Ribosome profiling
library preparation protocol for yeast

1. Whole cell extract preparation

(*Essentially prepared as in* Ingolia N, 2010 *Methods in Enzymology* with some modifications).

1.1. Grow cells in volume of 750 mL until turbidity OD_{600}=0.5-0.6.
1.2. Prewarm filter unit with 50 mL of media or sterile water at 30°C and collect cells by rapid filtration through 0.45 μm nitrocellulose membrane (use Millipore glass filtration unit for 90 mm diameter filters and a vacuum pump).
1.3. Immediately after filtration, while keeping under low vacuum scrape the cells off the membrane using metal spatula and transfer into a 50 mL tube filled with liquid N_2.
1.4. Place the tube (pierced cap on!!) to -85°C freezer, allow N_2 to evaporate, and recap with unpierced lid for storage.
1.5. Prepare required amount (2 mL/sample) of Polysome Lysis Buffer, freshly supplemented with DTT and cycloheximide (see Appendix 1). Freeze the buffer in the bottom of freezer milling vials (Spex Freezer Mill 6870) before addition of frozen cell pellets and run program: cycles 4, precool 6 min, run 2 min, cool 2 min, rate 14 CPS. Transfer the powder (pour out and tap on side of the vial using a metal spatula) to a new chilled 50 mL conical tube soaked into liquid N_2. Avoid spills of liquid nitrogen in the freezer mill vial, because the gas is sprayed out together with the powdered sample when opening the tube after milling.
1.6. Thaw the cell powder on ice. Spin the tube at 3000 x g for 5 min at 4°C.
1.7. Transfer supernatant to a chilled 1.5 mL tube on ice and clarify the lysate by centrifugation for 10 min at 20,000 x g at 4°C.
1.8. Transfer supernatant to a new 1.5 mL tube on ice, avoiding both, the pellet and the lipid layer on top.
1.9. Prepare aliquots (e.g. 150 μL), freeze in liquid N_2 and store at -80°C.
1.10. Measure RNA concentration in the lysate using Qubit™ RNA BR Assay Kit (ThermoFisher).

2. Nuclease digestion and monosome purification

Prepare in advance: succrose gradients 7-45% and precool (Section 3.1-3.2)

2.1. To a 30 μg aliquot (volumes adjusted to 150 μL) of lysate add 3 μL of RNase I (100U/μL, AM2294 ThermoFisher) and 25 U/mL final concentration of TURBO DNase (2U/μL, AM2238 ThermoFisher).
2.2. Incubate at 23°C in a thermomixer at 300 rpm for 40 min. Similarly, incubate 30 μg of RNase I-non-treated sample for polysome analysis, although add the RNase inhibitor before incubation.
2.3. Place samples on ice and add 6 μL of SUPERaseIN (20 U/μL) to stop the RNase treatment. Load onto sucrose gradient as soon as possible.

3. Sucrose gradient ultracentrifugation and fractionation

3.1. Prepare 7% and 45 % (w/v) sucrose solutions in Polysome Gradient Buffer (Appendix 1). Chill the ultracentrifuge rotor at 4 °C. Prepare gradients in Sw41 14 × 89 mm ultracentrifuge tubes. Mark the tubes according to the marker block and fill with 10% sucrose solution until the mark. Use a syringe to underlay 45% sucrose solution and fill the tube entirely. Gently insert the rubber cap into the top of the tube at an angle, with the hole entering the liquid last to allow air to escape. Remove excess of solution from the cap.

3.2. Form the gradient using a Gradient Master (BioComp Instruments) with rotation at 81.5°, speed 16, for 1:58. Load the gradients into the Sw41 buckets and return to 4°C for at least an hour.

3.3. From top of the gradient, remove the same volume of sucrose solution as that of the sample to be loaded and load the sample. Check the weight for good balance between opposing buckets!

3.4. Centrifuge gradients at 35,000 rpm for 3 hours at 4°C. Keep gradients at 4°C until ready to fractionate.

3.5. Fractionate sucrose gradients using Piston Gradient Fractionator (BioComp Instruments) and continuous A_{260} monitoring to identify the 80S ribosome peak. Analyze the undigested sample first to ensure that it contains intact polysomes and to determine the approximate location of the 80S ribosome peak. Then, fractionate the digested sample, which has a large 80S monosome peak.

3.6. Collect monosome fraction (~3 mL) into a 15 mL tube containing 2 mL of ice-cold ethanol. Adjust ethanol to 3x volume (e.g. 3 mL fraction + 9 mL EtOH) and precipitate O/N at -20°C.

3.7. Centrifuge tubes at 16,000 x g for 30 min at 4°C.

3.8. Remove all traces of supernatant, air dry pellet few minutes, and continue with TRI Reagent purification in step 4.1.

4. RNA purifications (Ribo-seq/RNA-seq)

Bring TRI Reagent to room temp.

for Ribo-Seq:

4.1. Add 1 mL of TRI Reagent (AM9738, ThermoFisher) to the RNA pellet from step 3.8., mix by vortexing.

4.2. Incubate for 5 min at room temperature.

4.3. Add 100 μL of BCP (B9673, Sigma-Aldrich).

4.4. Shake vigorously for 15 sec and incubate for 5-15 min

4.5. Centrifuge 12,000 x g for 15 min at 4°C and transfer aqueous phase to a fresh RNase-free tube.

4.6. Proceed with RNA Clean & Concentrator -25 Kit (R1017, Zymo Research) according to the manufacturer's general protocol.

4.7. Elute with 30 μL of nuclease-free water and measure RNA concentration using Nanodrop.

for RNA-Seq:

4.8. Purify RNA from an aliquot of the cell extract stored in step 1.10 using TRI Reagent. Add 1 mL of TRI Reagent per 100 µL of extract, mix by vortexing.
4.9. Incubate for 5 min at room temperature.
4.10. Add 100 µL of BCP.
4.11. Shake vigorously for 15 sec and incubate for 5-15 min
4.12. Centrifuge 12,000 x g for 15 min at 4°C and transfer 400 µL aqueous phase to a fresh RNase-free tube.
4.13. Proceed with RNA Clean & Concentrator -25 Kit according to the manufacturer's general protocol.
4.14. Elute with 50 µl of nuclease-free water and measure RNA concentration of 5x diluted samples using Nanodrop.
4.15. Proceed with in-tube DNase treatment. Prepare TURBO DNase reaction mix in an RNase-free tube:

RNA sample (10-12 µg)	44-43 µL
10X TURBO Buffer	5 µL
Turbo DNase (AM2238, Invitrogen)	1-2 µL
TOTAL	50 µL

4.16. Incubate at 37°C for 30 minutes. Then purify samples following RNA Clean & Concentrator -25 Kit protocol that retain RNAs>200 nt.
4.17. Elute with 25 µL of nuclease-free water.
4.18. Measure RNA concentration using Qubit RNA BR Assay Kit (Q10210, ThermoFisher) and preferably analyse RNA integrity using an automated electrophoresis system e.g. Bioanalyzer. 1-4 µg of total RNA is required.
4.19. Proceed with Ribo-Zero rRNA removal.

5. rRNA removal using Ribo-Zero

Applies to both, Ribo-Seq and RNA-Seq samples.

5.1. Follow Ribo-Zero protocol according to manufacturer's instructions, except omit the step 4. incubation at 50°C for 5 minutes. It helps to remove the undesired small rRNA fragments.
5.2. Use RNA Clean & Concentrator -5 Kit (R1015, Zymo Research) following the modifications below to purify Ribo-Zero treated samples:

a) Add following components and mix:

Reagent	RNA-Seq sample	Ribo-Seq sample
RNA Binding Buffer	100 µL	220 µL
100% Ethanol	100 µL	450 µL

b) Continue with purification according to the manufacturer's instructions.
c) Elute samples in 12 µL of nuclease-free water. Expect to recover 10 µl and quantify using NanoDrop.
d) Proceed with PAGE purification of Ribo-Seq samples to isolate ribosome protected fragments (RPFs).

Following steps in the protocol, modified from Ingolia *et al.*, 2012 *Nature Protocols*, are only for Ribo-Seq samples. RNA-Seq libraries prepare using ScriptSeq Complete Gold Yeast Kit (BGY1324, Illumina) according to the manufacturer's protocol.

6. PAGE Purification of RPFs

6.1. Prepare 15% PAA/7M Urea/TBE gel and Sample Loading Buffers according to Appendix 2

6.2. Prerun gel at 180V for 15 min in 1x TBE

6.3. Prepare samples (S1-S4), 18-RNA and 34-RNA oligomarker mix (M) and 10-bp Ladder (L) (10821015, Invitrogen):

a) Add 10 μL 2x Urea/TBE Sample loading buffer to Ribo-Seq samples from step 5.2.

b) Prepare 4x oligomarker (M) mix (10 μL per lane required) used in lanes between the samples:

10 μM 18-RNA	4 μL
10 μM 34-RNA	2 μL
Nuclease-free water	14 μL
2x Urea/TBE Loading	20 μL
TOTAL	40 μL

c) Prepare Ladder:

Nuclease-free water	4 μL
10-bp Ladder	1 μL
2X Urea/TBE loading	5 μL
TOTAL	10 μL

6.4. Denature Samples, Marker mix and Ladder at 80°C for 90 sec and place on ice. Rinse wells of the gel to remove urea and load samples. Preferably, do not load different RPF samples side by side on a gel to avoid cross-contamination. Outermost lanes leave empty to avoid "smiling". See Appendix 4 for an example of gel images.

6.5. Run the gel at 180V until bromophenol blue dye reaches at the bottom of the gel (~1.5 h).

6.6. Stain the gel using SYBR Gold dye (see Appendix 2). Mix 50 ml of chilled (4°C) 1xTBE with 5 μL of SYBR Gold. Incubate the gel in PP container for 5 min with gentle agitation. Place gel between sheets of a transparent plastic pocket and illuminate using SYBR compatible gel imaging system (do not use UV light!).

6.7. Excise the sample lane in a region delimited by 18 and 34 nt Markers and place in a non-stick RNase-free 1.5 ml tube. Similarly, excise bands from at least two Marker oligo lanes and pool them to one tube. The excised Markers will be processed similarly to samples, thereby guiding the excisions in the next gel purifications.

6.8. Extract RNA from gel slices using overnight gel extraction:

a) Crush gel slices using disposable RNase-free pestle for 1.5 mL tube.

b) Add 600 μL of RNA Gel Extraction Buffer (Appendix 2) and freeze the samples for 30 min on dry ice.

c) Incubate the samples overnight at room temperature on a thermomixer at 300 rpm.

d) Briefly centrifuge the gel extractions to collect the liquid at the bottom of the tube. Using 1 ml pipet (tip cut off) transfer 600 μL of the gel slurry to a Costar Spin-X

Centrifuge Tube Filter (8161, Corning) and spin at max speed for 30 sec to separate gel pieces, and transfer flow through into a nonstick RNase-free 1.5 mL tube.

6.9. Precipitate RNA by adding 2 µL of Glycogen (20 mg/mL), mixing well, and then adding 900 µL of isopropanol. Mix properly by turning over few times and vortexing.

6.10. Incubate >2 hours at -20°C.

6.11. Pellet RNA by centrifugation for 30 min at max speed (20 000 x g) at 4°C. Prepare 80% EtOH and chill on ice.

6.12. Keep sample on ice and carefully remove all liquid from the tube. Wash with 1 mL of 80% EtOH, be cautious not to release RNA pellet from the wall of the nonstick tube.

6.13. Centrifuge samples for 5 min at 12 000 x g at 4°C.

6.14. Keep the sample on ice until all the liquid is removed. Avoid disturbing the pellet. Lay tube on the side and allow to air-dry for 10 min.

6.15. Resuspend RNA in 43 µL of water and transfer to a new nonstick RNase-free tube. Proceed with 3' end repair.

7. 3' End Repair of RPFs (dephosphorylation)

7.1. Denature samples from step 6.15 for 90 sec at 80°C. Equilibrate to 37°C and set up reaction:

RNA sample	43
10X T4 PNK buffer	5
SUPERase·In (20 U/µL)	1
T4 PNK (10 U/µL)	1
TOTAL	50 µL

7.2. Incubate 1 h at 37°C.

7.3. Purify with RNA Clean & Concentrator -5 kit (R1015, Zymo Research). For that add 50 µL water, 200 µL of RNA Binding Buffer and 450 µL of absolute ethanol. Mix by vortexing, load to a spin column and continue according to the kit protocol.

7.4. Eluate in 11 µL of nuclease-free water. Expect to recover 9 µL.

8. Linker ligation

8.1. Add 1 µL of 100 µM preadenylated rA-NNNN-linker (see Appendix 1) to each tube from step 7.4 and denature for 90 sec at 80°C, and then equilibrate to room temperature.

8.2. Set up ligation reaction below and incubate for 2.5 h at room temperature:

RNA and linker	10
10X T4 Rnl2 buffer	2
50% PEG 8000	6
SUPERase·In	1
T4 Rnl2(tr), KQ enzyme (NEB)	1
TOTAL	20 µL

Use 20 µL pipette and mix properly, PEG is very viscous!!

8.3. Purify and concentrate using RNA Clean & Concentrator -5 kit. Add 80 µL of nuclease-free water, 200 µL of RNA Binding Buffer and 450 µL of absolute ethanol. Mix by vortexing, load to spin columns and continue according to the kit protocol.

8.4. Eluate in 10 µL of water. Expect to recover 9 µL.

8.5. Add 9 μL of 2X Urea/TBE Sample Loading Buffer and PAGE-purify ligation products (including ligated marker oligos (M_L) as described in Steps 6.1-6.15 using 12% denaturing PAA gel (Prepare gel in advance according to Appendix 2). M_L marker can be divided between two lanes to provide accurate demarcations for excision on RPF Sample lanes. Also excise M_L ligated products.

8.6. After precipitation resuspend RNA in 10 μL of nuclease-free water and transfer to 0.2 mL PCR tube.

9. Reverse Transcription

9.1. Add 2 μL of 1 μM (1 pmol/μL) reverse transcription primer to RNA from step 8.6. Denature for 2 min at 80°C and then place on ice. Cool cycler to 52°C.

9.2. Set up reverse transcription reaction and incubate it for 30 min at 52°C in thermal cycler:

Ligation and primer	12
5x First-strand buffer	4
10 mM dNTPs	1
0.1 M DTT	1
SUPERase·In	1
SuperScript IV enzyme	1
TOTAL	20 μL

9.3. Hydrolyze the RNA by adding 2.2 μL of 1 N NaOH to each reaction; incubate for 20 min at 98°C.

9.4. Add 20 µl of 3 M sodium acetate (pH 5.5), 2 µl of Glycogen, 156 µl of water, transfer to 1.5 ml nonstick tube, and add 400 µl of isopropanol to each reaction and precipitate cDNA as described in Steps 6.10-6.14.

9.5. Dissolve cDNA in 8μL of water.

9.6. Separate the reverse-transcription products from the unextended primer by 10 % denaturing PAGE as described in Steps 6.2-6.6. Instead of marker oligo, prepare 2 μL of 1 μM reverse transcription primer (Pr) in 3 μL of water and 5 μL of 2× Urea/TBE sample buffer. Split reverse transcribed markers between two lanes.

9.7. Excise the reverse-transcription products from the gel and place each in a clean nonstick RNase-free 1.5 mL tube.

9.8. Extract cDNA from the polyacrylamide gel using gel extraction protocols described in Steps 6.8-6.14. Use DNA Gel Extraction Buffer (Appendix 2) for overnight gel extraction and precipitation.

9.9. Dissolve first-strand cDNA in 15 μL of water, transfer to 0.2 ml tube, and proceed with circularization.

10. Circularization

10.1. Prepare circularization reaction:

First-strand cDNA	15
10X CircLigase Buffer	2
1 mM ATP	1
50 mM $MnCl_2$	1
CircLigase enzyme	1
TOTAL	20 μL

10.2. Incubate for 2 h at 60°C in a thermal cycler, followed by 10 min at 80°C for heat-inactivation.

10.3. Transfer reaction mix to 1.5 mL non-stick tube and precipitate DNA by adding 2 µL of Glycogen, 2.4 µL of 3M Sodium acetate pH 5.2, mix by vortexing and add 75 µL of absolute ethanol. Continue as described in Steps 6.10-6.14.

10.4. Dissolve DNA in 15 µL of water.

11. Library amplification and indexing

11.1. Prepare PCR reaction:

Nuclease-free water	6-7
10 µM riboseq frw library PCR pr	1
10 µM Scriptseq Index PCR pr	1
Circularized cDNA template	1-2
NEBNext Ultra II Q5 Mastermix	10
TOTAL	20 µL

Take 1 µL of circularized cDNA sample to PCR reaction, prepare also oligo markers and minus template control. Optimize number of PCR cycles (8-12 cycl) and amount of template to obtain at least 20 µL of each library in concentration range of 2-10 nM.

Run program:

98°C 30 sec
6-12 cycles { 98°C, 10 sec; 65°C, 75 sec }
65°C, 5 min
Hold 4°C

11.2. Add 10 µL of water, mix and add 8 µL of 5x Native TBE Sample Loading Buffer (Appendix 2) and load to two lanes of 8% Native TBE PAGE. Prepare 1 µL of 10-bp Ladder.

11.3. Run gel at 180V and purify amplified libraries (160-180 bp) as described in Steps 6.5-6.14 using DNA gel extraction buffer.

11.4. Dissolve in 10-30 µL of nuclease-free water.

12. Library QC

12.1. Measure concentrations of the libraries using Qubit High Sensitivity DNA Kit.
Library size estimation of RNA-Seq and molar concentration measurement is likely part of QC by a sequencing facility.

13. Sequencing

13.1. Platform: Illumina HiSeq2500.

13.2. Sequencing depth: 60M reads per Ribo-Seq sample, 40M reads per RNA-Seq sample.

13.3. Clustering: multiplexing, uneven clustering.

13.4. Sequencing primer: Illumina's Read 1 SP (Gives sense strand information for both library types, Appendix 3).

13.5. Read length: 1x 51 nt, single read for both Ribo-Seq and RNA-Seq.

13.6. At least two biological replicates per sample, no technical replicates required.

Appendix 1

Solutions

Polysome Lysis Buffer: 20 mM Tris-Cl (pH 8), 140 mM KCl, 1.5 mM $MgCl_2$, 1% (v/v) Triton X-100.

Polysome Gradient Buffer: 10 mM Tris-Cl (pH 8), 140 mM KCl, 15 mM $MgCl_2$, 0.5 mM DTT (add freshly). Sucrose solutions in Polysome Gradient Buffer (without DTT) can be prepared in bulk and stored at -20°C as 40 mL aliquots.

RNA Gel Extraction Buffer: 300 mM NaAc pH 5.5, 1 mM EDTA, 0.25% SDS (wt/vol). Store at room temperature.

DNA Gel Extraction Buffer: 300 mM NaCl, 10 mM Tris pH 8, 1 mM EDTA. Store at room temperature.

Oligonucleotides

(Oligo sequences are based on Ingolia et al., 2012 Nature Protocols and custom modified to introduce random nucleotides. Custom synthesized oligos were ordered from Integrated DNA Technologies).

18-RNA:

5’-CAACUGUAAACCGCUAUC-(Phos)-3’

34-RNA (NI-NI-19):

5’-AUGUACACGGAGUCGAGCUCAACCCGCAACGCGA-(Phos)-3’

rA-NNNN-linker:

5’rApp-NNNNCTGTAGGCACCATCAAT-3ddC

Reverse transcription primer (NNN-RT primer):

5’-(Phos)-NNNAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGTAGATCTCGGTGGTCGC-(SpC18)-CACTCA-(SpC18)-TTCAGACGTGTGCTCTTCCGATCTATTGATGGTGCCTACAG-3’

Frw library PCR primer:

5’-AATGATACGGCGACCACCGAGATCTACAC-3’

ScriptSeq™ Index PCR Primers (Set 1) (RSBC10948 Illumina) for library PCR and barcoding:

5’-CAAGCAGAAGACGGCATACGAGAT**NNNNNN**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT-3’

Appendix 2: Denaturing/Native PAGE of Nucleic acids

Hoefer MiniVE system		**Denaturing** PAA/7M Urea/TBE gels					
		10%		**12%**		**15%**	
		1 gel (10 ml)	2 gels (20 ml)	1 gel (10 ml)	2 gels (20 ml)	1 gel (10 ml)	2 gels (20 ml)
mQ	(mL)	up to 10	up to 20	up to 10	up to 20	up to 10	up to 20
Urea	(g)	4,2	8,4	4,2	8,4	4,2	8,4
40% AA:bis 19:1	(mL)	2,5	5	3	6	3,75	7,5
10X TBE	(mL)	1	2	1	2	1	2
10% APS	(μ L)	100	200	100	200	100	200
TEMED	(μ L)	6	12	6	12	6	12

		Native PAA/TBE gels	
		8%	
		1 gel (10 ml)	2 gels (20 ml)
mQ	(mL)	6,9	13,8
Urea	(g)	-	-
40% AA:bis 19:1	(mL)	2	4
10X TBE	(mL)	1	2
10% APS	(μ L)	100	200
TEMED	(μ L)	6	12

10X TBE: 0.89M TRIS, 0.89M Boric acid and 20 mM EDTA. Filter through 0.2 μm. Store at room temperature.

2X Urea/TBE Sample Loading Buffer: 1x TBE, 12% Ficoll, 0.01% Bromophenol Blue, 7M Urea (Do not add Xylene cyanol for Ribo-Seq RFPs). It takes O/N nutating to dissolve Ficoll 400. Store at +4°C for six months.

10x TBE	1 mL
Urea	4,2 g
Ficoll 400	1,2 g
0.1% BBlue	1 mL
mQ	up to 10 mL

5x Native TBE Sample Loading Buffer: 1x TBE, 15% Ficoll 400, 0.02% Bromophenol Blue. It takes O/N nutating to dissolve Ficoll 400. Store at room temperature.

10x TBE	1 mL
Ficoll 400	1.5 g
0.1% BBlue	2 mL
mQ	up to 10 mL

Pre-run gel for 15-30 min before sample loading to the denaturing gel. Wash wells of denaturing gels prior loading the samples using 1x TBE running buffer. Denature samples at 80°C for 90 sec. Load 10 μL (up to 20 μL) (0.2-0.3 μg of RNA Sample) to keep sharp bands and to avoid overloading. Run at constant 180 V.

SYBR Gold Staining: Mix 50 mL of chilled (4°C) 1x TBE with 5 µl of SYBR Gold. Incubate gel in PP container for 5 min with gentle agitation. Illuminate using Dark Reader or any SYBR compatible non-UV gel imaging system for preparative gels or under UV for non-preparative gels.

Appendix 3: Library architectures

Ribo-seq

AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT-18-34_nt_cDNA_of_RPF-
NNNNCTGTAGGCACCATCAATAGATCGGAAGAGCACACGTCTGAACTCAGTCACNNNNNNATCTCGTATGCCGTCTTCTGCTTG

Read 1 SP primer site
cDNA of Ribosome Protected Fragment
rA-NNNN-linker
Index (Barcode)

RNA-seq (SriptSeq)

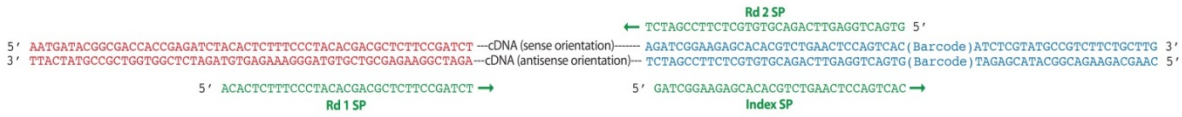
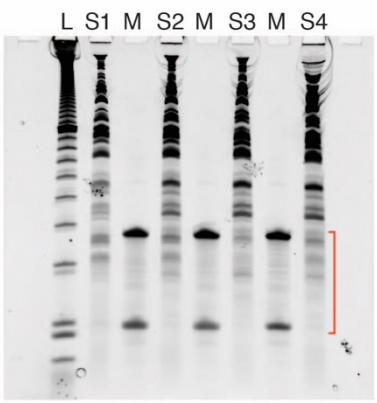


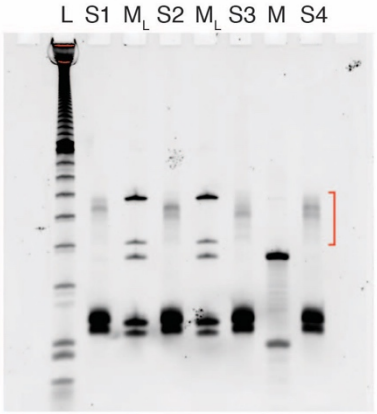
Figure 3. Sequencing a ScriptSeq™ v2 library.

Red	=	sequence incorporated by the Terminal Tagging process and PCR amplification.
Blue	=	sequence incorporated during reverse transcription and PCR amplification.
Black	=	sequence of the cDNA.
Rd 1 SP	=	sequence generated is that of the sense strand of the original fragmented RNA molecule.
Rd 2 SP	=	sequence generated is that of the antisense strand of the original fragmented RNA molecule.
Index SP	=	first nucleotide read is that of the Index or barcode.

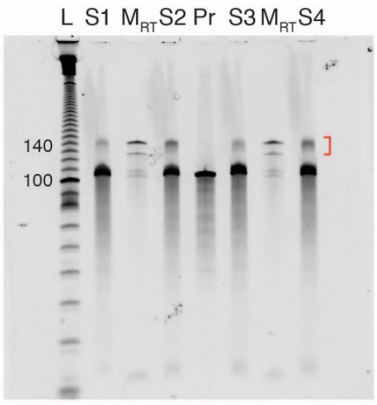
Appendix 4: Examples of gel images from Ribo-Seq library prep.



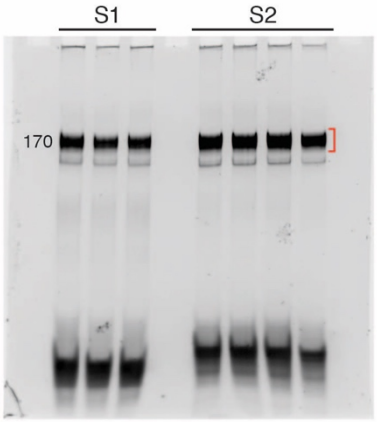
Gel 1: RPF purification



Gel 2: linker ligation



Gel 3: reverse transcription



Gel 4: amplification