Ribosome profiling: library preparations of 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 a wide 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. 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. 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. Store at -80°C until required.
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. Measure concentration of 200-fold dilution of extract in mQ water at A_{260} using a quartz cuvette. Blank using the same dilution of lysis buffer in mQ water.
1.10. Prepare aliquots (e.g. 150 μl), freeze in liquid N_2 and store at -80°C.

2. Nuclease digestion and monosome purification

Prepare in advance: succrose gradients 10-45% and precool (Section 3.1-3.2)

2.1. To a 20 A_{260} Units aliquot of lysate add 3 µl of RNase I (100U/μl, AM2294 ThermoFisher) and 25 U/ml final concentration of TURBO DNase (2 U/μl, AM2238 ThermoFisher).
2.2. Incubate 1 hour at room temperature on a nutating mixer. Similarly, incubate 20 OD units of RNase I non-treated sample for polysome analysis, although add the RNase inhibitor before incubation.
2.3. Add 6 μl of SUPERaseIN (20 U/μl) to stop the RNase reaction and place samples on ice. Load onto sucrose gradient as soon as possible.

3. Sucrose gradient ultracentrifugation and fractionation

3.1. Prepare 10% 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 continuous UV monitor to identify the 80S ribosome peak. Analyse 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)

Prepare fresh 75% ethanol. Bring TRI Reagent to room temperature.

Ribo-Seq:

4.1. Add 1 ml of TRI Reagent (AM9738, ThermoFisher) to the RNA pellet from step 3.5., 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.

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 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	1-2 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 (ThermoFisher) and analyze integrity using Bioanalyzer with RNA 6000 Nano Kit. 1-5 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 (Epicentre) 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), 26-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 26-RNA	4 μl
10 μM 34-RNA	2 μl
Nuclease-free water	14 μl
2x Urea/TBE Loading	20 μl

c) Prepare Ladder:

Nuclease-free water	4 μl
10-bp Ladder	1 μl
2X Urea/TBE loading	5 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 covered with aluminium foil for 5 min with gentle agitation. Illuminate using SYBR compatible gel imaging system (do not use UV light!).

6.7. Excise the sample lanes in a region delimited by 26 and 34 nt Markers and place in non-stick RNase-free 1.5 ml tubes. 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) Add 400 µl of RNA Gel Extraction Buffer (Appendix 2) and freeze the samples for 30 min on dry ice.

b) Incubate the samples overnight at room temperature with gentle agitation on a nutator.

c) Briefly centrifuge the gel extractions to collect the liquid at the bottom of the tube. Transfer 400 µl of eluate into a nonstick RNase-free 1.5 ml tube. Avoid carry over of the gel pieces, optionally run the eluate through a Costar Spin-X Centrifuge Tube Filter (8161, Corning) to remove all the gel pieces.

6.9. Precipitate RNA by adding 2 µl of Glycogen (20 mg/ml), mixing well, and then adding 600 µl of isopropanol. Mix properly by turning over few times and vortexing.

6.10. Incubate >1 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

7.1. Denature samples from step 6.15 for 90 sec at 80°C. Equilibrate to 37°C and set up a 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 by adding 50 µl of water, 200 µl of RNA Binding Buffer and 450 µl of absolute ethanol. Mix by vortexing, load onto the 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 miRNA cloning linker 1 (IDT) to each tube from step 7.4 and denature for 90 sec at 80°C, and then cool it to room temperature.

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

RNA and linker	10	6x mix
10X T4 Rnl2 buffer	2	12
50% PEG 8000	6	36
SUPERase·In	1	6
T4 Rnl2(tr) enzyme	1	6
TOTAL	20 µl	Transfer 10 µl of mix to each tube.

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 the spin column and continue according to the Kit protocol.

8.4. Elute 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 6.1-6.14 using 10% 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.

8.6. After precipitation resuspend RNA in 10 µl of nuclease-free water.

9. Reverse Transcription

9.1. Add 2 µl of 1 µM reverse transcription primer (Appendix 1) to RNA from step 8.6. Denature for 2 min at 80°C and then place on ice. Cool cycler to 48°C.

9.2. Set up reverse transcription reaction and incubate for 30 min at 48°C in a thermal cycler:

Ligation and primer	12	6x mix
5x First-strand buffer	4	24
10 mM dNTPs	1	6
0.1 M DTT	1	6
SUPERase·In	1	6
SuperScript III enzyme	1	6
TOTAL	20 µl	Transfer 8 µl of mix to each tube.

9.3. Hydrolyze the RNA by adding 2.2 µl of 1 N NaOH to each reaction and incubating 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, and 400 µl of isopropanol to each reverse-transcription 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 8 % denaturing PAGE as described in Steps 6.1-6.6. Instead of marker oligo, load 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 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 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 a 1.5 ml nonstick 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:

	1x	6x master
Nuclease-free water	10.3	61.8
5x Phusion HF buffer	2	12
10 mM dNTPs	0.2	1.2
10 μM riboseq frw library PCR pr	1	6
10 μM Scriptseq Index PCR pr	1	-
Phusion Hot Start II enzyme	0.5	3
Circularized Template	5	-
TOTAL	20 μl	Add 14 μl of mastermix to each reaction

Take 1 μl of circularized cDNA sample to a PCR reaction, prepare also a reaction for oligo markers and minus template control. Optimize the number of PCR cycles (8-16 cycles) and amount of template to obtain at least 20 μl of each library in concentration range of 2-10 nM. Fewer cycles are better to avoid PCR bias by overamplifying more abundant sequences.

Run program:

98°C 30 sec

8-16 cycles
- 98°C, 15 sec
- 65°C, 10 sec
- 72°C, 10 sec

Hold 4°C

11.2. Add 5 μl of 5x Native TBE Sample Loading Buffer and load to an 8% Native TBE PAA gel. Prepare also 1 μl of 10-bp Ladder.

11.3. Run gel at 180V and purify amplified libraries 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 to. Library size estimation of RNA-Seq and molar concentration measurement is mostly 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

(Prepare 10 ml of 10mg/ml Cycloheximide (C7698, Sigma-Aldrich) stock solution in mQ water. It is hydophobic compound and takes time to dissolve. Store at 4°C for up to few months).

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

Polysome Gradient Buffer: 10 mM Tris-Cl (pH 8), 140 mM KCl, 15 mM $MgCl_2$, 100 ug/ml Cycloheximide, 0.5 mM DTT (CHX and DTT add freshly).

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 from Ingolia et a., 2012 Nature Protocols. Custom synthesized oligos were ordered from Integrated DNA Technologies).

26-RNA (NI-NI-20):

5’-AUGUACACGGAGUCGACCCAACGCGA-(Phos)-3’

34-RNA (NI-NI-19):

5’-AUGUACACGGAGUCGAGCUCAACCCGCAACGCGA-(Phos)-3’

miRNA Cloning Linker 1 (IDT Integrated DNA Technology) for 3’ ligation:

/5rApp/CTGTAGGCACCATCAAT/3ddC/

Reverse transcription primer:

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

Frw library PCR primer:

5’-AATGATACGGCGACCACCGAGATCTACAC-3’

ScriptSeq™ Index PCR Primers (Set 1) (Epicentre) 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					
		8%		10%		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)						
Urea	(g)	4.2	8.4	4.2	8.4	4.2	8.4
40% AA:bis 19:1	(ml)	2	4	2.5	5	3.75	7.5
10X TBE	(ml)	1	2	1	2	1	2
10% APS	(µl)	100	200	100	200	100	200
TEMED	(µl)	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	(µl)	100	200
TEMED	(µl)	6	12

There are two commonly used TBE recipes and it is important to use the same for both, gel and running buffer:

1. **10x TBE** (93290-1L Sigma): 1.3M TRIS, 450 mM boric acid and 25 mM EDTA in water. Some companies have 0.89M Tris and 20 mM EDTA in 10x TBE.
2. **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. 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 covered with aluminium foil 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 architextures

Ribo-Seq

AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT—26-34 nt cDNA of RPF-
CTGTAGGCACCATCAATAGATCGGAAGAGCACACGTCTGAACTCAGTCACNNNNNNATCTCGTATGCCGTCTTCTGCTTG

Read 1 SP primer site
cDNA of Ribosome Protected Fragment (it can vary between 26-34 as were the size range between RNA oligos in gel purifications)
miRNA Cloning linker 1 (IDT Inc.)
Index (Barcode)

RNA-Seq (SriptSeq Complete Gold Kit, Epicentre)

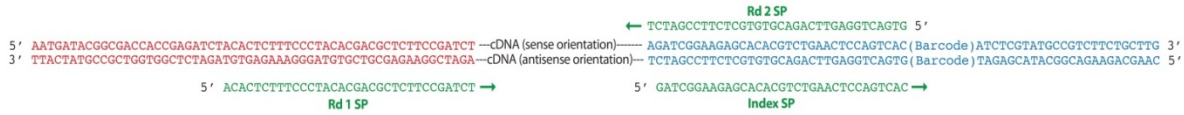
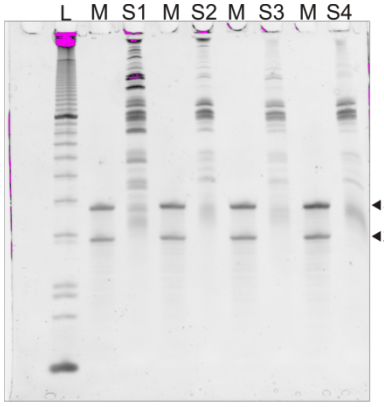


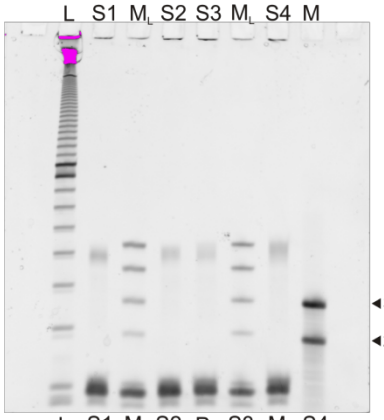
Figure 3. Sequencing a ScriptSeq™ v2 library.

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.

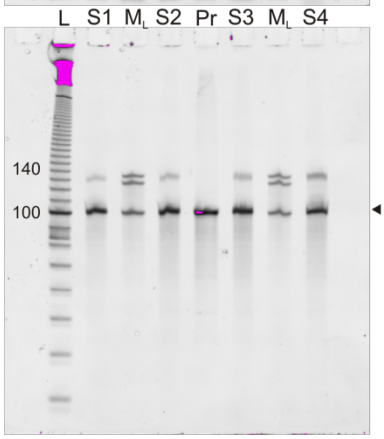
Appendix 4: Example of gel images from Ribo-Seq library prep



Gel 1: footprint purification



Gel 2: linker ligation



Gel 3: reverse transcription

Gel 4: amplification