Ribosome Profiling Protocol for *Kluyveromyces marxianus*

Materials

Strains

Kluyveromyces marxianus CBS 6556 (ATCC 26548/ NCYC 2597/ KCTC 17555).

Equipment

- Thermocycler (Applied biosystems 2720 Thermal Cycler)
- Nutator (Stuart see-saw rocker SSL4)
- Shaker (Heidolph Titramax 1010)
- Vortex Genie 2 (Scientific Industries)
- Refrigerated centrifuge
- Beckman ultracentrifuge and SW41ti swinging bucket rotor
- Ultracentrifuge polypropylene tubes (14 x 89 mm) (Beckman #331372)
- Ultracentrifuge polypropylene tubes (8 x 34 mm) (Beckman #343777)
- T-shaker (thermo-shaker for 1.5 mL tubes, T-shaker EuroClone)
- Retsch Mixer Mill (model: mm200)
- Retsch 10 mL stainless steel grinding jars and grinding balls
- Heat block (Stuart block heater SBH130D)
- Qubit Fluorometer 4.0
- Qubit assay tubes
- Micropipettes and filter tips (P2, P10, P20, P200 and P1000).
- RNase-free PCR tubes
- 1.5 mL RNase-free tubes
- 2.0 mL RNase-free tubes
- 15 mL RNase-free falcon tubes
- 50 mL RNase-free falcon tubes
- Gradient master (BIOCOMP #B108-2)
- Density Gradient Fractionation System (Brandel BR-188)
- TracerDaq USB-1208FS (Measurement Computing)
- UV Plate Reader (Spectramax M3, Molecular Devices)
- 0.45 µM Whatman cellulose nitrate filter membranes (GE life sciences #7184-004)

- Steriflip Sterile 50 mL Disposable Vacuum Filtration System 0.22µm (Millipore #SCGP00525)

Enzymes

- RNase I 10 U/µL (Lucigen #N6901K)
- T4 PNK 10 U/µL (NEB #M0201L)
- T4 RNA Ligase 2, truncated K227Q 200 U/µL (NEB #M0351L)
- Protoscript II 200U/µL (NEB #M0368L)
- 5' DNA Adenylation Kit (NEB #E2610L)
- CircLigase II 100U/µL (Epicentre #CL9025K)
- Phusion Polymerase 2U/µL (NEB #M0530)

Reagents

- Ultra-pure water
- Yeast Extract (Sigma-Aldrich #Y1625)
- Tryptone (Fluka analytical #9410)
- D-(+)-Glucose (Sigma-Aldrich #G7021)
- Cycloheximide ≥95% (HPLC) (Sigma-Aldrich #01810)
- SUPERase•In™ RNase inhibitor (Invitrogen #AM2696)
- PEG-8000 (supplied with T4 RNA ligase 2, truncated K227Q).
- dNTPs 10mM (NEB #N0447L)
- Streptavidin C1 Dynabeads (Invitrogen #65001)
- ssRNA low range ladder (NEB #N0364S)
- Oligo Clean & Concentrator kit (Zymoresearch)
- Qubit BR-RNA assay kit (Life Technologies #Q10210)
- Glycoblue coprecipitant (Ambion #AM9515)
- Oligo clean and concentrator (Zymoresearch #D4060)

Chemicals

- Magnesium Chloride (Sigma-Aldrich #M2670)
- Sodium chloride (Sigma-Aldrich #S7653)
- Sodium Hydroxide (Sigma-Aldrich #S8045)
- Trizol (Invitrogen #10296010)
- Sucrose (Sigma-Aldrich #S0389-500g)
- Ethanol (Sigma-Aldrich #51976)
- Chloroform (Sigma-Aldrich #C2432)
- Isopropanol (2-propanol, Sigma-Aldrich #I9516)

- Triton X-100 (Sigma-Aldrich #X100)
- DTT (Sigma-Aldrich #D9779)
- Potassium chloride (Sigma-Aldrich #P9541)
- Tris Base (Fisher Scientific #CAS 77-86-1)
- Urea (Fisher Scientific #BP169-500)
- APS (Sigma-Aldrich #A3678)
- Cesium Chloride (Sigma-Aldrich #C4036-1kg)
- TEMED (Sigma-Aldrich #T9281)
- EDTA (Sigma-Aldrich #EDS)
- Sodium dodecyl sulfate, SDS (Sigma-Aldrich #L4509)
- NaOAc (Sigma-Aldrich #S8625)
- 40% acrylamide/bis-acrylamide (19:1) (Sigma-Aldrich #A9926)
- Liquid nitrogen

Oligonucleotides

Oligonucleotides used in this study (same as McGlincy and Ingolia, 2017) were purchased from Integrated DNA Technologies (IDT) except for the biotinylated oligos for rRNA depletion which are sourced from Sigma.

RPF markers

- These are ordered as RNA oligonucleotides.
- The 3' ends of these oligos are phosphorylated, to allow these to be used as controls for dephosphorylation (with T4 PNK) and subsequent linker ligation.

32mer marker NI-800: 5′-AUGUACACUAGGGAUAACAGGGUAAUCAACGCGA/3Phos/
26mer marker, NI-801: 5′-AUGUUAGGGAUAACAGGGUAAUGCGA/3Phos/

Linkers

- These are ordered as DNA oligonucleotides.
- Each linker contains a unique 5 nt barcode to allow pooling of ligations before cDNA library preparation.

Name	Barcode	Sequence
NI-810	ATCGT	5′-/5Phos/NNNNNATCGTAGATCGGAAGAGCACACGTCTGAA/3ddC/
NI-811	AGCTA	5′-/5Phos/NNNNNAGCTAAGATCGGAAGAGCACACGTCTGAA/3ddC/
NI-812	CGTAA	5′-/5Phos/NNNNNCGTAAAGATCGGAAGAGCACACGTCTGAA/3ddC/

NI-813	CTAGA	5′-/5Phos/NNNNNCTAGAAGATCGGAAGAGCACACGTCTGAA/3ddC/
NI-814	GATCA	5′-/5Phos/NNNNNGATCAAGATCGGAAGAGCACACGTCTGAA/3ddC/
NI-815	GCATA	5′-/5Phos/NNNNNGCATAAGATCGGAAGAGCACACGTCTGAA/3ddC/
NI-816	TCTAG	5′-/5Phos/NNNNNTAGACAGATCGGAAGAGCACACGTCTGAA/3ddC/
NI-817	TCTAG	5′-/5Phos/NNNNNTCTAGAGATCGGAAGAGCACACGTCTGAA/3ddC/

RT primer

- The RT primer is ssDNA oligonucleotide, containing an 18-atom spacer modification (iSp18). This spacer is required during the final library PCR to amplify the library from circularized cDNA.
- Also included are 2 random nucleotides at the 5'end of RT primer.

Primer	Sequence
(RT-primer NI-802	/5Phos/NNAGATCGGAAGAGCGTCGTGTAGGGAAAGAG/iSp18/GTGACTGGAGTTCAGA(TGTGCTC

Biotinylated oligos for rRNA depletion

- These DNA oligonucleotides are biotinylated at the 5'ends and are purified via HPLC.

Name	Sequence	purification method
km_rRNA#1	/5Biosg/AAGGGTGCATCATCGACCGATCCTG	HPLC
km_rRNA#2	/5Biosg/GTTTCTTTACTTATTCAATTAAGCGGA	HPLC
km_rRNA#3	/5Biosg/TAAAGAATGGTACAGCTATAAATATT	HPLC
km_rRNA#4	/5Biosg/GCTCGAATATATTAGCATGGAATAATGGA	HPLC
km_rRNA#5	/5Biosg/TATAGAAGGATACGAATAAGGCGTC	HPLC
km_rRNA#6	/5Biosg/TTTCCACGTTCTAGCATTCAAAGTCCT	HPLC

cDNA library PCR primers

- The Illumina forward primer is the same in each reaction while each sample includes different reverse primer to allow multiplexing.
- Each reverse primer includes a unique barcode which allows for multiplexing of samples as follows, whereby JJJJJJ indicates a unique barcode:

5′-
CAAGCAGAAGACGGCATACGAGATJJJJJJGTGACTGGAGTTCAGACGTGTG

Primer	Sequence
Illumina forward primer	5′AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACG ACGCTC
Illumina reverse indexed primer (NI-799)	CAAGCAGAAGACGGCATACGAGATCGTGATGTGACTGGAGTTCA GACGTGTG
Illumina reverse indexed primer (NI-822)	CAAGCAGAAGACGGCATACGAGATACATCGGTGACTGGAGTTCA GACGTGTG
Illumina reverse indexed primer (NI-823)	CAAGCAGAAGACGGCATACGAGATGCCTAAGTGACTGGAGTTCA GACGTGTG
Illumina reverse indexed primer (NI-824)	CAAGCAGAAGACGGCATACGAGATTGGTCAGTGACTGGAGTTCA GACGTGTG
Illumina reverse indexed primer (NI-825)	CAAGCAGAAGACGGCATACGAGATCACTGTGTGACTGGAGTTCA GACGTGTG
Illumina reverse indexed primer (NI-826)	CAAGCAGAAGACGGCATACGAGATATTGGCGTGACTGGAGTTCA GACGTGTG

Solutions/Buffers

Polysome lysis buffer and Polysome buffer

- Keep on ice.
- Prepare this buffer excluding Cycloheximide (CHX) and DTT, freeze at -20^{o}C. On day of use, thaw on ice and add Cycloheximide from a 100 mg/mL in DMSO stock and DTT, keep on ice.
- For polysome buffer, substitute Triton X-100 with water as Triton X-100 is only required for lysis/lysate preparation.

Component	Volumes	Final Concentration
1M Tris-HCL 7.5	200 µL	20 mM
2M KCl	750 µL	150 mM
1M MgCl2	100 µL	10 mM
Cycloheximide (100mg/ mL)	10 µL	100 µg/ mL
1M DTT	10 µL	1 mM
20% Triton X-100	500 µL	1%
Ultra-pure water	8.43 mL	
Total Volume	10 mL	

Polysome gradient solutions

- Make polysome gradients fresh.
- First make a polysome gradient buffer (without sucrose) which is added to a 50 mL falcon tube containing the required weight of sucrose.
- Once buffer is added to the sucrose, vortex each solution rigorously until sucrose is dissolved.
- The 10% and 50% sucrose solutions can be left on a nutator for 10 minutes to reduce bubbles in the tubes and leave on ice until use.

1. Polysome Gradient Buffer

Component	Volume for 2 gradients	Volume for 4 gradients	Volume for 6 gradients	Concentration
1M Tris pH 7.5	500 µL	1 mL	1.5 mL	20 mM
2M KCl	1.75 mL	3.5 mL	5.25 mL	140 mM
1M $MgCl_2$	125 µL	250 µL	375 µL	5 mM
100 mg/ mL cycloheximide in DMSO	25 µL	50 µL	75 µL	100 µg/ mL
1M DTT	25 µL	50 µL	75 µL	1 mM
20 U/ µL SUPERase•In	25 µL	50 µL	75 µL	20 U/ mL
Ultra-pure water	22.55 mL	45.15 mL	67.73 mL	
Total Volume	25 mL	50 mL	75 mL	

2. 10% Sucrose (w/v):

Component	Amount for 2 gradients	Amount for 4 gradients	Amount for 6 gradients
RNase-free sucrose	1.5 g	3 g	4.5 g
Polysome gradient buffer	13.9 mL	27.8 mL	41.7 mL

3. 50% Sucrose (w/v):

Component	Amount for 2 gradients	Amount for 4 gradients	Amount for 6 gradients
RNase-free sucrose	7.45 g	14.9 g	22.35 g
Polysome gradient buffer	10.4 mL	20.8 mL	31.2 mL

60% CsCl Solution

To make 100 mL of 60% (w/v) CsCl:

1. Weigh 60g of CsCl.
2. Add ultra-pure water to 100 mL.
3. Vortex/shake until CsCl solution is fully dissolved.
4. Stain the solution with bromophenol blue by dipping the tip of a metal spatula into bromophenol blue powder and into the bottle of 60% CsCl until the solution becomes clear blue. Solution can be stored at room temperature.

Recipe for 15% PAGE gel with Bis-Acrylamide and Urea

- Note that 15% Urea-PAGE gel solution must be prepared without 10% APS and TEMED, these two chemicals are added immediately before making the gel.
- Stocks of this Urea-PAGE gel solution (without 10% APS and TEMED) can be prepared and stored at RT for 3 months. Protect PAGE solutions from light, store in dark cupboard or cover with foil.
- For all PAGE-gels, add 10% APS and TEMED to stock solutions in a 15 mL falcon tube, vortex briefly and immediately pour into a 0.75 mm gel cassette with a 10 mL pipette.

For a stock solution, add the following to an appropriately sized tube/bottle. Dissolve the solution in a 37^{o}C water bath (vortexing during the incubation can speed up the dissolving process). After the solution is dissolved, filter with steriflip and store at RT.

Component	Stock for 1 gel	Stock for 2 gels	Stock for 6 gels
40% acrylamide/bis acrylamide (19:1)	2.815 mL	5.63 mL	18.76 mL
Urea	3.6 g	7.2 g	24 g
10X TBE	750 µL	1.5 mL	5 mL
Ultra-pure water	950 µL	1.9 mL	6.33 mL
Total volume	7.5 mL	15 mL	50 mL

3X RNA loading dye

3X RNA loading dye is composed of 6M Urea, 25% sucrose and 1-2 grains of bromophenol blue.

RNA extraction buffer

RNA extraction buffer is composed of 300 mM NaOAc, 1mM EDTA and 0.25% SDS (v/v). Store at room temperature.

Component	Volume	Final Concentration
3M NaOAc pH 5.5	1 mL	300 mM
0.5M EDTA	20 µL	1 mM
10% SDS	250 µL	0.25% v/v
Ultra-pure water	8.73 mL	
Total volume	10 mL	

Recipe for 7.5% Urea-PAGE gel with Bis-Acrylamide and Urea

- This 7.5% Urea-PAGE gel is used for cDNA size selection after reverse transcription.
- Volumes for larger stock solutions are shown below (15 mL or 50 mL) which can be stored at RT (protected from light), adding 10% APS and TEMED immediately before gel loading.

Prepare the following Urea-PAGE gel solution.

40% acrylamide/bis-acrylamide (19:1)	1.41 mL	2.82 mL	9.4 mL
Urea	3.6g	7.2g	24g
10X TBE	0.75 mL	1.5 mL	5 mL
Ultra-pure water	2.36 mL	4.72 mL	15.73 mL
Total volume	**7.5 mL**	**15 mL**	**50 mL**

Dissolve in a water bath at 37^{o}C then filter with steriflip. Store at RT.
7.5 mL Gel = 7.48 mL stock + 18.75 µL 10% APS + 3.75 µL TEMED

DNA Extraction Buffer

DNA extraction buffer is composed of 300 mM NaCl, 1 mM EDTA and 10 mM Tris pH 8.0. Store at room temperature.

Component	Volume	Final Concentration
5M NaCl	600 µL	300 mM
0.5M EDTA	20 µL	1 mM
1M Tris pH 8.0	100 µL	10 mM
Ultra-pure water	9.28 mL	
Total volume	10 mL	

20X SSC (hybridization buffer)

- A 20X stock solution contains 3 M sodium chloride and 300 mM sodium citrate adjusted to pH 7.2, may be stored at room temperature.

For 100 mL stock solution (scale down if required):

1. Weigh out 17.53 g NaCl and 8.82 g Sodium Citrate.
2. Add 80 mL ultra-pure water and dissolve.
3. Adjust pH to 7.2 with HCl.
4. Bring the final volume to 100 mL.

8% polyacrylamide gel for cDNA library products

- This 8% PAGE gel does not require urea and is used for dsDNA products from the final library PCR stages.
- Add 10% APS and TEMED last, allow 30 minutes to polymerize at room temperature.

Component	8%
10X TBE solution	750 µL
19:1 acrylamide:bisacrylamide solution (40%)	1.5 mL
Water	5.25 mL
TEMED	15 µL
Ammonium Persulfate (10% w/v) (APS)	52.5 µL

Total Volume	7.57 mL

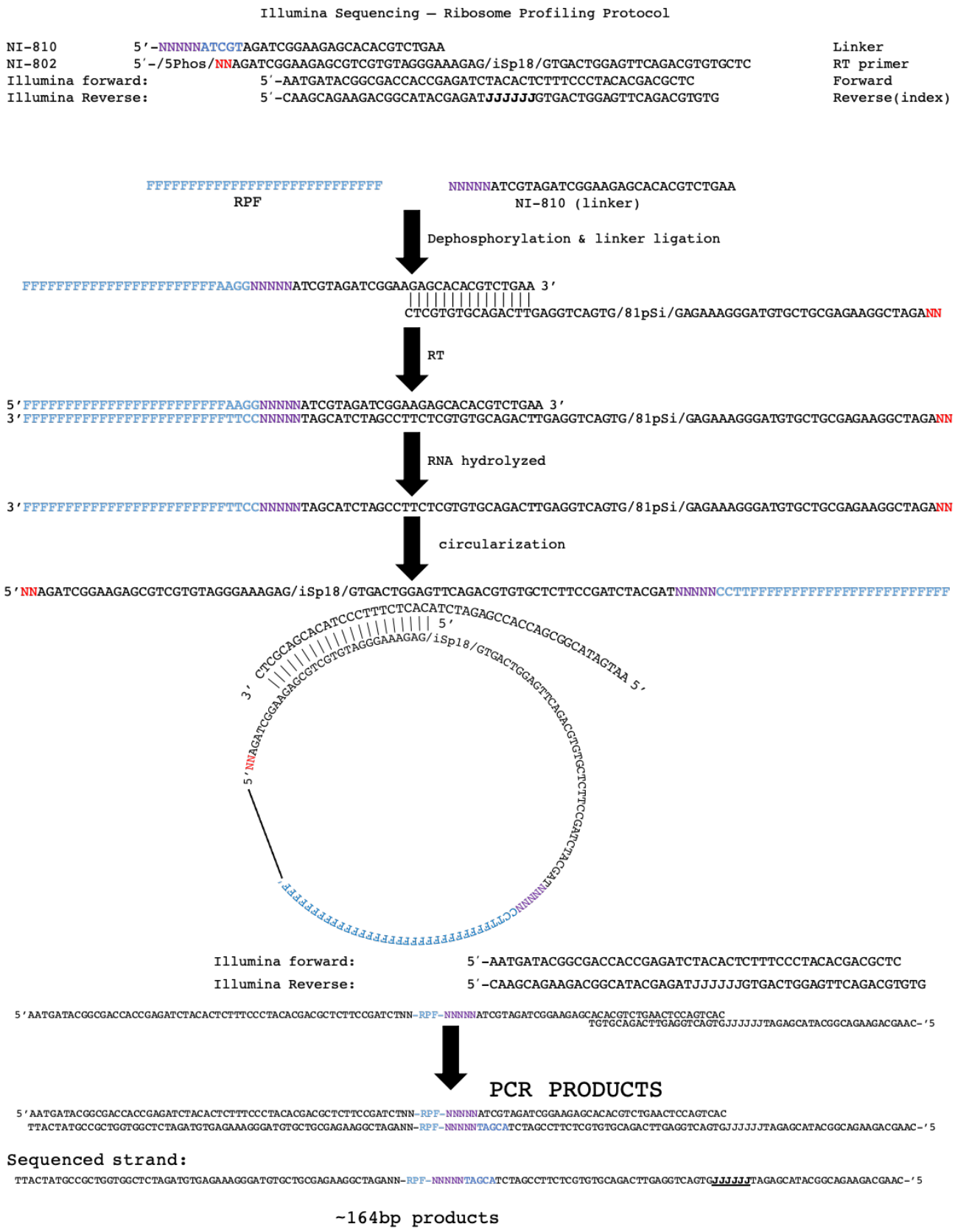

Figure 1. Molecular schematic of ribosome profiling library preparation. Oligos used in library generation are displayed at top of figure.

Protocol for ribosome profiling

Safety Precautions

While handling liquid nitrogen and chilled material, wear appropriate safety glasses and PPE, use cryogenic gloves to avoid burns, and adhere to all lab safety procedures.

1.1 Growth

- *Kluyveromyces marxianus* strain CBS 6556 (CBS-KNAW culture collection, Westerdijk Institute) was routinely used in this protocol but the method should work equally well with other strains. In the ribosome profiling data shown in this study, cultures were grown to early-log phase at A_{600} 0.6 - 0.8 in 150 mL synthetic minimal medium (Verduyn *et al.*, 1992) in 500 mL conical flasks with shaking.

1. 5 mL of YPD or synthetic minimal medium (Verduyn *et al.*, 1992) in a sterile 50 mL falcon tube is inoculated with a fresh colony of yeast and incubated overnight at 30^{o}C in a shaking incubator. Next day, transfer an appropriate volume of overnight culture to 150 mL media in a 500 mL Erlenmeyer flask so that the A_{600} is diluted to ~0.06.
2. Incubate the culture in a shaking incubator at 30^{o}C (or other) to ~0.6 – 0.8 A_{600} (log/exponential-phase). As the cells reach this desired OD600, prepare harvesting apparatus in following section.

1.2 Harvesting

- It is important to implement rapid harvesting of cells for ribosome profiling experiments. If cells are left for an extended period on exposed filter membrane, it may induce stress which can quickly alter global translation and compromise the experiment.
- In this protocol, a glass filter apparatus is used to rapidly remove media and trap cells on a filter membrane. The cells are immediately scraped off the membrane, flash frozen and stored in liquid nitrogen.
- The rapid filtration takes us ~10-12 seconds from pouring the culture onto the membrane and placing the scraper into the liquid nitrogen.

1. Before harvesting of cells, prepare the following equipment:
    a. Assemble the glass filter assembly and filter membrane.
    b. Prepare a 50 mL falcon tube for each sample by piercing the cap with a screwdriver to make a single hole which allows the evaporation of liquid nitrogen.

IMPORTANT: make sure to pierce the cap, failing to do so will result in tube explosion.

c. Place the falcon tube into a rack inside a styrofoam box and fill the falcon tube 3/4 with liquid nitrogen.

2. Quickly pour the 150 mL culture into the glass filter assembly and turn on the vacuum.
3. As the media becomes drained, have a cell scraper ready and when media is drained, quickly remove the clamp/upper glass funnel and scrape the cells off the membrane, scrape the membrane enough so that all of the membrane becomes scraped at least once. A clear visible accumulation of yeast on the scraper will be present.
4. Immediately place the cell scraper into the liquid nitrogen-filled falcon tube and leave it for 30 seconds to freeze. Using another pre-chilled cell scraper, detach the cell pellet from the original scraper. If processing multiple samples in quick succession proceed to step 6 and when all samples are collected, remove tubes from -80^{o}C, add liquid nitrogen to the pellet and proceed to step 5.
5. Using a P1000, slowly add 2 mL of polysome lysis buffer (PLB) to the falcon tube so that the PLB forms frozen droplets alongside the cell pellet, add more liquid nitrogen if needed.
6. Place the pierced cap on the tube and place upright in -80^{o}C for storage. The excess liquid nitrogen will boil off through the pierced cap. The frozen cells can be stored for extended periods.

1.3 Lysis

- Here, cryogenic mechanical lysis is used to break the cells and release ribosomes/polysomes.
- Cryo-milling ensures ribosomes are unable to translocate and reduces RNA degradation and upon thawing ribosomes are exposed the cycloheximide which will reduce translocation.

1. Place the unscrewed grinding jars and grinding balls into a liquid nitrogen filled styrofoam box. Add enough liquid nitrogen to ensure the jars and balls are submerged. Allow the jars and balls to cool until the rapid boiling of liquid nitrogen stops.
2. Using a large metal tongs, remove a single grinding jar and ball. Pour excess liquid nitrogen if present in the jars back into the styrofoam box.

3. Quickly add frozen cell pellet/PLB mixture and a grinding ball to the grinding jar and seal the jar.
4. Re-chill the jar in liquid nitrogen until boiling stops.
5. Loosen the grinding jar ¼ turn, place in a mixer mill and mill for 3 minutes at 15 Hz. Loosening the jar ¼ will allow any liquid nitrogen and gases escape from the jar during the 3-minute milling process.
6. Tighten the jar and return to liquid nitrogen.
7. Repeat for 5 more cycles.
8. Remove the jars from the mixer mill and chill the tips of two spatulas in liquid nitrogen.
9. Partly fill a 50 mL falcon tube with liquid nitrogen and place it upright in a liquid nitrogen bath. Open the grinding jar and recover the lysate powder into a 50 mL falcon tube using liquid nitrogen chilled spatulas, using a separate chilled spatula for each sample to avoid contamination.
10. Pierce the cap of the tube containing yeast lysate and place it upright in a -80^{o}C freezer, until the liquid nitrogen evaporates. Samples can be stored at -80^{o}C for extended periods.

1.4 Lysate Clarification

- It is critical here to use a clean workspace, filter tips, with RNase-free gloves and tubes. Do this for all steps when working with RNA.
- Always keep RNA samples on ice between steps.

1. Thaw the yeast lysate powder gently (on ice or in a cold room) and transfer a 2 mL tube. Typical recovered yield should be ~1.5 mL.
2. Immediately centrifuge at 3,000 x g, 4^{o}C for 5 minutes.
3. Recover supernatant into 2 mL RNase-free tube.
4. Further clarify the supernatant by spinning at 20,000 x g, 4^{o}C for 10 minutes and recover the supernatant again in a 1.5 mL tube.

1.5 Lysate RNA quantification

- To quantify RNA concentration, use a Qubit fluorometer with BR (Broad-Range) assay kit or other.

1. Mix 199 µL RNA BR buffer and 1 µL BR reagent for each sample in a 1.5 mL tube.
2. Transfer 199 uL solution to Qubit assay tube,
3. Add 1 µL sample to the Qubit assay tube with 199 µL solution.

4. Briefly vortex and spin down on a microfuge.
5. Incubate at room temperature for 2 minutes.
6. Take readings on Qubit (select correct assay: RNA-BR (Broad Range)).

1.6 Aliquot Lysates

- For ribosome profiling, multiple aliquots of 30 µg of total RNA into 1.5 mL tubes can be made and stored in -80^{o}C. This leaves multiple backup samples should something go wrong.
- Additionally, aliquoted lysates provide extra material which can be used for RNA-seq to measure relative mRNA abundance.
- For an example of typical RNA yields from an experiment with 150 mL cultures (2% glucose minimal medium) of *K. marxianus* grown between A_{600} 0.6 - 1.0 see below.
- Each 150 mL culture should generally provide ~500-900 µg RNA.

	concentration (µg/ mL)	Volume (µL) for 30 µg	polysome buffer (µL)	total RNA yield (µg) from 1.5 mL lysate
sample 1	557	54	146	835
sample 2	338	89	111	507
sample 3	338	89	111	507
sample 4	598	52	148	897
sample 5	571	53	147	856
sample 6	455	66	134	682

1. After RNA quantification, calculate the volume of clarified lysate required to aliquot 30 µg. In addition, note the volume of each sample.
2. Once 30 µg material has been dispensed into a tube, immediately drop it in a dewar flask of liquid nitrogen.
3. To collect the tubes, one may use a styrofoam box and a plastic sieve to quickly collect all tubes and place them into a freezer box.
4. Store the lysate stocks at -80^{o}C indefinitely.

2.1 Preparation of sucrose gradients

- Sucrose gradients for monosome isolation can be formed via two methods:
    - Using a Gradient master (Gradient Master 108, Biocomp) to quickly form reproducible gradients.
    - Bench-top gradient formation if a Gradient master is not available; however, this takes 4 hours for gradients to form.

- Alternatively, ribosomes can be pelleted in 1 M sucrose cushion as described in the Ingolia Protocol 2017 (McGlincy and Ingolia, 2017).

Gradient master method (preferred)

1. Prepare 10% and 50% sucrose solutions as described in the recipe section.
2. Place a polypropylene tube in the gradient master SW41ti metal marker supplied with the gradient master and using a fine-tip permanent marker, mark a line around the polypropylene tube.
3. Using a 50 mL syringe, carefully dispense 10% sucrose solution until the solution is raised to be in line with the marker.
4. Using a 10 mL syringe, carefully dispense 50% sucrose solution under the 10% sucrose and dispense until the 10%-50% sucrose border is lined up with the marker.
5. Carefully place the caps on each tube, using a syringe or P1000, carefully remove excess sucrose solution from the cap.
6. Turn on the gradient master and level the circular platform using the DOWN and UP options. When level, select DONE.
7. Select LIST, then SW41, navigate to “Short Sucr 10-50% wv 14S” and select USE and then RUN to begin gradient formation.
8. Place the tubes in a cold room for 45 minutes to allow gradients to cool before loading samples.

Bench-top method (alternative)

1. Prepare 10% and 50% sucrose solutions as described in solutions/buffers section.
2. Dispense 5.5 mL 50% sucrose solution to each polypropylene tube.
3. Gently layer 5.5 mL 10% sucrose on top of the 50% sucrose solution.
4. Seal the tubes with parafilm and gently lay the tubes horizontally for 4 hours.
5. Place the tubes vertically in the cold room for 45 minutes to allow gradients to cool before loading.

2.2 RNase Digestion

1. Thaw 30 µg RNA samples on ice.
2. Dilute each 30 µg sample to 200 µL with ice-cold PB (polysome buffer, see solutions/buffers section).
3. To each sample, add 1.5 µL (10 U/µL) RNase I and incubate for 45 minutes at room temperature with agitation (T-shaker).
4. Place the samples on ice and proceed directly with ultracentrifugation.

2.3 Ultracentrifugation

- For this protocol, use sucrose gradients and fractionation to isolate the monosome which contains the ribosome/RPF.
- Alternatively, if the above method is not available, have also pelleted monosomes using smaller scale ultracentrifugation. This alternative method is also described below.

Preferred method: Sucrose Gradient Ultracentrifugation

1. Turn on ultracentrifuge and input the following settings:
    - Rotor: SW41-Ti
    - Temperature: 4^{o}C
    - Speed: 36,000 RPM
    - Time: 3 hours (3:00)
    - Acceleration: 5
    - Deceleration: 5
2. Pre-cool the ultracentrifuge by selecting the pre-cool option, the swinging bucket rotor and tubes are stored in a cold room.
3. In a cold room, remove 200 µL from the top of each gradient (200 µL is the volume to be added) and gently layer 200 µL of the sample using a P200.
4. Balance each gradient tube within 20 mg (0.02 g).
5. Place rotor in the ultracentrifuge and slide the door to close.
6. Press ENTER followed by START to begin the run.
7. Once the run is completed, allow air back into the rotor chamber by pressing Vacuum.
8. Once the pressurization is complete, slide open the centrifuge door and carefully remove the rotor and place it back on the rotor stand.
9. Carefully remove each tube and place on the provided rack.
10. Tubes can be left in the cold room or a fridge during the fractionation step.

Alternative method: Ribosome Pelleting Ultracentrifugation

1. Pre-cool the ultracentrifuge.
2. Pre-cool the TLA120.1 rotor in a cold room or fridge.
3. To each tube, pipette 200 µL of each sample.
4. Weigh and balance each tube with 10 mg.
5. Run at 120,000 rpm for 2 hours at 4^{o}C.

6. Using a clean metal tongs, carefully remove each tube and using a P200, carefully remove the supernatant. Use a P10 to remove small volumes of remaining supernatant. A glassy pellet should be visible.
7. With this method, resuspend the pellet in 250 µL ultra-pure water and proceed directly to RNA extraction via Trizol protocol according to manufacturer's instructions.

2.4 Density Gradient Fractionation and Monosome Collection

- In this protocol, a tracerDaq is connected to a Windows desktop and Brandel UV detector, which provides a digital output of absorbance readings. Alternatively, the Brandel UV detector has a paper output which can be used as an alternative.

1. Assemble the fractionator equipment and prewarm the UV lamp, the lamp will be ready when the red light turns green.
2. Select the following settings on the pump and absorbance detector:
    a. 1.5 mL/min flow speed
    b. 0.2 Sensitivity
    c. Baseline speed of 60 (if using paper graph output)
3. Using the glass syringe provided with the fractionator, uptake 60% CsCl solution and insert into the pump.
4. Once the lamp indicator is green, fill a polypropylene tube with Ultra-pure water, insert tube into the detector component and pierce the tube.
5. Pierce the polypropylene tube by raising the needle just below the polypropylene tube, then use the screw to raise the needle and pierce the tube.
6. Select normal & forward to begin pumping CsCl into the tube. The CsCl will raise the tube contents through the detector unit and out through the outflow tube. For this tube filled with ultra-pure water, a flat "blank" line will eventually form.
7. Using rapid reverse mode on the pump, remove some CsCl back into the syringe. Leave a noticeable amount remaining in the polypropylene tube so that the needle is submerged in CsCl.
8. Lower the needle using the screw, then lowering the needle stand, remove the polypropylene tube and discard.
9. When the tube is removed, start the forward pump to remove any air bubbles until CsCl comes through, then stop. Samples are now ready to be processed.
10. Once liquid comes through the outflow tube, carefully insert it into the 1st well (A1) of a 96 well plate, hold for 12 seconds and move to the next and so on.

11. Disassemble all parts and wash with distilled water and leave them on a layer of tissue to dry on the bench. For the inflow tube (thick), wash with water. For the thinner outflow tube, use a P1000 to wash through 1 mL pure ethanol, then 1 mL water, then 2 x 1 mL empty pipette to push through any remaining liquid.
12. From the 96 well plate, the monosome fraction is selected using a UV reading from a plate reader. With readings from 260 nm, select the well with peaks corresponding to 80S. See supplementary figure 2 for an example of a trace from the fractionation.
13. Transfer the well(s) containing the monosome fraction to an RNase-free 1.5 mL tube. Typically, this is 2-3 wells, each containing ~300 µL.
14. Proceed with the Trizol protocol to isolate RNA with the manufacturer's instructions, resuspending in 12 µL of ultrapure water.

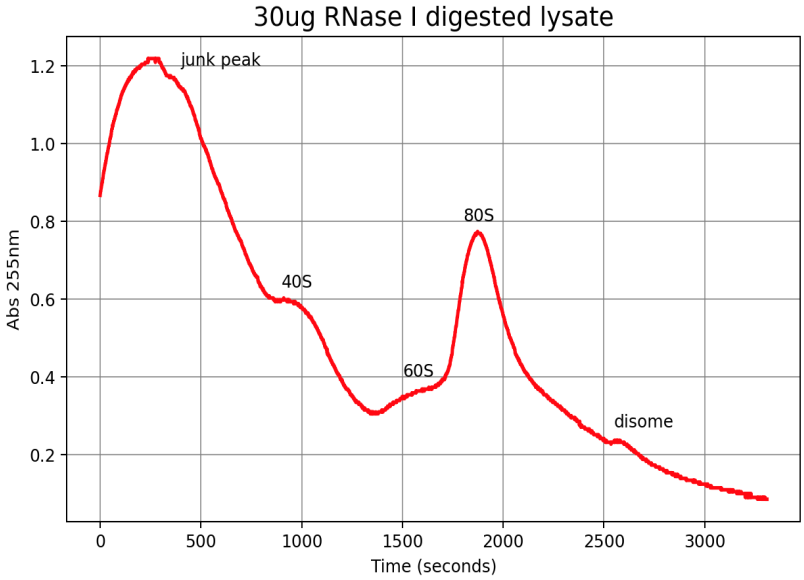

Figure 2. Sucrose gradient fractionation absorbance distribution of 30 µg RNase I digested material.

2.5 RPF Size Selection (Urea-PAGE gel)

- After polysome RNase digestion and monosome isolation, the RPFs need to be size selected and purified. This is done with a size selection gel using a marker 26-34nt to select these range of fragments.
- In yeast, RPFs are ~28nt in length and the use a 26-32nt marker allows excision of RPFs from a PAGE gel.
- For all gels required in this protocol, generally make a homemade gel mix described in the recipe table below. These stocks can be stored at room temperature for 2-3 months.

- Pre-cast gels are also available commercially which should be adequate for these experiments.
- Important note: The 28 and 32 nt RPF markers, which are used in high concentration, can contaminate libraries. It is recommended that careful dispensing of these markers is important to avoid contamination. In addition, allow empty wells between both marker and sample lanes to avoid cross contamination.

1. Prepare 15% Urea-PAGE gel stock solution as described in reagents. For a single gel, combine 7.478 mL Urea-PAGE stock solution (recipes), 18.75 µL APS and 3.75 µL TEMED to a 15 mL tube, vortex and using a 10 mL pipette, immediately add to 0.75 mm Bio-Rad gel plate sandwich and slowly insert 10 well comb. Leave to polymerize for 2 hours.
2. Set up the electrophoresis tank and add 1x TBE buffer.
3. Insert the polymerized gel and using a 10 mL syringe, clean the wells with buffer from the tank with 1X TBE 3 times. Be careful to not damage the gel separating the wells.
4. Pre-run at 300 V constant (~15 mA) for 20-30 minutes (1x TBE)
5. Clean again with syringe.
6. Prepare marker: 1 µL marker + 7 µL water + 4 µL 3X RNA loading dye.
7. Prepare RPF sample: 12 µL RPF sample + 6 µL 3X RNA loading dye.
8. Denature marker and RPFs for 90 seconds at 80^{o}C.
9. Load markers and RPF samples, leaving at least one lane between samples.
10. Run gel for 70 minutes at 300 V constant (~15 mA).
11. Carefully remove gel and place into a tank with 20 mL 1X TBE and 2 µL SYBR Gold.
12. Place on a nutator for 2 minutes.
13. Visualize gel with Bio Rad GelDoc or equivalent. Figure 3 shows a typical gel from this experiment.

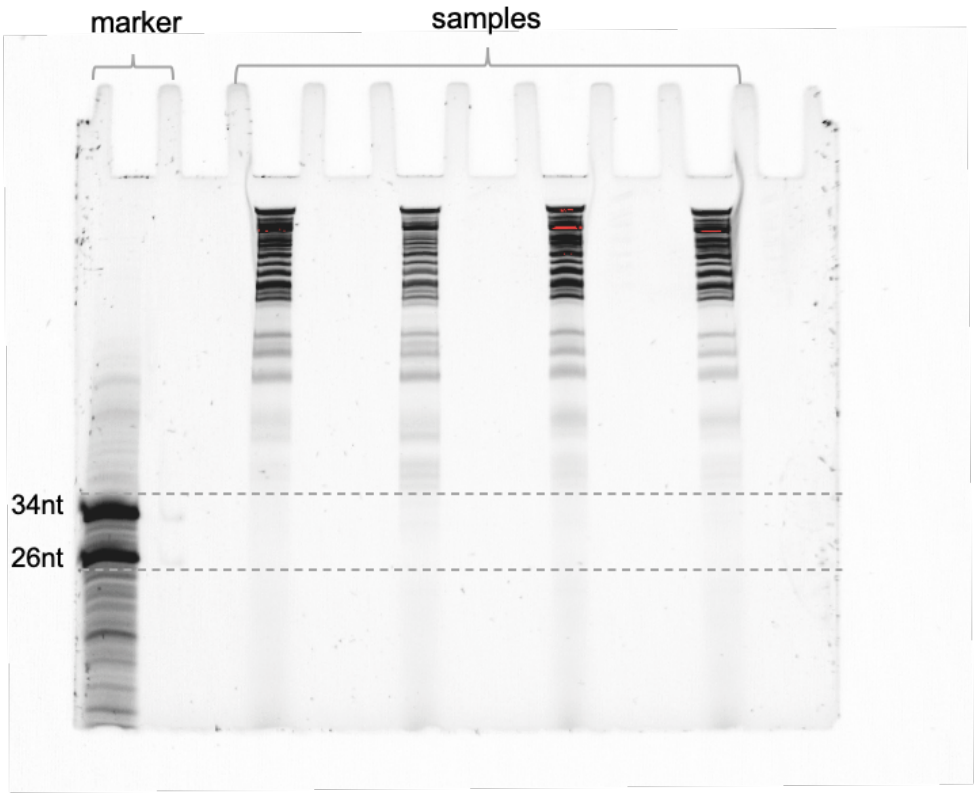

Figure 3. 15% Urea-PAGE gel electrophoresis for RPF size selection with maker (left) and four loaded RNase I digested samples. Horizontal dashed gray lines represent the area for size selection across four footprint samples.

2.6 RPF gel extraction

1. Cut out required gel section representing the RPFs. This can be done by printing an actual size image of the gel and marking the regions to be excised. The gel is then placed on a glass plate over the printed image and a scalpel is used to excise the region of interest. Alternatively, the gel can be placed under blue LED light and sliced using RPF markers as a guide.
2. Place each gel slice in a RNase-free 1.5 mL tube.
3. Add 500 µL RNA elution buffer.
4. Shake overnight on shaker at room temperature to extract RNA from slices at 1000 rpm.
5. Briefly centrifuge and collect the liquid, transfer to a new RNase-free 1.5 mL tube.
6. Add 500 µL of ice-cold isopropanol
7. Add 1.5 µL glycoblue.
8. Briefly vortex.

9. Precipitate in -20°C overnight or -80°C for 1 hour.
10. Thaw on ice and pellet by centrifugation for 30 minutes at 20,000g and 4°C.
11. Remove supernatant and wash pellet in 1 mL cold 80% ethanol.
12. Centrifuge again.
13. Carefully pipette all liquid from the tube and place it sideways on a rack in a chemical hood. Leave for 5 minutes to air dry remaining ethanol.
14. Resuspend in 4 µL ultra-pure water.
15. Store at -20°C overnight or -80°C indefinitely.

3.1 Enzymatic pre-adenylation of linker using Mth RNA ligase

- Linker oligonucleotides requires enzymatic pre-adenylation prior to ligation to RNA fragments with T4 Rnl2(tr) K227Q, as this enzyme can only ligate adenylated linkers.

1. In a PCR tube, add the following:
    a. 1.2 µL linker oligonucleotide at 100 µM
    b. 2 µL 10X 5' DNA adenylation action buffer
    c. 2 µL 1 mM ATP
    d. 12.8 µL water
    e. 2 µL Mth RNA Ligase
2. Incubate for 1 hour at 65°C, then heat-inactivate the enzyme by incubation at 85°C for 5 minutes.
3. Add 30 µL water to the sample and then purify using the Oligo Clean & Concentrator kit according to the manufacturer's instructions, except elute in 6 µL nuclease-free water.
4. Store at −20°C and avoid repeated freeze–thaw.

3.2 Dephosphorylation and Linker Ligation

- The purpose of dephosphorylation with T4 PNK is to "heal" the 3' ends of RPFs as nuclease digestion introduces a 3' cyclic phosphate which is incompatible for 3' ligation to the pre-adenylated barcoded linker.
- As a positive control for dephosphorylation and linker ligation, use the 26 and 34 nucleotide RNA markers. These markers (like RPFs) require dephosphorylation prior to linker ligation and are thus suitable controls. See Figure 4B for positive control gel.
- To aid understanding of how this library preparation works, a molecular schematic of library preparation is provided in Figure 1.
- To avoid size-selection gel for ligation products, McGlincy and Ingolia 2017 use yeast 5' deadenylase and RecJ exonuclease to digest un-ligated linkers from the sample,

meaning no separation of ligated products from un-ligated-linkers via size selection is required. However, in this protocol, a size-selection gel is preferred as it allows monitoring of efficiency of the ligations.

- An example of a size selection gel to separate un-ligated linkers from ligated RPFs is provided in Figure 4A.

1. Set up each phosphorylation reaction as follows (for positive controls with RPF markers, substitute RNA sample with 0.5 µL RPF markers (100 µM) and 3 µL ultra-pure water):

Component	Volume (µL)	Final
RNA sample	3.5	NA
T4 PNK buffer (10X)	0.5	1X
T4 PNK (10 U/µL)	0.5	5 U
SUPERase•In	0.5	10 U
Total volume	5	

2. Incubate for 1 hour at 37°C
3. Prepare the linker ligation reaction by adding components as follows directly to the dephosphorylation reaction, bringing the total volume to 10 µL.

Component	Volume (µL)	Final
Dephosphorylated RNA	5	
50% w/v PEG-8000	3.5	17.5%
10X T4 RNA ligase buffer	0.5	1X
Pre-adenylated linker (20 µM)	0.5	1 µM
T4 Rnl2(tr) K227Q (200 U/µL)	0.5	100 U
Total volume	10	

4. Incubate the ligation reaction for 3 hours at 22°C (RT).
5. Run a 15% PAGE gel and size select for ligated products by following the previous RPF size selection gel and overnight extraction protocol, eluting ligated products with 11 µL ultra-pure water. In addition to ligation samples, load the ssRNA low range ladder (NEB) as a guide for size selection. Ligated products will be present as a band between the 50-80 nt ssRNA markers.

3.3 Pooling and purification of ligations

- At this point, each individual sample contains a unique barcode within its linker. This allows for pooling of up to 8 individual samples with separate barcodes. This is advantageous as it reduces sample to sample variability during cDNA library preparation. Other advantages include increased material allows product bands to be more easily visualized on polyacrylamide gels.
- If doing a gel purification step of ligations, you may pool individual samples before loading onto gel.

1. Each sample to be pooled contributes 11 µL volume. Calculate the total sample volume: #samples x 11 µL.
2. If sample is <50 µL, bring total volume to 50 µL with ultra-pure water.
3. To the sample, add twice the volume of oligo binding buffer.
4. Add ethanol equal to 8 times the original total sample volume. eg, six samples (66 µL) use 528 µL ethanol.
5. Load samples onto the Zymo spin-column nested in a collection tube. Load no more than 800 µL at once (8 samples max).
6. Centrifuge the column for 30 seconds at 12,000 x g and discard the flow-through.
7. Add 750 µL DNA wash buffer and spin at 12,000 x g for 30 seconds.
8. Centrifuge again (with no wash) for 1min at max speed to remove any residual wash buffer.
9. Transfer the Zymo-Spin column into a 1.5 mL RNase-free tube and add 10 µL water.
10. Centrifuge for 30 seconds at 12,000 x g and recover RNA in the eluted liquid.
11. Store overnight at -20°C or indefinitely at -80°C.

3.4 Size selection of ligation products

- The ligated RPFs are separated from the linker and non-ligated RNA fragments using a size selection gel.
- An example of PAGE separation of ligation products is visualized in Figure 4A, including RPF marker ligation in Figure 4B.
- RPFs (~28 nt) ligated to 33 nt linker produces a ligated product ~61 nt in size.

1. Carry out excision of ligation products as described previously for RPF size selection.
2. Isolate RPFs from gel slices as described previously, except resuspend RNA in 10 µL ultra-pure water.

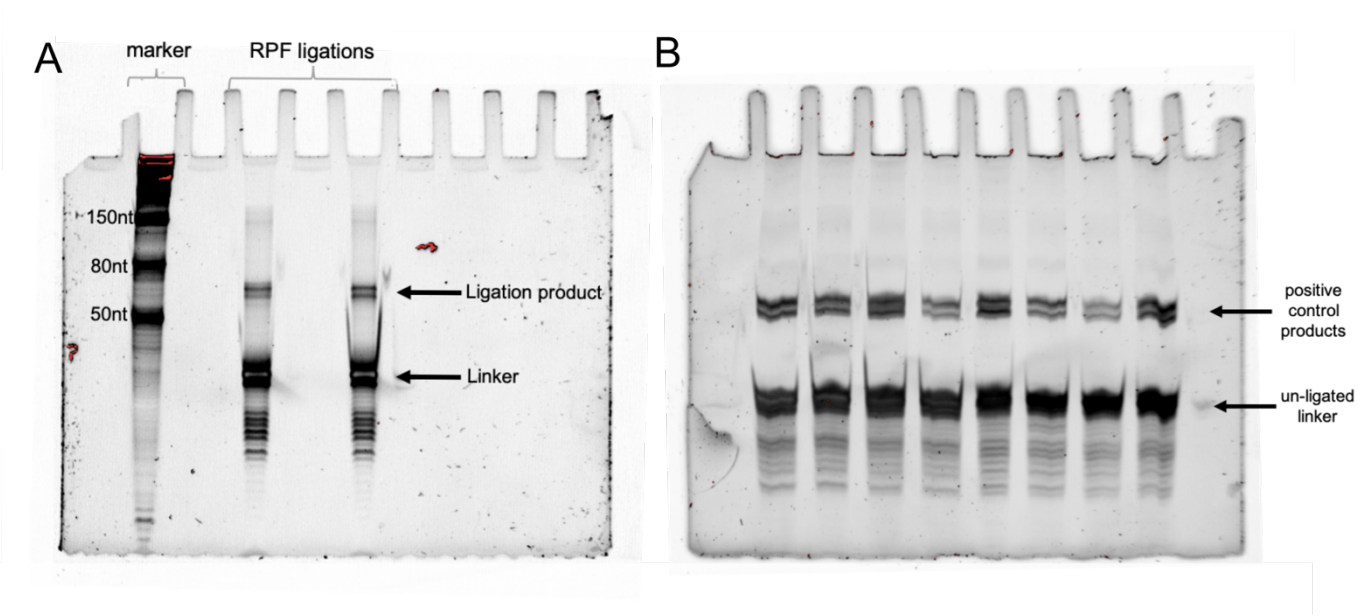

Figure 4. Polyacrylamide gel electrophoresis separation of ligation products. A. PAGE-gel separation of RPF samples with ssRNA marker. B. PAGE gel separation of positive control ligation products.

3.5 Reverse Transcription

- As a marker here, use the NEB Low Range ssRNA ladder.
- The expected product size is ~111 nt.
- Temperatures have been optimized as described in McGlincy and Ingolia 2017 to reduce non-templated addition of nucleotides to cDNA products.

1. Add 2 µL reverse transcription primer (NI-802) at 1.25 µM to all RNA samples, bringing sample volume to 12 µL.
2. Denature for 5 min at 65ºC in a PCR machine and then place on ice.
3. Cool the PCR machine to 50ºC.
4. Set up the following reaction (if using Superscript, replace 1 µL Protoscript II with 1 µL Superscript III 200U/µL):

Component	Volume (µL)	Final
RNA sample and primer	12	NA
5X Protoscript II buffer	4	1X
dNTPs	1	1
0.1M DTT	1	5 mM
SUPERase•In (20U/ µL)	1	20 U
Protoscript II (200U/ µL)	1	20 U

5. Incubate for 30min at 50ºC (Protoscript II) or 55ºC (Superscript III).
6. Hydrolyze the RNA template by adding 2.2 µL 1M NaOH to each tube and incubate at 70ºC for 20 minutes.
7. Add 28 µL water, bringing total volume to 50 µL.
8. Purify sample using oligo clean and concentrator kit, except elute in 8 µL water.
9. Proceed to RT gel for size selection of RT products.

3.6 Size selection of RT products

- Similar to previous PAGE gels, but 7.5%, not 15%.

- This gel separates the un-extended primer from the RT product see Figure 5 for example.

1. Prepare 7.5% PAGE gel as described in solutions/reagents.
2. Once polymerized, pre-run at 15mA (300V) for 20mins in 1X TBE.
3. Denature samples 90 seconds at 80^{o}C.
4. Each sample: 8 µL cDNA + 4 µL 3X loading dye.
5. Use NEB ssRNA low range ladder as marker.
6. Run gel for 40 minutes.
7. Stain as previously described and visualize on GelDoc.
8. Cut out slice representing cDNA products and elute in 750 µL of DNA extraction buffer.
9. Place on shaker overnight at RT.
10. Transfer elution to 1.5 mL tube.
11. Add one volume of isopropanol and 1.5uL glycoblue.
12. Vortex briefly.
13. Precipitate for one hour in -80^{o}C and resuspend in 12 µL ultra-pure water.
14. Thaw on ice and centrifuge at 20,000 x g for 20 minutes.
15. Discard supernatant and add 1 mL of ice-cold 80% ethanol.
16. Centrifuge again and discard supernatant.
17. Resuspend in 12 µL ultra-pure water.
18. Store cDNA products at -20^{o}C or proceed directly to circularization reaction.

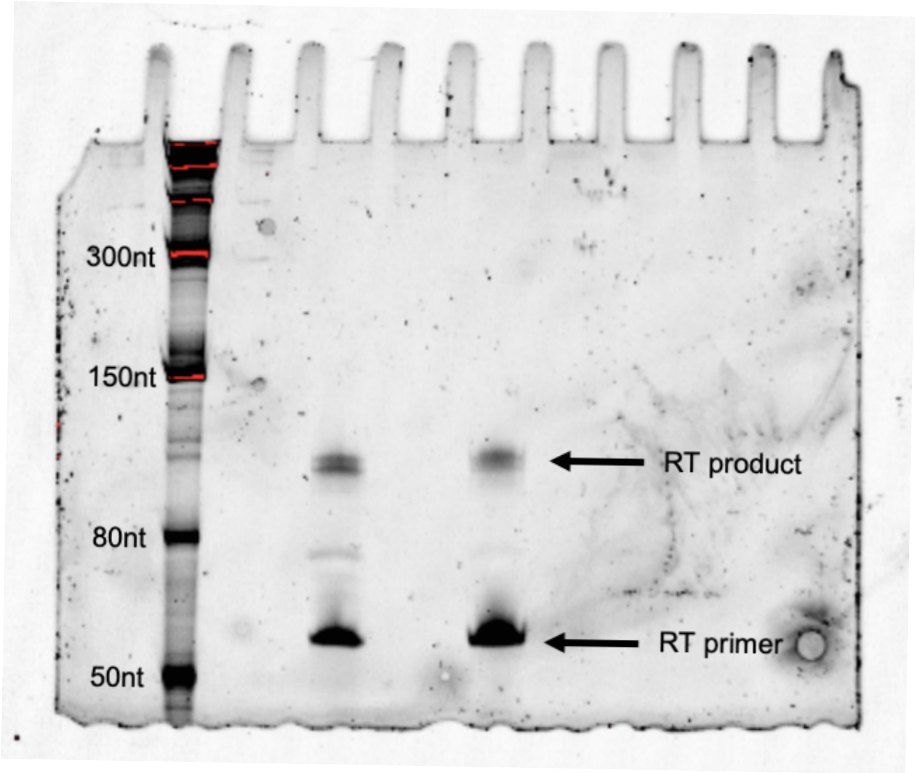

Figure 5. 7.5% Urea-PAGE gel separation of reverse transcription product and primer. NEB ssRNA ladder is used as ladder.

3.7 cDNA Circularization

Component	Volume (µL)	Final
cDNA	12	NA
CircLigase II 10X buffer	2	1 X
5 M Betaine	4	1 M
50 mM $MnCl_2$	1	2.5 mM
CircLigase II (100U/µL)	1	100 U

1. Transfer 12 µL RT product to a PCR tube.
2. Prepare circularization reactions as shown in table.
3. Incubate for 1 hour at 60°C in a thermal cycler.
4. Heat inactivates the enzyme at 80°C for 10 minutes.
5. The circularization products may be stored at -20°C.
6. At this point, proceed to rRNA removal via subtractive hybridization.

3.8 rRNA depletion

- Table below describes rRNA depletion oligos. These are DNA oligos which are biotinylated at the 5'end and purified via HPLC.

name	sequence	purification method
km_rRNA#1	/5Biosg/AAGGGTGCATCATCGACCGATCCTG	HPLC
km_rRNA#2	/5Biosg/GTTTCTTTACTTATTCAATTAAGCGGA	HPLC
km_rRNA#3	/5Biosg/TAAAGAATGGTACAGCTATAAATATT	HPLC
km_rRNA#4	/5Biosg/GCTCGAATATATTAGCATGGAATAATGGA	HPLC
km_rRNA#5	/5Biosg/TATAGAAGGATACGAATAAGGCGTC	HPLC
km_rRNA#6	/5Biosg/TTTCCACGTTCTAGCATTCAAAGTCCT	HPLC

1. To make 100 µL of subtractive oligo mix:
   - Prepare a 100 µM stock of each of the 6 oligos. Store at -20°C.
   - Add 10 µL of each oligo to a tube with 40 µL water.
   - Final concentration for oligos will be 60 µM, with 10 µM of each oligo.
2. Combine in a PCR tube:
   a. 5 µL circularization reaction (use 5 µL of previously prepared 20 µL circularization reaction)
   b. 1 µL subtractive oligo mix
   c. 1 µL 20X SSC
   d. 3 µL water in a PCR tube
3. Place the PCR tube in a thermal cycler and denature for 90 seconds at 100°C. Then anneal at by reducing the temperature by 0.1°C s^{-1} to 37°C (i.e., reduce to 37°C by 0.1°C per second). Incubate for 15 minutes at 37°C.
4. Vortex the streptavidin C1 Dynabeads to resuspend beads in storage solution.
5. Transfer the total required volume of beads to a clean tube in a magnetic stand: use 25 µL beads per reaction, plus an additional 12.5 µL beads (37.5 µL for single depletion reaction).
6. Leave for one minute and carefully aspirate all the liquid.
7. Remove from rack and add 1 volume 1X bind/wash buffer.
8. Repeat twice.
9. Place the beads on a magnetic rack for 1 minute to isolate beads and aspirate the final wash solution.

10. Resuspend in 0.4 volumes of 2X bind/wash buffer (15 µL for single sample).
11. Transfer one 10 µL aliquot of beads per subtraction reaction into another tube.
12. Place bead aliquots in the T-shaker at 37°C and equilibrate for 15 minutes.
13. Transfer 10 µL of subtraction reaction directly from the PCR tube in the thermal cycler (step 2) to a bead aliquot in the T-shaker. Incubate for 15 minutes at 37°C with mixing at 1,000 rpm.
14. Place tubes on a magnetic rack and isolate beads for 1 minute. Recover 17.5 µL eluate from the depletion and transfer to the new tube.
15. Add 1.5 µL glycoblue, 6 µL of 5 M NaCl and 74 µL water, followed by 150 µL isopropanol. Leave for 30 minutes on ice.
16. Centrifuge at 20,000 x g for 30 mins.
17. Remove supernatant and wash pellet with 1 mL ice-cold 80% ethanol.
18. Centrifuge and remove supernatant.
19. Air dry for a few minutes in chemical hood until residual ethanol evaporates.
20. Resuspend in 10 µL water.

3.8 Final library PCRs

- For the final library PCR, Phusion high fidelity polymerase is used.
- The aim of initial trial PCRs is to determine the optimal cycle number, that is, the lowest PCR cycle to reduce PCR duplicates.
- For each PCR, it is important to have a particular ratio of template to volume, ideally no more than 5%. This strategy ensures that the primer concentration remains greater than 10 times the concentration of extended PCR product, and thus that in later cycles primer annealing and extension predominates over re-annealing of the two template strands.
- Once the optimal cycle number has been determined (see Figure 6A for trial PCR gel), one can increase the scale of PCR reaction and size select the final library PCR products (see Figure 6B for optimum cycle PCR).
- The forward primer is NI-798. Reverse primers are NI-799, 822–826, each include a different barcode to demultiplex samples.

Set up trial PCR reactions as follows:

- For each sample, prepare a 10 µL PCR reaction with 0.5 µL circularized template.
- Trial cycles: 8, 10, 12, 14 (scale as needed).

Component	10 µL reaction	50 µL reaction	Final concentration
5X Phusion HF buffer	2	10	1X
dNTPs 10 mM each	0.2	1	0.2 mM each
10µM Forward Primer (NI-798)	0.5	2.5	500 nM
10µM Reverse Primer	0.5	2.5	500 nM
Circularized cDNA template	0.5	2.5	-
Ultra-pure water	6.2	31	-
Phusion polymerase (2U/µL)	0.1	0.5	1 U

Stage	Temperature	Time and Cycles	
Initial Denaturation	98oC	30 Seconds	
Denaturation	98oC	10 Seconds	7-14 cycles
Annealing	65oC	10 Seconds	
Extension	72oC	5 Seconds	
Final Extension	72oC	5 Minutes	
Hold	4oC	Until removed.	

The forward primer is NI-798. Reverse primers are NI-799, 822–826

Once the optimal desired PCR cycle has been determined, prepare a 50 µL reaction with 2.5 µL template.

3.9 Final PCR gel

1. Prepare 8% PAGE-gel solution in Solutions/Buffers section.
2. Assemble gel tank and add 1X TBE.
3. Insert polymerized gel into tank and gel wells as described previously.
4. As a marker, use 1 µL of 100 bp ladder (dsDNA).
5. To a 50 µL PCR reaction, add 10 µL 6X DNA loading dye (for trial PCRs, add 2 µL of 6X DNA loading dye to 10 µL trial PCR).
6. Load gel by dividing the 50 µL reaction into 3 separate wells.
7. Run gel for 40 minutes at 180 V.
8. Stain gel for 3 minutes with 1X SYBR gold in 20 mL 1X TBE.

3.10 Library DNA extraction

1. Cut product bands and continue with an overnight gel extraction by adding 750 µL DNA gel extraction buffer.
2. Leave on a shaker overnight.
3. Spin down tubes briefly and transfer the buffer to fresh 1.5 mL tube.
4. Add 1.5 µL glycoblue and 750 µL isopropanol. Vortex briefly.
5. Spin for 30 minutes at 20,000 x g for 30 minutes at 4^{o}C and carefully discard supernatant.
6. Add 750 µL ice-cold 80% ethanol and spin again for 15 minutes and discard the supernatant.
7. Let the pellet air dry for 5 minutes in a vacuum hood.
8. Resuspend pellet in 15 µL ultra-pure water.
9. Store at -20^{o}C.

Once products have been size selected and eluted, measure library concentration with Qubit (HS-dsDNA assay) or equivalent. ng/µL may be converted to nM using the following formula:

DNA concentration (nM) = (ng/µL)/(660 g/mol x library size) x 10^{6}

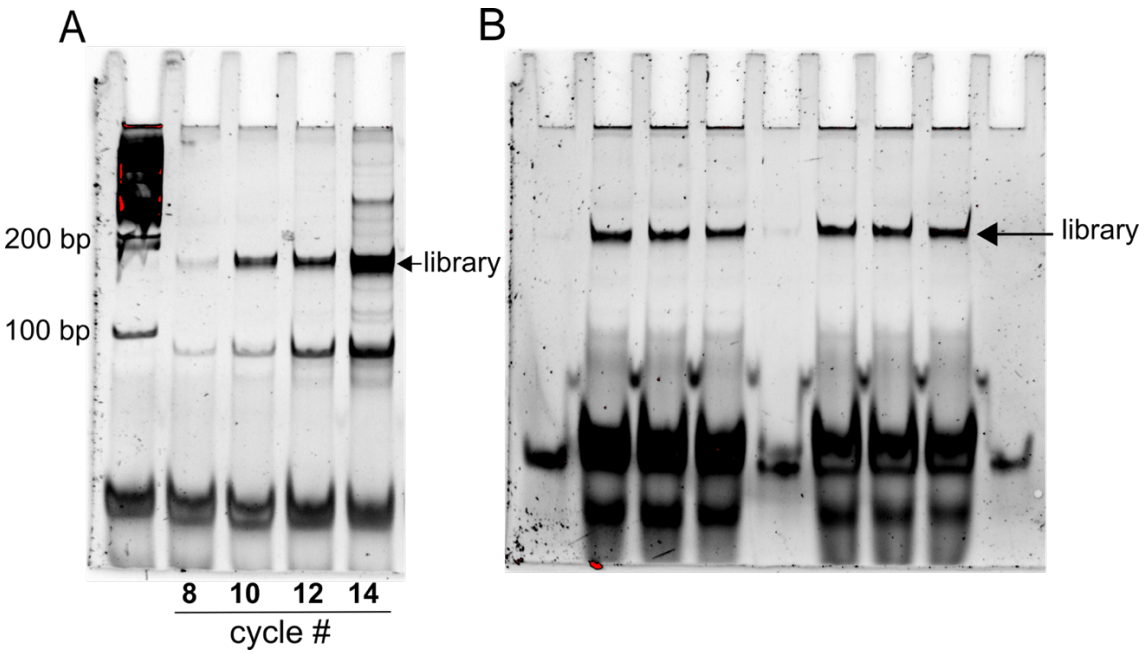

Figure 6. Final cDNA library PCR. A. Trial PCR with 8, 10, 12 and 14 cycles, leftmost lane contains 100 bp dsDNA ladder. 100 bp dsDNA used as ladder. B. PCR amplification with 8 cycles divided into 3 wells per sample.

3.10 QC of Libraries

- Libraries for next generation sequencing are often loaded onto an Agilent Bioanalyzer to determine correct library size, prior to loading onto a flow cell.
- For our experiments, libraries are sequenced by the Genomics & Cell Characterization Core Facility (GC3F), University of Oregon on an Illumina HiSeq4000.
- Figure 7 shows an example of Agilent Bioanalyzer of library size distribution for a Ribo-Seq library.

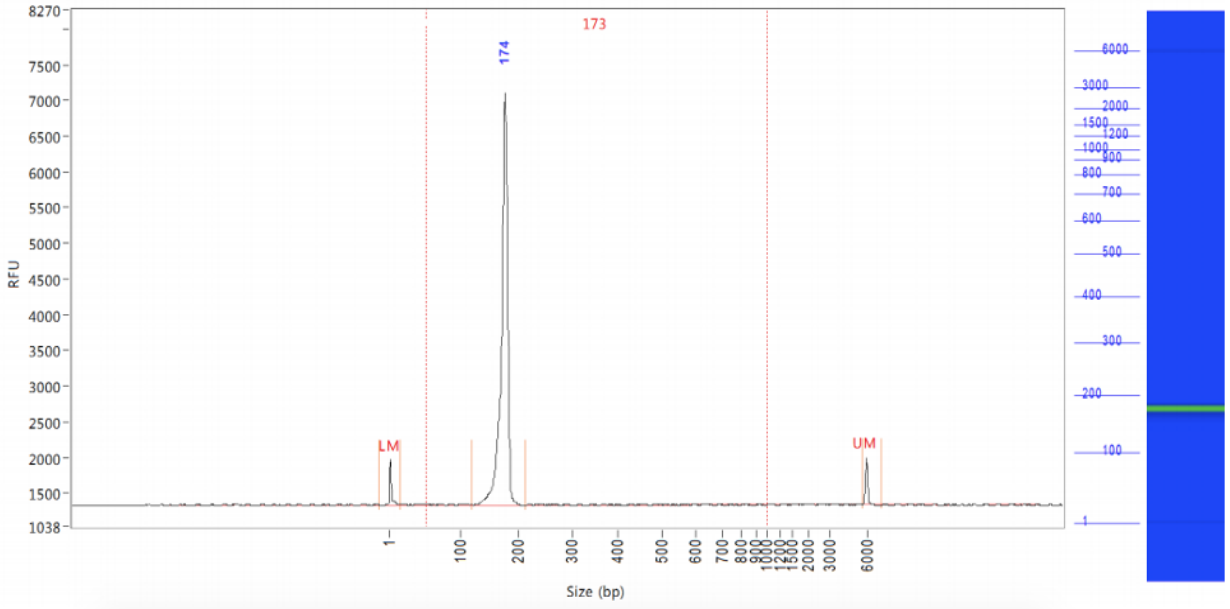

Figure 7. Bioanalyzer distribution of library size including lower and upper bioanalyzer markers. Ribosome profiling library is present at ~174 size (bp).

Bioinformatic Analysis

- For reference genome, *K. marxianus* DMKU3-1042 (Lertwattanasakul *et al.*, 2015) is used. This includes the recently reannotated the DMKU3-1042 genome using ribosome profiling data which included over 150 novel genes and multiple gene annotation corrections (Bioarchive citation).
- All analysis of ribosome profiling and RNA-seq data is performed with Ubuntu server.
- The following software for processing the ribosome profiling data is provided before and usually installed via the conda environment package manager (https://conda.io/) (Anaconda software distribution, *Conda*).
    - FastQC to determine basic quality metrics such as read lengths, sequencing quality, GC content and more (Simon Andrews, Babraham institute).
    - Cutadapt is used to demultiplex and trim adapter sequences, specifically the Illumina adapters (Martin, 2011).
    - Bowtie is an aligner used to remove rRNA contaminants from the library followed by alignment to the *K. marxianus* strain DMKU3-1042 genome (Langmead *et al.*, 2009).
    - Samtools converts the alignment SAM file to a sorted and indexed BAM alignment file (Li *et al.*, 2009).
    - HT-seq counts the number of RPFs aligned to each gene using a GTF file (Anders, Pyl and Huber, 2015).
        - From HT-SEQ, a count file is generated which can be used to determine sample correlations and for differential gene expression analysis.
    - bedtools coverts the BAM file to a forward and reverse strand genome alignment coverage file (Quinlan and Hall, 2010).
    - bedGraphToBigWig converts the coverage files to BigWig format which can be loaded onto a genome browser such as GWIPs-Viz (Michel *et al.*, 2014).
    - For differential gene expression analysis, use DESeq2 (Love, Huber and Anders, 2014).
- Below, an example of commands used to process data via a Linux OS (Ubuntu) server is provided. Using these commands, one should be able to clip sequencing adapters, remove other rRNA contaminants and align sequences to the genome. A genome alignment is a precursor to generation of a counts table, which details the number of RPFs per gene for a specific condition, these tables are commonly used to determine reproducibility of replicates and for differential expression analysis. In

addition, the genome alignment can be used to generate a bigWig file, which can be displayed as a private track on GWIPS-Viz.

- Figure 8 displays structure of a single read including UMIs, RPF, barcode and common adaptor. These reads are typically provided from a sequencing centre, which with clip Illumina adapters before providing the resulting reads to a customer.

Demultiplex and clip adapter, a fasta file describing barcodes is required (example below).

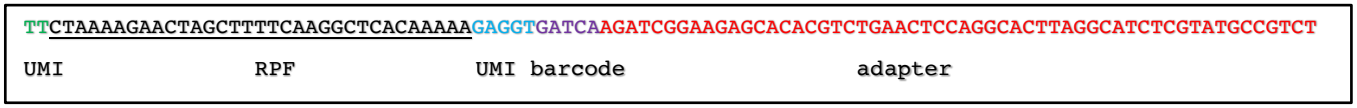

Figure 8. Structure of a single FASTQ sequence from HiSeq4000, generated with the ribosome profiling protocol described. During data processing, sequences upstream and downstream of the ribosome protected fragment “RPF” are clipped to allow alignment to the genome or transcriptome.

cutadapt -e 0.15 --no-indels -g file:barcodes.fasta -o "trimmed-{name}.fastq" test.fastq

>CTAGA_barcode
CTAGAAGATCGGAAGAGCACACGTCTGAA
>CGTAA_barcode
CGTAAAGATCGGAAGAGCACACGTCTGAA

Once demultiplexed, clip barcode and UMI.
cutadapt -u 2 -u -5 -m 25 -M 32 -j 12 -o example.fastq example.fastq

Remove remaining rRNA contaminants
bowtie -p 8 --un $1.temp path/to/rRNA/index $1 > non_coding.fastq

Align remaining reads to the genome
bowtie -p 2 -n 2 -m 1 -S path/to/genome/index example.fastq > genome_aligned.fastq

Convert sam to bam
samtools view -@ 32 -Sb example.sam > example.bam

Sort bam file
samtools sort -O bam example.bam > example_sorted.bam

Generate an index file

```
samtools index example_sorted.bam
```

Generate a counts table of mapped reads per gene (need for differential expression analysis)

```
htseq-count --format bam example_sorted.bam path/to/gtf > example_counts.tsv
```

Create coverage files. Here, split the forward and reverse strand reads. The chrominfo.tsv is a file describing chromosome names and length (example below).

```
bedtools genomecov -ibam $1_sorted.bam -strand + -g chromInfo.tsv -bg > example_FR.cov
bedtools genomecov -ibam $1_sorted.bam -strand - -g chromInfo.tsv -bg > example_RV.cov
```

```
chrI    1745387
chrII   1711476
chrIII  1588169
chrIV   1421472
chrV    1353011
chrVI   1197921
chrVII  963005
chrVIII 939718
chrM    46308
```

Create bigWigs for genome browser

```
bedGraphToBigWig example_FR.cov chromInfo.txt example_FR.bigWig
bedGraphToBigWig example_RV.cov chromInfo.txt example_RV.bigWig
```

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Anders, S., Pyl, P. T. and Huber, W. (2015) 'HTSeq--a Python framework to work with high-throughput sequencing data', *Bioinformatics (Oxford, England)*. 2014/09/25, 31(2), pp. 166–169. doi: 10.1093/bioinformatics/btu638.

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