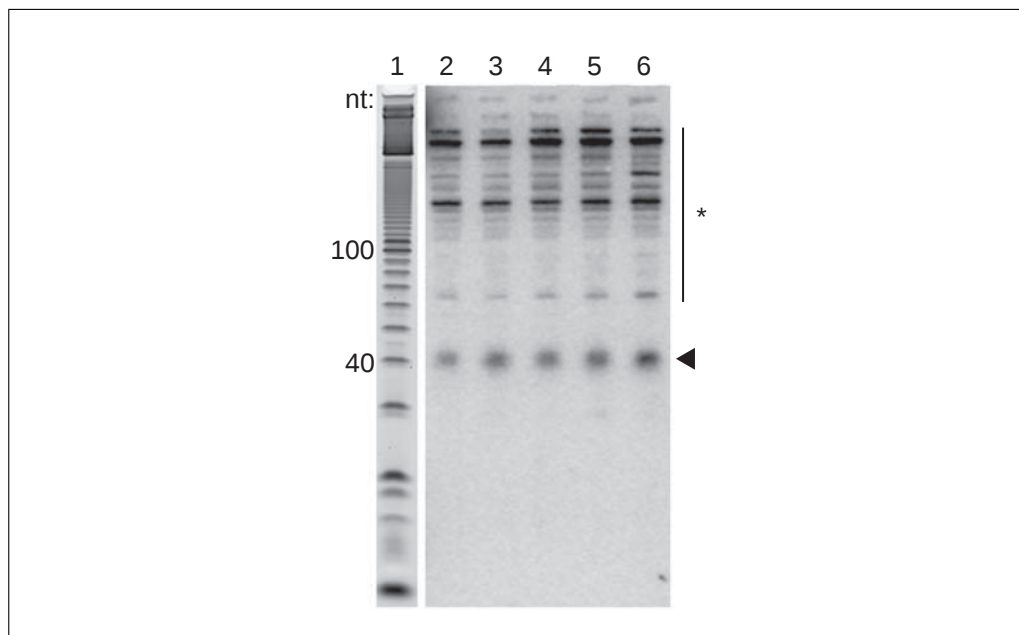


Figure 4.28.5 Northern blot hybridization to visualize footprints. Lane 1 shows the denatured 10-bp DNA ladder from the gel stained with SYBR gold before blotting. Lanes 2-6 show *COX2* probe hybridization to RNA extracted after mitoribosome immunoprecipitation from a series of samples. The arrowhead indicates hybridization to mitoribosome footprints. The asterisk indicates non-specific cross-hybridization.

Probe can be stored for up to a week at -20°C . Longer storage time is possible, but specific activity will be reduced, as the half-life of ^{32}P is 14 days.

Hybridize probe to RNA blot

12. Block non-specific interactions by pre-hybridizing blot without probe in 15 ml Church's buffer with formamide for at least 1 hr at room temperature.
13. Just before adding probe, denature by incubating 2 min at 100°C and immediately ice for at least 2 min.

This step is critical because hybridization is performed at room temperature.

14. Add 100 μl probe to buffer in hybridization tube and swirl by mixing.

Take care to not pipet probe directly onto blot.

15. Hybridize overnight (at least 8 hr) at room temperature.
16. Properly dispose of buffer with probe by pouring into radioactive liquid waste container.

Diluted probe can alternatively be transferred to a 50-ml conical tube and stored at 4°C to be used again. Before use, heat to 50°C and cool to room temperature.

17. Rinse blot once in $3\times$ blot wash buffer, wash in $3\times$ blot wash buffer 20 min at 30°C , then wash in $2\times$ blot wash buffer 20 min at 30°C .

An additional wash in $2\times$ or $1\times$ blot wash buffer may be necessary.

18. Remove blot from hybridization tube and wrap in plastic wrap.

Be sure no liquid can escape, as it will damage phosphorimager screen.

19. Expose face up to storage phosphor screen 5 hr to overnight.
20. Image on Typhoon using Storage Phosphor acquisition mode.

21. Align ladder from SYBR gold-stained image with northern image using pencil marks at wells (Fig. 4.28.5).

ISOLATE mRNA FOR RNA SEQUENCING

This protocol describes how to isolate and process mRNA from mitoribosome profiling lysates to generate RNA fragments that can be used in Basic Protocol 2 to make stranded RNA sequencing libraries. Importantly, ribosomal RNA (rRNA) depletion is used to enrich mRNA in place of poly(A) selection in order to not bias against recovery of mitochondrial mRNAs, which are not polyadenylated in *S. cerevisiae*. First, lysate is deproteinized and DNA is removed. An optional step is included for small RNA depletion (Nilsen, 2012) to remove transfer RNA (tRNA), which otherwise makes up >50% of the library. This step should be used with caution and only if quality of RNA is carefully monitored. Next, rRNA is depleted and the remaining RNA is fragmented and size selected at which point it is ready for library generation as described in Basic Protocol 2.

Additional Materials (also see Basic Protocol 2)

20% (w/v) SDS (RNase-free)
20 mg/ml proteinase K
24:1 chloroform/isoamyl alcohol
100% ice-cold ethanol
10× RQ1 RNase-free DNase reaction buffer (Promega, cat. no. M6101)
1 U/μl RQ1 RNase-free DNase (Promega, cat. no. M6101)
Ribo-Zero Gold rRNA Removal Kit for yeast (Illumina, cat. no. MRZY13)
2× alkaline fragmentation buffer (see recipe)

Heat blocks: 42°C, 37°C, 70°C, 80°C
NanoDrop UV/Vis Spectrophotometer

CAUTION: Chloroform/isoamyl alcohol is harmful if swallowed, causes skin and eye irritation, and is potentially carcinogenic. It should be used with appropriate safety measures such as protective gloves, glasses, clothing, and sufficient ventilation. All waste should be handled according to hazardous waste regulations.

Isolate total RNA from lysate

1. Start with reserved lysate from step 20 in Basic Protocol 1.
2. Bring volume to 390 μl with 10 mM Tris·Cl, pH 7.0. Allow sample to reach room temperature.
3. Add 10 μl 20% SDS, vortex on medium-high setting 10 sec.
4. Add 2 μl 20 mg/ml proteinase K, vortex on medium-high setting 10 sec, incubate 20 min at 42°C.
5. Add 40 μl 3 M sodium acetate. Vortex 10 sec.
6. Add 600 μl 125:24:1 acid-phenol/chloroform/isoamyl alcohol (pH 4.5).
7. Shake vigorously by hand 10 sec, then vortex at top speed 10 sec. Centrifuge 5 min at 10,000 × g, room temperature.
8. Transfer aqueous (top) layer to a fresh 1.5-ml tube, being careful to leave behind any debris, interphase, or organic material.

Collect organic (bottom) phase for appropriate disposal in phenol/chloroform waste.

9. To remove residual phenol, add 500 μ l 24:1 chloroform/isoamyl alcohol to reserved aqueous phase.
10. Repeat steps 7 and 8.
11. Add 800 μ l ice-cold 100% ethanol. Vortex 10 sec. Precipitate RNA as described above (Basic Protocol 1, steps 38 to 41).

Ethanol is used in place of isopropanol at this step because it may precipitate less phenol.

12. Resuspend pellet in 40 μ l 10 mM Tris·Cl, pH 7.0.

Gently tap tube, let sit at room temperature up to 30 min with periodic tapping, and pipet up and down to ensure all pellet is dissolved.

13. Dilute 1 μ l to 1:10 and quantify using NanoDrop, “RNA” settings.

Remove remaining DNA

14. To 40 to 60 μ g material as quantified by NanoDrop, add 10 mM Tris·Cl, pH 7.0 to bring volume to 25 μ l.

If the optional small RNA depletion step will be carried out it is ideal to use 60 μ g here.

15. Add 35 μ l DNase mix: 26 μ l RNase-free water, 6 μ l 10 $\times$ DNase buffer, and 3 μ l RQ1 DNase. Mix and incubate 30 min at 37°C.
16. Add 240 μ l RNase-free water.
17. Add 300 μ l 125:24:1 acid-phenol/chloroform/isoamyl alcohol (pH 4.5). Extract RNA as in steps 7 and 8 above.
18. To reserved aqueous phase, add 30 μ l 3 M sodium acetate and 0.7 μ l LPA. Vortex 10 sec.
19. Add 600 μ l ice-cold 100% ethanol. Vortex 10 sec. Precipitate RNA as described above (Basic Protocol 1, steps 38 to 41).
20. Resuspend pellet in 31 μ l 10 mM Tris·Cl, pH 7.0.
21. Dilute 1 μ l to 1:10 and quantify using NanoDrop. Concentration should be 1 to 2 μ g/ μ l.

Deplete small RNAs

Optional: For high-quality RNA this step removes tRNA alone and dramatically increases number of mRNA-mapping reads, without bias against short mRNA transcripts (M. T. Couvillion, unpub. data). However, if RNA is fragmented, this step may deplete mRNA fragments <100 nt. Gel visualization of total, small, and large RNA fractions is encouraged for quality control (Fig. 4.28.6).

22. Combine 15 μ l RNA (concentration at least 1 μ g/ μ l) with PEG precipitation mix: 9 μ l RNase-free water, 3 μ l 50% PEG 8000, 3 μ l 3 M sodium chloride. Mix and incubate 30 min on ice.
23. Centrifuge 30 min at 20,000 $\times$ g, 4°C to pellet RNAs >100 nt.
24. Discard supernatant containing tRNA and other small RNAs.

Alternatively, ethanol precipitate small RNAs for analysis as in Figure 4.28.6.

25. Add 800 μ l 70% ethanol at room temperature, vortex 5 sec. Centrifuge 1 min at 20,000 $\times$ g, room temperature.
26. Remove supernatant, being careful not to disturb pellet.

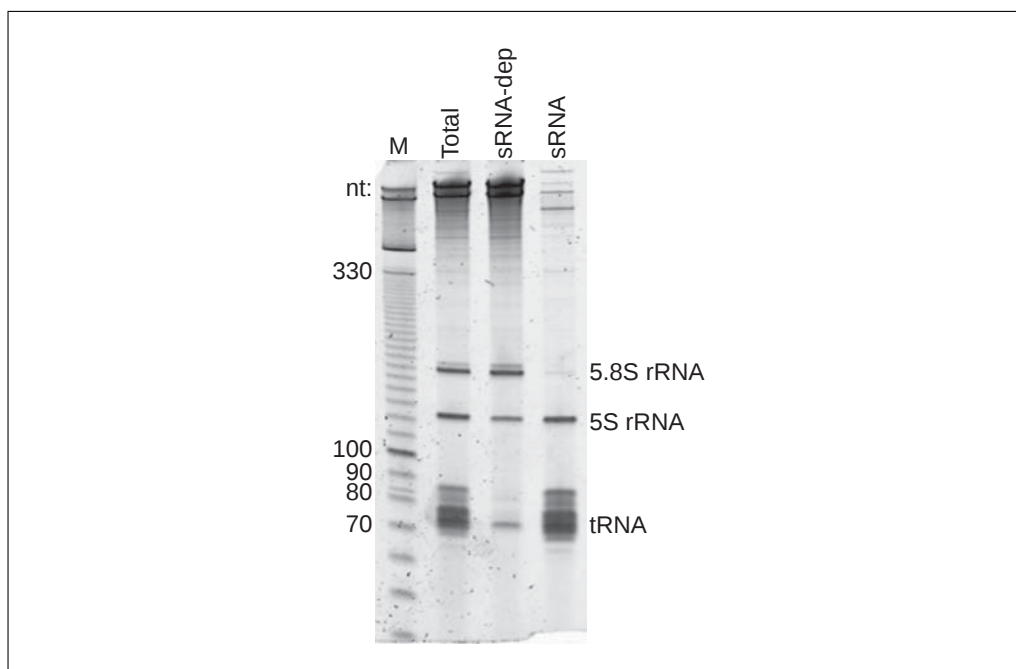


Figure 4.28.6 Quality control step to visualize fractions after small RNA depletion. Total RNA (Total; 0.7 μ g loaded), large RNA to be carried through library generation (sRNA-dep), and the PEG/NaCl supernatant (sRNA) resolved on a 6% TBE-urea polyacrylamide gel.

To remove as much supernatant as possible, centrifuge again briefly to collect ethanol wash from the walls and aspirate liquid using a fine tip.

27. Resuspend pellet in 21 μ l 10 mM Tris·Cl, pH 7.0.
28. Dilute 1 μ l to 1:5 and quantify using NanoDrop.

Deplete ribosomal RNA

29. Apply 5 μ g RNA (total or small RNA-depleted) to Ribo-zero kit according to manufacturer's instructions.
30. After depleting rRNA, bring volume to 180 μ l with RNase-free water.
31. Add 20 μ l 3 M sodium acetate and 0.7 μ l LPA. Vortex 10 sec.
32. Add 600 μ l ice-cold 100% ethanol. Vortex 10 sec. Precipitate RNA as described above (Basic Protocol 1, steps 38 to 41).
33. Resuspend pellet in 16 μ l 10 mM Tris·Cl, pH 8.0.

Create fragmentation curve to determine fragmentation time

If multiple samples are being prepared in parallel, it is only necessary to perform the following fragmentation test with a single sample. Perform fragmentation in a thermocycler with heated lid.

34. Combine 4 μ l RNA and 36 μ l RNase-free water. Add 40 μ l 2 $\times$ alkaline fragmentation buffer, mix, and aliquot into four PCR tubes, 20 μ l each.
35. Incubate 0, 10, 20, or 30 min at 95°C in PCR machine.
36. Transfer each sample to a 1.5-ml tube, add 340 μ l water, 40 μ l 3 M sodium acetate, and 0.7 μ l LPA. Vortex 10 sec.
37. Add 500 μ l room temperature isopropanol. Vortex 10 sec. Precipitate RNA as described above (Basic Protocol 1, steps 38 to 41).

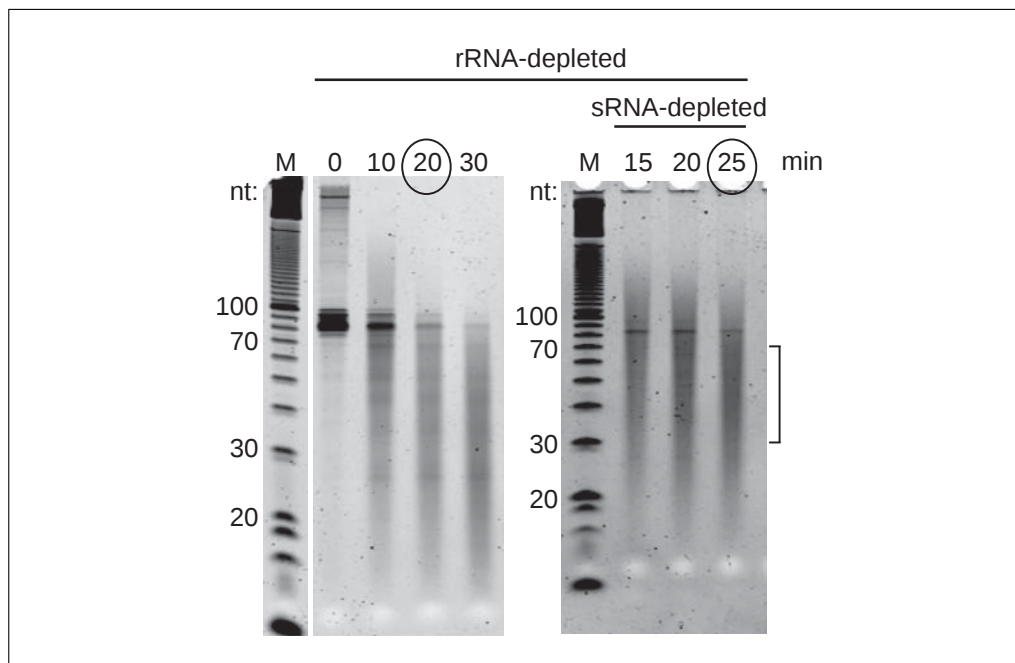


Figure 4.28.7 Representative examples of fragmentation curves. Purified mRNA, with or without small RNA depletion was incubated in alkaline fragmentation buffer for the indicated time at 95°C. The incubation time chosen is indicated. Bracket indicates target region for fragments.

Perform gel electrophoresis to visualize fragmentation curve

38. Resuspend pellet in 10 μ l 1 $\times$ urea RNA loading buffer.
39. Prepare 0.2 μ g 10-bp ladder in a final volume of 10 μ l 1 $\times$ urea RNA load buffer.
40. Prepare and run samples and ladder, as described above, on 15% TBE-urea polyacrylamide gel at 200 V, 1 hr.

Load each sample in one well of a 1-mm thick 12-lane gel

41. Stain and image gel as previously described (Basic Protocol 2, step 6).

Fragment RNA

Choose fragmentation time based on the incubation time in which most fragments lie in the range of 30 to 70 nt (Fig. 4.28.7).

42. Combine 8 μ l RNA and 72 μ l RNase-free water. Add 80 μ l 2 $\times$ alkaline fragmentation buffer, mix, and aliquot into eight PCR tubes, 20 μ l each.

Conditions should be kept identical to those used in the fragmentation curve

43. Incubate in thermocycler for time determined above at 95°C.
44. Pool all eight aliquots per sample into a 1.5-ml tube, add 335 μ l water, 55 μ l 3 M sodium acetate, and 0.7 μ l LPA. Vortex 10 sec.
45. Add 690 μ l room temperature isopropanol. Vortex 10 sec. Precipitate RNA as described above (Basic Protocol 1, steps 38 to 41).

Size select fragmented RNA and perform rapid gel extraction

46. Resuspend RNA, run, stain, and image gel as described above.
47. Excise fragment in the range of 30 to 70 nt using the 10-bp ladder as a guide.

48. Perform rapid gel extraction as described, except resuspend gel pieces in 495 μ l RNase-free water and after elution add 55 μ l 3 M sodium acetate and 0.7 μ l LPA. Precipitate with 690 μ l room temperature isopropanol.

A larger elution volume is used to accommodate the extra gel mass.

49. Prepare sequencing libraries identically to procedure described in Basic Protocol 2 for ribosome footprints, beginning with dephosphorylation of 3' ends (i.e., Basic Protocol 2, step 15).

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes unless noted as RNase-free in which cases use DEPC-treated or commercial RNase-free water and RNase-free reagents. For common stock solutions, see APPENDIX 2.

Alkaline fragmentation buffer, 2 $\times$ (RNase-free)

100 mM sodium carbonate-bicarbonate buffer, pH 9.2 (12% 100 mM Na₂CO₃ and 88% 100 mM NaHCO₃)

Store in 200 μ l aliquots, tightly capped, for 1 year at room temperature.

Blot wash buffer, 2 $\times$

2 $\times$ SSC

0.1% SDS

Store 6 to 12 months at room temperature.

Blot wash buffer, 3 $\times$

3 $\times$ SSC

0.1% SDS

Store 6 to 12 months at room temperature.

Church's hybridization buffer with formamide

0.5 M phosphate buffer, pH 7.2 (68.4% Na₂HPO₄, dibasic and 31% NaH₂PO₄, monobasic)

1 mM EDTA

7% SDS

1% BSA

Heat and stir to dissolve

Add formamide to 15% (v/v)

Store in 15 ml aliquots for 2 years at -20°C .

DNA gel extraction buffer

10 mM Tris $\cdot$ Cl, pH 8.0 (APPENDIX 2)

0.3 M NaCl

1 mM EDTA

Store, tightly capped, 2 years at room temperature.

DNA loading buffer, 10 $\times$

10 mM Tris $\cdot$ Cl, pH 7.0 (APPENDIX 2)

50% glycerol

100 mM EDTA

Bromophenol blue (avoid measuring; transfer powder with pipet tip until desired color is achieved)

Store 2 years at 4°C .

Lysis buffer (RNase-free)

10 mM Tris·Cl, pH 8.0 (*APPENDIX 2*)
 50 mM NH₄Cl
 10 mM MgCl₂
 0.5% lauryl maltoside (*n*-dodecyl β-D-maltoside; Sigma-Aldrich, cat. no. D4641)
 0.25 mM DTT
 1.5× EDTA-free protease inhibitor cocktail (Sigma-Aldrich, cat. no. 11873580001)
 Prepare fresh on day of use.

SDS-PAGE sample buffer, 5×

300 mM Tris·Cl, pH 7.0 (*APPENDIX 2*)
 10% SDS
 20% β-mercaptoethanol
 50% glycerol
 Bromophenol blue (avoid measuring; transfer powder with pipet tip until desired color is achieved)
 Store in 1-ml aliquots 2 years at −20°C.

TE buffer, 1×

10 mM Tris·Cl, pH 8.0 (*APPENDIX 2*)
 1 mM EDTA

***Urea RNA loading buffer, 2×* (RNase-free)**

8 M urea
 30 mM EDTA
 Bromophenol blue (avoid measuring; transfer powder with pipet tip until desired color is achieved)
 Store 6 months at −20°C.

Wash buffer (RNase-free)

10 mM Tris·Cl, pH 8.0 (*APPENDIX 2*)
 50 mM NH₄Cl
 10 mM MgCl₂
 0.1% Triton X-100
 Store 6 to 12 months at 4°C.

COMMENTARY

Background Information

Ribosome profiling relies on deep sequencing of ribosome footprints to provide codon-resolution information about ribosome positions and a quantitative readout of expression (Ingolia et al., 2009). Mitochondrial ribosomes (mitoribosomes) are less abundant and more fragile than cytosolic ribosomes (cytoribosomes), and protect a longer stretch of mRNA (~40 nt; Couvillion et al., 2016), necessitating a dedicated procedure to examine mitochondrial translation. The method traditionally used to monitor mitochondrial translation is metabolic labeling, which relies on incorporation of labeled amino acids under conditions that inhibit cytosolic translation (Fox et al., 1991). While mitoribosome profiling is more labor-intensive, it

overcomes many limitations of metabolic labeling. First, it provides high temporal and positional resolution, which is critical for many applications as translational responses can occur on a rapid time scale. Second, it can be performed on cells in any condition and is not confounded by labeled amino acid uptake efficiency. Finally, it does not rely on inhibition of cytosolic translation, which we have found affects mitochondrial translation (Couvillion et al., 2016).

Basic Protocol 2, in which mitoribosome footprints are converted to a sequencing library, yields codon-resolution data that reports on the distribution of mitoribosomes across mitochondrial mRNAs, allowing within-sample comparisons of protein synthesis. While *distributions* among the

messages can be compared across samples, absolute increase or decrease of ribosome occupancy must be evaluated using the Alternate Protocol in which mitoribosome footprints are quantified by northern blotting, with normalization to cell number. Both the Basic and Alternate Protocols report on the combined effects of RNA levels and translation. To isolate translation regulation, translation efficiency must be calculated by normalizing to RNA abundance. We also provide a Support Protocol for isolating and preparing mRNA for RNA sequencing. Combining these protocols provides a powerful means to investigate *S. cerevisiae* mitochondrial translation in stress, aging, mutants, and mitochondrial disease models.

Critical Parameters

Culture growth

S. cerevisiae metabolism, and thus potentially mitochondrial translation, is highly sensitive to growth conditions. It is important to be consistent by using cells fresh from the freezer (within 1 week), to note culture inoculation and collection density, and flask to culture volume. Additionally, we found it necessary to check the pH of culture media (pH 5.0) to maintain consistent growth rate, especially in glycerol-containing media.

Cell collection

Because translational responses can be rapid, it is critical to move cells from the condition of interest to liquid nitrogen as quickly as possible. This requirement precludes mitochondrial purification, during which cells must be incubated for long periods to break down the cell wall. Filtration is the collection method of choice for its speed. The cell number collected should be kept constant between samples to be compared.

Solubilization of mitoribosomes

Yeast mitoribosomes are substantially more fragile than cytoribosomes and their subunits are prone to dissociation in typical yeast lysis conditions (Couvillion et al., 2016). At the same time, translating mitoribosomes are membrane associated (Ott, Amunts, & Brown, 2016), so solubility and subunit association must be balanced. The critical parameter in subunit association is a low ratio of monovalent (NH_4^+ , Tris^+) to divalent (Mg^{2+}) cations in the buffer (Vignais, Stevens, Huet, & Andre, 1972).

RNase digestion/gel excision

Footprint capture during gel excision as well as data quality depend on precise nuclease digestion. If any parameters or conditions are modified, nuclease must be titrated and footprints visualized by northern blotting prior to gel excision and library generation. When size selecting footprints from the gel, it is ideal to excise as small a region as possible without leaving behind any footprint fragments. The larger the region excised, the greater the rRNA contamination will be in the final sequenced library. It is also possible to deplete rRNA fragments if more footprint reads are needed (Ingolia et al., 2012), but typically this is not necessary. In contrast, rRNA depletion should always be performed in preparation for RNA-seq, as described in the Support Protocol.

Quantification of northern blot signal

Digital images of northern blots can be quantified by densitometry using free software such as ImageJ (<https://imagej.nih.gov/ij/>). Relative signal should only be compared between bands on the same blot from a single probe, and only when equal cell numbers were collected for each sample and each step in the protocol yielded similar efficiency, including RNA precipitation in the final step. One indicator of success is equal intensity of rRNA fragments visible in the gel after SYBR gold staining (e.g., see Figure 4.28.2). However, it is possible that different strains or cells in different states may yield more or less of these rRNA fragments for biologically relevant reasons.

Troubleshooting

Troubleshooting problems and their possible solutions may be found in Table 4.28.2.

Anticipated Results

The vast majority of the RNA extracted after mitoribosome immunoprecipitation is RNase-digested rRNA (mitochondrial and cytosolic), which appears in a characteristic banding pattern in the gel (see Figure 4.28.2). The mobility of the footprint fragments cannot be visualized without northern blotting (see Figure 4.28.5). Because of this, recovered RNA mass is not a useful indicator of footprint abundance. Instead, success should be gauged using immunoblotting by efficiencies of MrpS17-FLAG solubility and immunoprecipitation, which are expected to be >60%. Mitoribosome profiling libraries

Table 4.28.2 Troubleshooting Guide for Mitoribosome Profiling and Related Protocols

Problem	Possible cause	Solution
Low recovery of footprints	Low starting cell number	Start with more cells; some strains or mutants may have less mitochondrial translation
	Low solubility	Increase buffer volume in cryogenic lysis
	Mitoribosome dissociation	Check lysis buffer conditions for ratio of monovalent to divalent cations Do not vigorously shake (aerate) lysate
	RNA degradation	Be sure all reagents are RNase-free and work area is free of dust; use barrier tips
Poor separation or smearing of RNA in gels	Incomplete ethanol/salt removal	Wash pellet thoroughly with vortexing in 70% ethanol; remove all traces of ethanol wash
	Incomplete resuspension of pellet	Mark position of pellet in tube before it dries
	Urea accumulation in gel wells prior to loading	Use a syringe to clear wells of urea within 2 min of loading
	RNA not fully denatured	Heat RNA in urea load buffer and place immediately on wet ice
High fraction of rRNA reads in mitoribosome library	Gel slice too big in initial size selection	Excise a more narrow range
	Mitoribosome dissociation	Check lysis buffer conditions for ratio of monovalent to divalent cations Do not vigorously shake (aerate) lysate
Poor signal on northern blot	Probe specific activity too low	Check labeling protocol and ^{32}P stock
	Probe not full denatured	Boil probe for at least 2 min and place immediately on wet ice just before use
	High background	Repeat wash step with 2× or even 1× blot wash buffer
Poor/inconsistent fragmentation for RNA-seq	RNA is in too high a concentration of buffer	Tris concentration should be 1 mM before addition of 2× alkaline fragmentation buffer
	Condensation is forming during heating	Perform fragmentation in thermocycler with heated lid

typically have 15% to 25% footprint reads, 40% to 60% cytosolic rRNA, 20% to 30% mitochondrial rRNA, and <1% tRNA and nuclear mRNA-mapping reads. Mitoribosome footprint reads should map precisely to open

reading frames with their 5' ends beginning ~18 nt upstream of start codons. RNA sequencing libraries made without small RNA depletion typically have 20% mRNA-mapping reads, 65% tRNA mapping reads, and 1%

rRNA reads. When small RNA depletion is included, libraries are 40% to 50% mRNA-mapping reads, 15% to 20% tRNA, and 20% to 25% rRNA.

Time Considerations

All steps for the protocols presented in this unit can be completed in 8 days if only one or two samples are being processed. Day 1: Culture, collect, and cryogenically lyse cells. Day 2: Immunoprecipitate mitoribosomes and associated RNA; extract total RNA. Days 3 to 4: Perform immunoblot and northern blot to verify quality of immunoprecipitation. Day 5: Fragment total RNA; size select footprints and total RNA. Days 6 to 8: Library generation.

The protocols are flexible with many stopping points, and not every experiment will require every protocol in this unit. For an experienced investigator up to six samples can be processed in parallel, but >8 days will be required to complete all steps.

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