

Ribosome profiling and RNA-seq protocol

Citation: Subtelny et al., *Nature* (2014)

This protocol is an updated version of the protocol posted by Huili Guo (8/8/10), which detailed the approach she used for ribosome profiling and mRNA-seq in Guo et al., *Nature* 466: 835–840 (2010); available at <http://bartellab.wi.mit.edu/protocols.html>. In this version of the protocol the sample collection section has been expanded, minor changes were made to library preparation, and a ribosomal RNA subtraction step has been added for ribosome profiling samples. Much of this protocol is as it appears in Huili's original protocol.

Section 1: Harvesting samples for parallel ribosome profiling and RNA-seq

1.1a Harvesting cultured mammalian cells

Cells grown as a monolayer can generally be harvested as described below.

Day(s) before:

Grow cells as appropriate for the cell type and/or growth condition being used. Generally actively dividing cells should be split the day prior to harvesting such that the cells will be ~70% confluent when harvested.

Day of harvesting:

Arrest translation with cycloheximide (CHX)

1. Add cycloheximide to the cell culture media (100 µg/mL final)
2. Incubate cells at 37 C for 10 min

Harvest cells

1. Transport cells to cold room
2. Remove media on ice
3. Wash 2x with ice-cold PBS (+100 µg/mL CHX)
4. Add 1 mL lysis buffer [+ 2 mM DTT, 100 µg/mL CHX, 500 U/mL RNasin Plus (Promega), and cOmplete EDTA-free protease inhibitor (1 mini tablet per 10 mL; Roche)] to a 15 cm dish, rotating to coat evenly
5. Scrape the dish and transfer into an eppendorf tube
6. Shear 4x with a 26-gauge needle, very gently
7. Centrifuge at 1300 x g for 10 min
8. Transfer supernatant to a fresh tube

Generally at this point the lysate is flash frozen in liquid nitrogen, after which it can be stored at –80 C. When ready to proceed, thaw frozen samples on ice in the cold room.

1.1b Harvesting zebrafish embryos

Embryos from blastula to gastrula stages have been harvested as described below.

Day of harvesting:

Harvest embryos

1. When embryos are at the appropriate stage, remove chorion (either manually or with pronase according to standard protocols)
2. Wash 2x with room temperature E3 medium
3. Add cycloheximide to E3 medium (100 µg/mL)
4. Incubate embryos at room temperature for 5 min
5. Using a p200 pipetman, transfer the embryos into an eppendorf tube on ice
6. Immediately add lysis buffer [+ 2 mM DTT, 100 µg/mL CHX, 500 U/mL RNasin Plus, and cOmplete EDTA-free protease inhibitor (1 mini tablet per 10 mL); for every embryo being harvested add 1 µL lysis buffer]
7. Flash freeze in liquid nitrogen, store at –80 C.
8. When ready to proceed, thaw samples on ice in cold room
9. Perform steps 6–8 of 1.1a (Harvest cells)

Note: unlike in 1.1a, I do not freeze these samples in liquid nitrogen at this point so only proceed past step 7 if you are ready to work with the lysate.

1.1c Harvesting frog embryos

Embryos from blastula to gastrula stages have been harvested as described below

Day of harvesting:

Harvest embryos

1. When embryos are at the appropriate stage, wash 2x with room temperature 0.1X MBS
2. Transfer embryos to an eppendorf tube using a wide-bore transfer pipette
3. Remove buffer until it is at the level of the embryos
4. Add lysis buffer [+ 2 mM DTT, 100 µg/mL CHX, 500 U/mL RNasin Plus, and cOmplete EDTA-free protease inhibitor (1 mini tablet per 10 mL); for every embryo being harvested add 5 µL lysis buffer]
5. Flash freeze in liquid nitrogen, store at –80 C.
6. Proceed from step 8 of 1.1b (Harvest embryos)

Note: unlike in 1.1a, I do not freeze these samples in liquid nitrogen at this point so only proceed past step 5 if you are ready to work with the lysate.

1.1d Harvesting yeast

Both *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* have been harvested as described below

Day of harvesting:

Harvest yeast

1. Assemble a filtration apparatus
2. When yeast have reached desired growth state, pre-wet a filter with growth media

3. With vacuum on pour in culture and allow media to drain
4. Immediately before the last drops of media are drained through, scrape the cell paste from the filter directly into liquid nitrogen
5. Transfer frozen cell paste to -80 C (can be stored at this point)
6. Pre-chill a mortar and pestle in a dry ice–ethanol bath
7. Pour liquid nitrogen into the mortar, allowing it to chill until doing so does not result in vigorous bubbling
8. Transfer frozen cell paste to mortar
9. Grind to a very fine powder, refilling with liquid nitrogen as needed
10. Transfer the powdered sample back to -80 C
11. When ready to proceed, thaw samples on ice in cold room
12. Perform steps 6–8 of 1.1a (Harvest cells)

Note: The amount of lysis buffer used will depend on how large of a culture you harvest. For a 1 L culture grown to mid-log phase we used 5 mL lysis buffer.

Note: Unlike in 1.1a, I do not freeze these samples in liquid nitrogen at this point so only proceed past step 10 if you are ready to work with the lysate.

1.1e Harvesting primary mammalian tissue

Mouse liver has been harvested as described below

Day of harvesting:

Harvest tissue

1. Excise desired tissue
2. Rinse exterior of tissue 2x in ice-cold PBS
3. Flash freeze in liquid nitrogen, store at -80 C
4. Proceed from step 6 of 1.1d (Harvest cells)

Note: The amount of lysis buffer used will depend on the tissue being harvested. For most of a mouse liver we used 5 mL lysis buffer.

Note: Once this sample is processed to the point of being a lysate I do not freeze it, so only proceed to this point if you are ready to work with the lysate.

Buffers

Lysis buffer

- 10 mM Tris-HCl (pH 7.4)
- 5 mM $MgCl_2$
- 100 mM KCl
- 1% Triton X-100
- Filter-sterilize and store at 4 C

Section 2: RNA-seq – RNA isolation and fragmentation

2.1 RNA extraction

1. Thaw a lysate prepared as described in section 1
2. Extract RNA from the lysate with TRI reagent (according to the manufacturer's protocol)

2.2a Poly(A) selection

1. Perform poly(A) selection with oligo(dT)-Dynabeads (according to the manufacturer's protocol; Life technologies)
2. Precipitate poly(A)-selected RNA

2.2b rRNA depletion

For samples that tend to have short poly(A) tails, rRNA depletion may be preferable instead of poly(A) selection. We use rRNA depletion for our yeast samples.

1. Perform rRNA depletion with product of choice (for *S. cerevisiae* we have used both Ribo-Zero yeast from Epicentre and RiboMinus yeast from Life technologies, for *S. pombe* we used RiboMinus yeast; use according to manufacturer's protocol)
2. Precipitate rRNA-depleted RNA

2.3 RNA fragmentation by partial alkaline hydrolysis

Resuspend RNA in 10 μ L water.

	Vol (μ L)
RNA	10
2x fragmentation buffer	10
Total	20

Incubate at 95 C for ~20 min.

After incubation, stop the hydrolysis as follows:

1. Chill sample on ice, spin down, return to ice
2. Add 280 μ L 0.3 M NaOAc (pH 5.2)
3. Add 4 μ L GlycoBlue and 750 μ L 100% EtOH
4. Precipitate
5. Proceed to library generation (section 4)

Buffers

2x fragmentation buffer

- 2 mM EDTA
- 12 mM Na₂CO₃
- 88 mM NaHCO₃
- Final pH should be ~9.3

Section 3: Ribosome profiling – ribosome footprinting

3.1 Gradient preparation

Gradients are prepared about an hour before needed using a Gradient Master (Biocomp).

1. Allow 10% and 50% gradient buffers to come to room temperature
2. Add DTT (2mM), CHX (100 µg/mL), and SUPERase•In (20 U/mL; Ambion) to both gradient buffer solutions.
3. Pour and form gradients according to the manufacturer's protocol
4. Move to cold room and allow to chill for at least 45 min prior to use

3.2 Nuclease digestion

To lysate (either freshly prepared or thawed)

1. Add RNase I (0.2–1.0 U/µL lysate, Ambion)
2. Incubate at room temperature for 30 minutes, with gentle shaking
3. Place tube on ice and load onto a gradient as soon as possible

Note: For all new cell or sample types, it is good to first run a series of RNase I concentrations to determine the amount needed for optimal digestion, we generally first run gradients for 0, 0.2, 0.5, or 1.0 U/µL to determine this.

3.3 Centrifugation

Pre-chill centrifuge to 4 C prior to use.

1. Layer the desired amount of digested extract onto the chilled gradient from 3.1
2. Weigh and balance all gradients
3. Centrifuge in SW-41 Ti rotor, 36,000 rpm for 2 hr at 4 C, acceleration mark '1', deceleration mark '7'
4. After centrifugation, move buckets to cold room

3.4 Fraction collection

Gradients are fractionated using the Piston Gradient Fractionator (Biocomp). Turn on all components about 30 min prior to use. We fractionate gradients according to the manufacturer's instructions (Speed: 0.3 mm/sec). We collect a fraction that contains only the entire monosome peak based on the visual offset displaying what portion of the gradient is being collected.

3.5 Release/filtration

Note: It is unclear exactly how much this step helps reduce rRNA contamination, this step can be considered optional. See Huili's protocol for a more thorough description of the logic of this step.

1. Load monosome fraction onto an Ultra-4 centrifugal filter with an Ultracel-100 membrane (Amicon)
2. Centrifuge at 1900 x g for 60 min at 4 C to concentrate sample to ~100 µL
3. Add 1220 µL ice-cold release buffer (+ 2 mM DTT and 20 U/mL SUPERase•In) to the retentate
4. Incubate on ice for 10 min

5. Transfer filter unit to a new 15-mL falcon tube and centrifuge at 1900 x g for 30 min at 4 C
6. Separate the release filtrate into two equal-volumes and proceed to proteinase K digest

3.6 Proteinase K/SDS digest

To each release filtrate aliquot:

1. Add x μ L 10% SDS (to final 1%)
2. Add y μ L proteinase K (to final 200 μ g/mL; Roche)
3. Mix
4. Incubate at 42 C for 30 min

3.7 Phenol/chloroform extraction

To each proteinase K-digested fraction:

1. Add equal volume acid phenol, pH 4.5, and phenol/chloroform extract
2. To final aqueous phase, add 0.1x vol NaOAc (3 M, pH 5.2), 4 μ L GlycoBlue, and 2.5x vol 100% EtOH
3. Precipitate
4. Proceed to library generation (section 4)

Buffers

10% or 50% gradient buffer for sucrose gradients

20 mM HEPES-KOH (pH 7.4)
5 mM MgCl₂
100 mM KCl
Either 10% or 50% sucrose (w/v)
Filter-sterilize and store at 4 C

Release buffer

20 mM HEPES-KOH (pH 7.4)
100 mM KCl
1 mM EDTA

Section 4: Library generation

This part of the protocol is essentially unchanged from Huili's original protocol. There are a few minor changes throughout, including using a different 5' adaptor and 3' PCR primer.

4.1 Size selection

Ribosome profiling:

Ribosome-protected fragments (RPFs) are usually ~30 nt in length. Adding 27 and 33 nt pCp-labeled markers to each sample and cutting tightly on them helps reduce rRNA contamination from rRNAs running outside this range.

RNA-seq:

Labeled Decade marker is run alongside the samples to estimate the size range to cut. The same pCp-labeled markers are added to these samples to follow ligation efficiencies.

Decade marker labeling

Label Decade markers (Ambion) according to the manufacturer's protocol

pCp labeling of marker oligos (26-mer and 32-mer)

	Vol (μL)
Marker oligo RNA (10 μM)	1.6
[5'- ³² P]-pCp (3000 Ci/mmol, 10 mCi/mL)	5
10x T4 ligation buffer	2
ATP (40 μM)	3
T4 RNA ligase 1 (20 U/ μL ; NEB)	2
H ₂ O	6.4
Total	20

Incubate at 16 C overnight.

After overnight ligation:

1. Add 20 μL 2x loading dye to ligation reaction
2. Gel-purify, elute in 0.3 M NaCl, precipitate eluted RNA

Following ligation these RNAs are 27 and 33 nt.

Size selection by denaturing polyacrylamide gel

1. Resuspend sample in H₂O to ~5 μL
2. Add 10,000 total counts of 27-mer and 33-mer (5,000 counts each) to each sample
3. Add 2x loading dye
4. Run on a 10% urea-polyacrylamide gel
5. For RPFs: Cut out 27–33 nt tightly on the labeled markers
For RNA-seq: Cut out 27–40 nt (lower based on 27-mer, upper based on Decade; this range can be expanded to capture more material)
6. Elute in 0.3 M NaCl and then precipitate

4.2 3' dephosphorylation

After size selection, resuspend RNA in H₂O

	Vol (μL)
RNA	5
1.5x MES-NaOH buffer, pH 5.5	16.67
PNK (10 U/μL; NEB)	1.25
H ₂ O	2.08
Total	25

Incubate at 37 C for 6 hr

After incubation:

1. Add 35 μL H₂O to dephosphorylation reaction
2. Desalt with Micro Bio-Spin P30 column (Biorad)
3. Top up to 400 μL H₂O
4. Add equal volume of phenol pH 8.0, and phenol/chloroform extract
5. To final aqueous phase, add 0.1x vol NaOAc (3 M, pH 5.2), 4 μL GlycoBlue, and 2.5x vol 100% EtOH
6. Precipitate

4.3 3' ligation

After 3' dephosphorylation, resuspend RNA in H₂O

	Vol (μL)
RNA	5
3' adenylated adaptor (100 μM)	1
10x T4 ligation buffer	1
T4 RNA ligase 1 (20 U/μL; NEB)	1
H ₂ O	2
Total	10

Incubate at 22 C for 2.5 hr

After incubation:

1. Add 2x loading dye
2. Gel-purify on a 10% urea-polyacrylamide gel, cut out expected size range (+21 nt to original size-selected fragments)
3. Elute in 0.3 M NaCl and then precipitate

4.4 5' phosphorylation

After 3' ligation, resuspend RNA in H₂O

	Vol (μL)
RNA	5
10x PNK buffer (NEB)	1
[³² P]γ-ATP (6000 Ci/mmol, 150 mCi/mL)	0.2
PNK (10 U/μL; NEB)	1.5
H ₂ O	2.3
Total	10

Incubate at 37 C for 1 min

After incubation, add:

	Vol (μL)
Cold ATP (4 mM)	3.5
10x PNK buffer (NEB)	0.5
Total	10+4

Incubate at 37 C for 30 min

After incubation:

1. Add 46 μL H_2O to phosphorylation reaction
2. Desalt with Micro Bio-Spin P30 column
3. Top up to 400 μL H_2O
4. Add equal volume of phenol pH 8.0, and phenol/chloroform extract
5. To final aqueous phase, add 0.1x vol NaOAc (3 M, pH 5.2), 4 μL GlycoBlue, and 2.5x vol 100% EtOH
6. Precipitate

4.5 5' ligation

After 5' phosphorylation, resuspend RNA in H_2O

	Vol (μL)
RNA	4.8
5' adaptor (100 μM)	2.6
10x T4 ligation buffer	1
ATP (4 mM)	0.6
T4 RNA ligase 1 (20 U/ μL ; NEB)	1
Total	10

Incubate at 22 C for 18 hr

After incubation:

1. Add 2x loading dye
2. Gel-purify on a 10% urea-polyacrylamide gel, cut out expected size range [+34 nt (or +26 nt if old 3' adaptor is used) to size range after 3' ligation]
3. Elute in 0.3 M NaCl and then precipitate

4.6 Reverse transcription

After 5' ligation, resuspend RNA in H_2O

	Vol (μL)
RNA	5
RT primer/5' PCR primer (100 μM)	1
H_2O	9.6
Total	15.6

Incubate at 65 C for 10 min

After incubation, add:

	Vol (μL)
5x First strand buffer	6.4
10x dNTPs (2 mM each dNTP)	7
0.1 M DTT	3
Total	15.6+16.4

Incubate at 48 C for 3 min

After incubation:

1. Remove 3 μL for RT-minus control
2. Add 1 μL SuperScript II (200 U; Life technologies) to RT-plus reaction
3. Incubate at 44 C for 1 hr

Hydrolyze RNA template

	RT-plus vol (μL)	RT-minus vol (μL)
1 M NaOH	5	0.5

Incubate at 90 C for 10 min

After incubation, add:

	RT-plus vol (μL)	RT-minus vol (μL)
1 M HEPES-NaOH (pH 7.0)	25	2.5

Desalt with Micro Bio-Spin P30, then either store at -20 C or proceed to PCR.

4.7 PCR

	Vol (μL)
RT reaction	4
5x Phusion High Fidelity buffer (NEB)	10
10x dNTPs	6.3
3' PCR primer (150 nM)	1
Phusion polymerase (2 U/ μL ; NEB)	0.5
H ₂ O	27.2
Total	49

Perform PCR:

1. 98 C for 30 sec
2. 94 C for 30 sec
3. 60 C for 30 sec
4. 72 C for 15 sec
5. Repeat steps 2–4 three more times
6. 72 C for 10 min

After these cycles, add:

	Vol (μL)
5' PCR primer (25 μM)	0.5
3' PCR primer (25 μM)	0.5
Total	49+1

Cycle as before for enough cycles to first see a product. We perform an initial PCR to determine the cycle at which it amplifies, and then perform a second PCR up to that cycle number. RNA-seq samples generally amplify at cycles 2-8; ribosome profiling samples generally amplify at cycles 6-12. Precipitate PCR.

4.8 Formamide gel purification

1. Resuspend PCR sample in 10 μL 1x formamide loading dye
2. Prepare 10 nt ladder [2 μL 10 bp DNA marker (diluted to 0.1 $\mu\text{g}/\mu\text{L}$; Life technologies) in 8 μL 1x formamide loading dye
3. Heat samples at 85 C for 10 min
4. Gel-purify on 90% formamide, 8% acrylamide gel
5. Stain with SYBR Gold (5 $\mu\text{L}/50$ mL 1x TBE; Life technologies)
6. Cut and elute band at ~150 nt for ribosome profiling samples; RNA-seq samples will run slightly higher
7. Ethanol-precipitate *without* adding GlycoBlue
8. After precipitation, remove ethanol, wash with 70% ethanol
9. Remove ethanol, air dry for ~5 min
10. Resuspend in 10 μL 10 mM Tris (EB)

Buffers

1.5x MES-NaOH buffer

150 mM MES-NaOH, pH 5.5
 15 mM MgCl_2
 15 mM β -mercaptoethanol
 450 mM NaCl
 Store at -20 C

10x T4 ligation buffer

500 mM Tris-HCl, pH 7.8
 100 mM MgCl_2
 100 mM DTT
 Store at -20 C

Oligos used

>26-mer marker oligo

5' AGCGUGUACUCCGAAGAGGAUCCAAA 3'

>32-mer marker oligo

5' GGCAUUAACGCGAACUCGGCCUACAAUAGUGA 3'

>3' adenylated adaptor (21.340x)

5' AppTCGTATGCCGTCTTCTGCTTGddC 3'

>Old 5' adaptor (26.71)

5' GUUCAGAGUUCUACAGUCCGACGAUC 3'

>New 5' adaptor (34.71)

5' GUUCAGAGUUCUACAGUCCGACGAUCNNNNNNNNN 3'

>RT primer/5' PCR primer (18.206)

5' CAAGCAGAAGACGGCATA 3'

>3' PCR primer

5' AATGATACGGCGACCACCGACAGGTTGGAATTCTCGGGTGCCAAGGAACTCCA
GTCACxxxxxxATCCGACAGGTTTCAGAGTTCTACAGTCCGA 3'

Note: xxxxxx represents the barcode

*For formulation of gels, loading dyes and preparation of the 3' adenylated adaptor,
please see the Bartel lab Solexa Library Protocol*

Section 5: rRNA subtractive hybridization

The rRNA subtraction protocol was originally developed by Huili, and has been expanded to include subtractive hybridization oligos for additional species. The logic of this step is that most rRNA contamination in ribosome profiling libraries tends to come from a small number of rRNA fragments that are very abundant in the library. Hybridizing biotinylated oligos to these rRNAs and removing them can decrease the rRNA contamination, often substantially.

The biotinylated oligos listed below target abundant rRNA species that have been problematic in human, mouse, *S. cerevisiae*, and zebrafish libraries. We have not applied this to frog libraries yet, but are intending to do so. These probes generally perform well in samples of the indicated species, but if you are profiling a different species or are having issues reducing rRNA contamination with these probes you should look at your sequencing results to determine which rRNAs are problematic for you and design new probes against them.

Human

>Oligo 1

5' CCG GCT ATC CGA GGC CAA CCG /3Bio/ 3'

>Oligo 2

5' CGA TCA GAA GGA CTT GGG CCC /3Bio/ 3'

>Oligo 3

5' GCG CTT GGC GCC AGA AGC GAG /3Bio/ 3'

>Oligo 4

5' GGC GCC GAG AGG CAA GGG GCG /3Bio/ 3'

>Oligo 5

5' CTA CAG CAC CCG GTA TTC CCA /3Bio/ 3'

>Oligo 6

5' TCA GAC AGG CGT AGC CCC GGG /3Bio/ 3'

>Oligo 7

5' AGT AGT GGT ATT TCA CCG GCG /3Bio/ 3'

Mouse

>Oligo 1

5' CGA GGT TAT CTA GAG TCA CCA /3Bio/ 3'

>Oligo 2

5' TCC TAG CTG CGG TAT CCA GGC G /3Bio/ 3'

>Oligo 3

5' TAG AAT TAC CAC AGT TAT C /3Bio/ 3'

>Oligo 4

5' TCA GAA GGA CTT GGG CCC CCC /3Bio/ 3'

>Oligo 5

5' TTG GCG CCA GAA GCG AGA GCC /3Bio/ 3'

>Oligo 6

5' ATA AAT GCA CGC ATC CCC C /3Bio/ 3'

>Oligo 7

5' CTA TCC GGG GCC AAC CGA G /3Bio/ 3'

S. cerevisiae

Note: These were designed for *S. cerevisiae*, but we've used them successfully for *S. pombe*.

>Oligo 1

5' GAT CGG TCG ATT GTG CAC CTC /3Bio/ 3'

>Oligo 2

5' CGC TTC ATT GAA TAA GTA AAG /3Bio/ 3'

>Oligo 3

5' GAC GCC TTA TTC GTA TCC ATC /3Bio/ 3'

Zebrafish

>Oligo 1

5' ATG CTC ACC ACT ATA CCA CCA /3Bio/ 3'

>Oligo 2

5' CTC AGA CAG GCG TGG CCC CGG /3Bio/ 3'

>Oligo 3

5' CGC CAC AGG TCC CTC GCC CTC /3Bio/ 3'

>Oligo 4

5' CCT CAG ACA GGC GTG GCC CCG /3Bio/ 3'

>Oligo 5

5' AAT TCT ACC ACT GAA CCA CCA /3Bio/ 3'

>Oligo 6

5' ATC CTA ACC ACT AGA CCA CCA /3Bio/ 3'

>Oligo 7

5' CGT ACG CCA CAG GTC CCT CGC /3Bio/ 3'

>Oligo 8

5' GTG ATA ACC ACT ACA CTA CGG /3Bio/ 3'

>Oligo 9

5' GCC ACA GGT CCC TCG CCC TCG /3Bio/ 3'

>Oligo 10

5' AGT ACG TTT GAT CGT CGG ACT /3Bio/ 3'

5.1 Subtraction of contaminating rRNA species

After 3' ligation, resuspend RNA in H₂O

	Vol (μL)
RNA	10
Oligos	x
20x SSC	10
H ₂ O	up to 100

The amount of each oligo to add for each species:

Human	Vol (μL)
Oligo 1	10
Oligo 2	6
Oligo 3	2
Oligo 4	2
Oligo 5	1.5
Oligo 6	1.5
Oligo 7	1.25

Mouse	Vol (μL)
Oligo 1	5
Oligo 2	5
Oligo 3	2.5
Oligo 4	6
Oligo 5	2
Oligo 6	2.5
Oligo 7	1.25

<i>S. cerevisiae</i>	Vol (μL)
Oligo 1	13
Oligo 2	9
Oligo 3	2.25

Zebrafish	Vol (μ L)
Oligo 1	7
Oligo 2	5
Oligo 3	2
Oligo 4	1
Oligo 5	1
Oligo 6	4.25
Oligo 7	1
Oligo 8	1
Oligo 9	1
Oligo 10	2

After combining all components, incubate at 70 C for 5 min then slow-cool to 37 C.

While slow-cooling is taking place, prepare MyOne C1 streptavidin beads (Life technologies):

1. Resuspend MyOne C1 beads and transfer 500 μ L to an eppendorf tube
2. Wash 3x with 500 μ L B&W buffer (1x)
3. Wash 2x with 500 μ L solution A
4. Wash 2x with 500 μ L solution B
5. Resuspend MyOne C1 beads in 100 μ L B&W buffer (2x)
6. After slow-cooling is finished, spin down annealed RNA:oligo mixture
7. Add RNA:oligo mixture to resuspended MyOne C1 beads, pipette to mix
8. Incubate at room temperature for 15 min with rotation
9. Place tube on magnet for 2 min, transfer \sim 200 μ L supernatant to a fresh tube
10. Divide supernatant into two aliquots and add 284 μ L H₂O, 4 μ L GlycoBlue, and 2.5x vol 100% EtOH to each
11. Precipitate
12. After precipitation, resuspend and run on a gel as in 4.3, steps 2–3

5.2 Proceed to section 4.4 for 5' phosphorylation

Buffers

B&W buffer (2x)

- 10 mM Tris-HCl (pH 7.5)
- 1 mM EDTA
- 2 M NaCl
- 0.01% Tween-20

B&W buffer (1x)

- 5 mM Tris-HCl (pH 7.5)
- 500 μ M EDTA
- 1 M NaCl
- 0.01% Tween-20

Solution A

0.1 M NaOH

0.05 M NaCl

Solution B

0.1 M NaCl

Section 6: Miscellaneous procedures

6.1 Gel purification

To each sample:

1. Add 2x loading dye
2. Heat to 90 C for 5 min
3. Run on 10 % urea-polyacrylamide gel
4. Cut out gel piece containing RNA of desired size range
5. Elute RNA from the gel piece by adding 440 μ L 0.3 M NaCl, rotating the tube at 4 C overnight
6. Remove supernatant (~400 μ L), add 4 μ L GlycoBlue and 1 mL 100% ethanol
7. Precipitate at -20 C for at least 2 hr
8. After precipitation, pellet RNA by centrifuging at 4 C for 30 min
9. Remove all ethanol, air-dry pellet for 3 min and resuspend in H₂O as needed for subsequent procedures

6.2 Phenol/chloroform extraction

To each sample:

1. Add equal volume of phenol (at appropriate pH), vortex for 1 min
2. Centrifuge at 4 C for 10 min
3. Transfer aqueous phase to a new tube
4. Add equal volume of chloroform, vortex for 1 min
5. Transfer aqueous phase to a new tube
6. Add 0.1x vol NaOAc (3 M, pH 5.2), 4 μ L GlycoBlue, 2.5x 100% EtOH
7. Precipitate at -20 C for at least 2 hr
8. After precipitation, pellet RNA by centrifuging at 4 C for 30 min
9. Remove all ethanol, air-dry pellet for 3 min and resuspend in H₂O as needed for subsequent procedures