mRNA-Seq and Ribosome Profiling protocol

Citation: Guo et al., *Nature* 466: 835–840 (2010)

This protocol describes the procedures used to perform mRNA-Seq and ribosome profiling on mammalian cells. In general, you would want to split each biological sample such that mRNA-Seq and ribosome profiling can be carried out in parallel for the same sample. If you are performing mRNA-Seq or ribosome profiling only for a given sample, skip to section 2 or 3, respectively.

Structure

In this protocol, some steps are specific to mRNA-Seq/ribosome profiling and indicated so. The library generation steps are largely common for both procedures. The last section includes steps for miscellaneous procedures used in the protocol.

Section 1: Harvesting cells for parallel mRNA-Seq and ribosome profiling

1.1 Harvesting of cells/biological material

The example below is written for cells grown in monolayer culture.
(e.g. untransfected HeLa cells)

Estimate of number of cells required:
mRNA-Seq: Use enough cells to get ~15-20 µg total RNA
Ribosome profiling: This varies depending on cell type/size, but as an example, I usually use ~10 million HeLa cells per sucrose gradient

Day before:
Split cells such that dish will be 70-80% confluent on day of harvesting.

Day of harvesting:
Arrest translation with cycloheximide (CHX)

1. Spike medium that cells are growing in with CHX (final 100 µg/ml, therefore NOTE the volume of media in the dish/container beforehand!)
2. Return cells to 37 °C for 10 min

Harvesting cells

1. Transport cells to cold room
2. Remove media on ice
3. Wash 2x with ice-cold PBS (+100 µg/ml CHX)
4. Scrape cells off culture dish in PBS (+100 µg/ml CHX),
5. Transfer to eppendorf tube and divide cell suspension into separate portions for mRNA-Seq and ribosome profiling accordingly
6. Centrifuge at 330 x g for 5 min to pellet cells
7. Remove supernatant

8a. To cell pellet used for mRNA-Seq: lyse cells with TRI reagent (done according to manufacturer's instructions → proceed to step 1 in section 2.1

8b. To cell pellet used for ribosome profiling: lyse cells with 1 ml lysis buffer → proceed to step 6 in section 3.2

Section 2: mRNA-Seq – mRNA fragmentation

Estimate of number of cells required:
mRNA-Seq: Use enough cells to get ~15-20 µg total RNA

2.1 RNA extraction and Poly(A) selection

1. Extract total RNA with TRI reagent (done according to manufacturer's instructions)
2. Perform poly(A) selection with oligo(dT)-Dynabeads from Invitrogen (done according to manufacturer's instructions)
3. Precipitate poly(A)-selected RNA

2.2 mRNA fragmentation by partial alkaline hydrolysis

After poly(A) selection, alkaline hydrolysis can be done with as little as 500 ng of input poly(A)-selected mRNA, in total reaction volume of 20 µl

Resuspend RNA pellet in 10 µl water

	Vol
RNA	10
2x fragmentation buffer	10
	20 µl

→ 95 °C, ~20 min
Stopping hydrolysis:
To the 20 µl fragmentation reaction:

1. Chill immediately on ice, spin down condensation, back on ice
2. Add immediately, 280 µl of stop/ppt solution
3. Add 750 µl 100% EtOH → precipitate

2.3 Proceed to library generation (section 4)

Buffers

2x fragmentation buffer

	Vol	Final conc
0.5 M EDTA	0.5	2 mM
0.1 M Na_2CO_3	15	~10 mM
0.1 M $NaHCO_3$	110	~90 mM
	For total of 125 µl	

→ this will give final pH ~9.3

Stop/ppt solution

	Vol	Final conc
3 M NaOAc, pH 5.2	134.5	0.3 M
15 mg/ml GlycoBlue	4.5	
H_2O	1121	
	For total of 1260 µl	

Section 3: Ribosome Profiling – ribosome footprinting

Estimate of number of cells required:
Ribosome profiling: This varies depending on cell type/size, but as an example, I usually use ~10 million HeLa cells per sucrose gradient

3.1 Gradient preparation

Gradients are prepared by horizontal diffusion for a few hours at 4 °C. Therefore, this is done the DAY BEFORE the gradients need to be used.

1. Prepare 10% and 50% gradient buffers by adding DTT (2 mM) and CHX (100 µg/ml) fresh
2. Pipette 5.4 ml 10% gradient buffer into polyallomer tube (Beckman Coulter: cat# 331372)
3. Underlay 5.4 ml 50% gradient buffer using a 21-gauge, 2-inch needle
4. Seal top of tube with parafilm
5. Gently tilt tube to horizontal position and leave in cold room for 6.5 hrs to set up the linear gradient by horizontal diffusion
   (Save remaining 10% gradient buffer (~3 ml) for calibration of the gradient fractionator the next day → store at 0–4 °C overnight)
6. After 6.5 hrs, tilt tube slowly back to vertical position
7. Leave in cold room till usage the next day, gradient formed will be stable till then

3.2 Harvesting of cells/biological material

The example below is written for cells grown in monolayer culture.
(e.g. untransfected HeLa cells)

Day before:
Split cells such that dish will be 70-80% confluent on day of harvesting

Day of harvesting:
Arrest translation with cycloheximide (CHX):

1. Spike medium that cells are growing in with CHX (final 100 µg/ml, therefore NOTE the volume of media in the dish/container beforehand!)
2. Return cells to 37 °C for 10 min

Harvesting cells

1. Transport cells to cold room
2. Remove media on ice
3. Wash 2x with ice-cold PBS (+100 µg/ml CHX)
4. Lyse with 1 ml lysis buffer (e.g. for one 15-cm dish) → for each plate, turn the dish to make sure that the lysis buffer covers every part of the dish at some point
5. Scrape cells off the dish, collect into eppendorf tube
6. Shear 4x with 26-gauge needle, VERY gently
7. Centrifuge at 1300 x g for 10 min
8. Remove supernatant to fresh tube

(At this point, lysates can be snap-frozen in liquid nitrogen and stored at –80 °C, for processing at a later date)

Proceed as follows if samples had been snap-frozen:

1. Thaw frozen samples on ice in the cold room (for 1 ml volume, this should take ~2 hrs)

3.3 Nuclease digestion

To fresh lysate, or snap-frozen lysate that has been thawed:

1. Add RNase I (0.5–1 U/µl lysate, Ambion)
2. Incubate at room temperature for 30 min, with gentle shaking/rotation
3. After digest, place tube on ice and load straight onto gradient

NOTE: For any new cell type tried, it is good practice to first run a gradient with undigested sample to get a sense of the "normal" polysome profile, and then optimize the amount of nuclease to add from there.

3.4 Centrifugation

Centrifuge should be switched on 30-45 min PRIOR to usage, for pre-chilling to 4 °C

1. Layer ~800 µl of digested extract onto 11-ml 10–50% linear sucrose gradient
2. WEIGH AND BALANCE CAREFULLY (with remaining extract, or lysis buffer)
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 (when the rotor has come to a complete stop), do NOT retrieve samples immediately → wait at least 5 min before doing so, otherwise it will be difficult to remove tubes from swinging buckets

3.5 Fraction collection

NOTE: This part is easier shown than described in words.

Fraction collector and chart recorder should be switched on at least 30 min BEFORE usage

1. Set baseline with ~3 ml 10% gradient buffer (left over from previous day of gradient pouring – step 5 in section 3.1)
2. Set up 15-ml Falcon tubes with parafilm and 2-ml microcentrifuge screw cap tubes (ALWAYS prepare more tubes than necessary!)
3. Remove sample tube from swinging bucket carefully with forceps, try not to touch sample
4. Fix tube to fractionator
5. Collect fractions by upward displacement with 60% sucrose (spiked with bromophenol blue for easy visualization of displacement front)
6. Place collected fractions on ice, till all gradients are fractionated
7. WASH with ~3 ml H_2O in between gradients
8. WASH with AT LEAST 3x 50 ml H_2O after all collection done

Settings

Sensitivity: 0.2

Pump speed: 0.75 ml/min

Chart recorder speed: 60 cm/hr

At this speed, should get 15 fractions of ~750 µl each per gradient

3.6 Release/filtration

(Optional: proceed straight to section 3.7 if not doing)

NOTE: The rationale behind the steps in this section is reduce ribosomal RNA contamination by making use of EDTA to separate the ribosomal subunits, thus releasing the mRNA fragments,

after which a 100 kDa membrane filter is used to trap the ribosomal subunits and any non-covalently associated ribosomal RNA fragment. However, we are not sure how effective this step is in reducing the rRNA contamination – even with this step, rRNA contamination can still range from 50% to >90%. As we found that the contaminants tend to come from a few locations in the ribosomal RNA sequences, we are currently trying to use subtractive hybridization to remove major contaminants during the library generation process, instead of performing this release/filtration step.

Pool monosome fractions (usually two fractions) for each gradient and:

1. Load onto Ultra-4 centrifugal filters with Ultracel-100 membranes (Amicon)
2. Centrifuge at 1900 x g for 30 min at 4 °C to concentrate sample
3. Add 1220 µl ice-cold release buffer to the retentate (should get ~100 µl retentate after centrifugation)
4. Incubate 10 min on ice
5. Transfer filter unit to new 15-ml falcon tube and centrifuge at 1900 x g for 15 min at 4 °C
6. Separate the release filtrate into two equal-volume aliquots and proceed to proteinase K digest

3.7 Proteinase K/SDS digest

The following steps can be applied to the release filtrate (from section 3.6), or to the collected fractions (from section 3.5, if not doing the release/filtration step).

To each release filtrate aliquot/fraction:

1. Add x µl 10% SDS (to final 1%)
2. Add y µl proteinase K (to final 200 µg/ml, Roche)
3. Invert to mix
4. Incubate at 42 °C, 30 min

(At this point, digested fractions can be stored at –80 °C, or you can proceed straight to phenol/chloroform extraction)

3.8 Phenol/chloroform extraction

To each proteinase K-digested fraction:

1. Add equal volume of acid phenol, pH 4.5, and phenol/chloroform extract
2. To final aqueous phase, add 0.1x vol NaOAc (3 M, pH 5.2), 2.5x vol 100% EtOH, GlycoBlue → precipitate

3.9 Proceed to library generation (section 4)

Buffers

1. 10% or 50% gradient buffer for sucrose gradients

   20 mM HEPES-KOH (pH 7.4)
   5 mM $MgCl_2$
   100 mM KCl
   Either 10% or 50% sucrose (w/v)
   → Filter-sterilize, store at 4 °C

Add fresh just before pouring:

2 mM DTT
100 µg/ml cycloheximide (Sigma, cat# C4859)
20 U/ml SUPERase•In (Ambion)

2. 60% (w/v) sucrose displacement solution (spiked with bromophenol blue)
   ➔ Filter-sterilize, store at 4 °C

3. Lysis buffer

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

   Add fresh before use:

   2 mM DTT
   500 U/ml RNasin (Promega)
   100 µg/ml cycloheximide (Sigma, cat# C4859)
   Protease inhibitor (1X complete, EDTA-free, Roche)

4. PBS (supplemented with 100 µg/ml CHX) – make fresh

5. Release buffer

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

   Add fresh before use:

   2 mM DTT
   20 U/ml SUPERase•In (Ambion)

Section 4: Library generation – for mRNA-Seq and ribosome profiling

This part of the protocol largely follows the same scheme as the Bartel lab small RNA cloning protocol (Solexa Library Protocol). The main differences are that modifications have to be made to the 5′ and 3′ ends of the size-selected mRNA fragments because fragments generated by alkaline hydrolysis (in the mRNA-Seq protocol) or RNase I digest (in the ribosome profiling protocol) both have 3′ phosphates and 5′ hydroxyls, in contrast to the 3′OH and 5′P of miRNA ends. Both mRNA-Seq fragments and ribosome-protected fragments can be put through the same library generation procedure.

4.1 Size selection

Ribosome profiling:
Ribosome-protected fragments (RPFs) are usually ~30 nt in length. Previous runs have indicated that the main contaminating rRNA fragments are at ~26 nt and ~35 nt. Thus I use 26-mer and 32-mer RNA marker oligos (these would be 27 nt and 33 nt after pCp labeling) to follow the entire library generation process.
mRNA-Seq:
I run labeled Decade markers alongside the samples to estimate the size range to cut, but I also spike in some marker oligos to follow ligation efficiencies at later steps.

Decade marker labeling
Label Decade markers (Ambion) according to manufacturer's instructions

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

	Vol
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_2O	6.4
	20 μl

→ 16 °C, O/N

After overnight ligation,

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

Size selection by denaturing polyacrylamide gel
For each sample RNA (resuspended in H_2O):

1. Add 10 K count (in total) of 26-mer and 32-mer labeled marker oligos (now 27 bases and 33 bases long respectively)
2. Add 2x loading dye
3. Run on 10% urea-polyacrylamide gel, till dye front just about reached bottom of gel

4a. For RPFs: Cut out 27–33 nt, make the cut tightly around the labeled markers
4b. For mRNA-Seq fragments: Based on the labeled Decade markers, cut out a 20-base size range (e.g. I have done 25–45 nt, and 35–55 nt)

5. Elute overnight in 0.3 M NaCl → precipitate RNA with GlycoBlue

NOTE: In step 4a, it is important to make the cut TIGHTLY around the RPFs to exclude as much of the flanking rRNA fragments as possible. In the paper, we did not make this cut tightly enough, which resulted in high levels of rRNA contamination (60–93%) in the initial RPF libraries. This led us to attempt a second size selection by first amplifying the libraries for an additional six cycles and then using an additional formamide-polyacrylamide gel to exclude more of the flanking PCR products derived from rRNA contaminants. This additional step reduced rRNA contamination to 40–54%. Making a tight cut in step 4a will avoid the need for this second size selection gel.

4.2 3′ dephosphorylation

Resuspend RNA pellet (after size selection) in H_2O

	Vol
RNA	5
1.5x MES-NaOH buffer, pH 5.5	16.67
PNK (10 U/µl, NEB)	1.25
H_2O	2.08
	25 µl

→ 37 °C, 6 hr

After 6-hr incubation, to each dephosphorylation reaction:

1. Add 5 µl H_2O to top up to 30 µl
2. Desalt with G-25 microspin column
3. Top up to 400 µl with 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), 2.5x vol 100% EtOH, GlycoBlue → precipitate

4.3 3′ ligation

Resuspend RNA pellet (after 3′ dephosphorylation) in H_2O

	Vol
RNA	5
3′ adenylated adaptor (100 µM)	1
10x T4 ligation buffer	1
T4 RNA ligase 1 (20 U/µl, NEB)	1
H_2O	2
	10 µl

→ 22 °C, 2.5 hr

NOTE: Do NOT use the T4 ligation buffer from NEB, which contains ATP

To each ligation reaction:

1. Add 2x loading dye
2. Gel-purify on 10% urea-polyacrylamide gel, cut out expected size range (+ 21 nt to original size-selected fragments), using marker oligos and/or Decade markers as guide
3. Elute overnight → precipitate RNA with GlycoBlue

4.4 5′ phosphorylation

Resuspend RNA pellet (after 3′ ligation) in H_2O

	Vol
RNA	5
10x PNK buffer (NEB)	1
[^{32}P]γ-ATP (6000 Ci/mmol, 150 mCi/ml)	Trace
PNK (10 U/µl, NEB)	1.5
H_2O	Top up to 10
	10 µl

→ 37 °C, 30 sec – 1 min

	Vol
Cold ATP (4 mM)	3.5
10x PNK buffer (NEB)	0.5
	10 + 4 = 14 µl

→ 37 °C, 30 min

After 30 min incubation, to each 5′ phosphorylation reaction:

1. Add 16 µl H_2O to top up to 30 µl
2. Desalt with G-25 microspin column
3. Top up to 400 µl with 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), 2.5x vol 100% EtOH, GlycoBlue → precipitate

4.5 5′ ligation

Resuspend RNA pellet (after 5′ phosphorylation) in H_2O

	Vol
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
	10 µl

→ 22 °C, 18 hr

To each ligation reaction:

1. Add 2x loading dye
2. Gel-purify on 10% urea-polyacrylamide gel, cut out expected size range (+ 26 nt to the size range after 3′ ligation), using marker oligos and/or Decade markers as guide
3. Elute overnight → precipitate RNA with GlycoBlue

4.6 Reverse transcription/Splicing by Overlap Extension PCR (SOE-PCR)

This part of the protocol largely follows the analogous section in the Bartel lab small RNA cloning protocol (Solexa Library Protocol).

Reverse transcription

Resuspend RNA pellet (after 5′ ligation) in H_2O

	Vol
RNA	5
RT primer/5′ PCR primer (100 µM)	1
H_2O	9.6
	15.6 µl

→ 65 °C, 10 min

Add in order:

	Vol
5x First strand buffer (Invitrogen)	6.4
10x dNTPs (2 mM for each dNTP)	7
0.1 M DTT	3
	15.6 + 16.4 = 32 µl

→ 48 °C, 3 min

To each tube:

1. Remove 3 µl for RT-minus control
2. Add 1 µl SuperScript II (200 U, Invitrogen) to RT-plus reaction

→ 44 °C, 1 hr

3. Hydrolyze RNA template by adding 1 M NaOH

	RT-plus	RT-minus
1 M NaOH	5 µl	0.5 µl

→ 90 °C, 10 min

4. Add 1 M HEPES-NaOH (pH 7.0) to neutralize

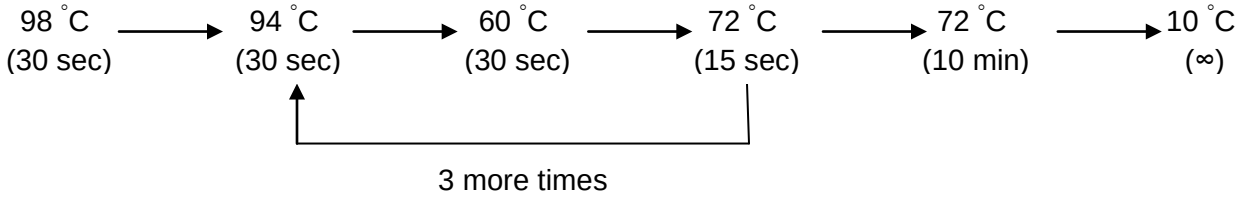
	RT-plus	RT-minus
1 M HEPES-NaOH (pH 7.0)	25 µl	2.5 µl

5. Desalt with G-25 microspin column
6. Proceed straight to SOE-PCR or store at –20 °C

SOE-PCR1 (to allow for extension)

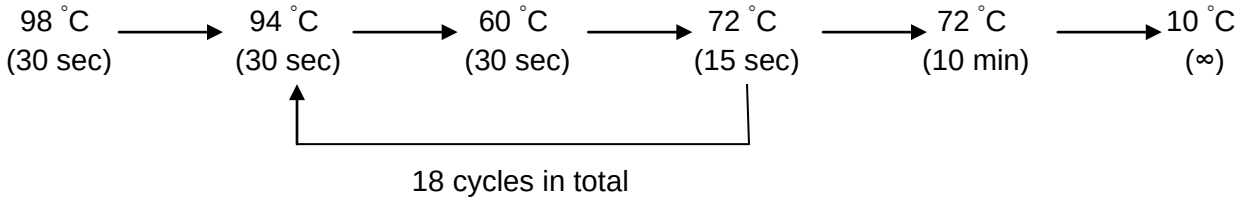
	RT-plus	RT-minus
RT reaction	8	4
5x Phusion High Fidelity buffer (NEB)	20	10
10x dNTPs	12.6	6.3
3′ PCR primer (150 nM)	2	1
Phusion polymerase (2 U/µl, NEB)	1	0.5
H_2O	54.4	27.2
	98 µl	49 µl

→ Split RT-plus into 2 tubes of 49 µl each



SOE-PCR2 (for amplification)
To each tube of PCR reaction, add:

	Vol
5′ PCR primer (25 μM)	0.5
3′ PCR primer (25 μM)	0.5
	49 + 1 = 50 μl



Ethanol-precipitate each PCR reaction without GlycoBlue

4.7 Formamide gel purification and sample submission

1. Resuspend each DNA pellet in 15 μl 1x formamide loading dye
2. Prepare 10 nt ladder: 2 μl 10 bp DNA marker (diluted to 0.1 μg/μl, Invitrogen) in 13 μl 1x formamide loading dye
3. Heat samples and marker for 10 min at 85 °C and gel-purify on 90% formamide, 8% acrylamide gel
4. Stain with SYBR Gold (Invitrogen) (5 μl/50 ml 1X TBE)
5. Cut and elute band at ~100 nt for ribosome profiling samples. mRNA-Seq samples will run slightly higher, depending on the initial size-selection (RT-minus sample will run at ~44 nt).
6. Ethanol-precipitate without adding GlycoBlue.
7. After precipitation, remove ethanol, wash once with 70% ethanol
8. Let dry for 10 min with cap open, and another 20 min with cap closed (but poke hole in cap)
9. Resuspend in 10 μl 10 mM Tris (EB) and submit sample for sequencing

Buffers

1. 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

2. 10x T4 ligation buffer

   500 mM Tris-HCl, pH 7.8
   100 mM $MgCl_2$
   100 mM DTT

   ➔ Store at –20 °C

Oligo's used

>26-mer marker oligo
5′ AGCGUGUACUCCGAAGAGGAUCCAAA 3′

>32-mer marker oligo
5′ GGCAUUAACGCGAACUCGGCCUACAAUAGUGA 3′

>3′ adenylated adaptor (21.340x)
5′ AppTCGTATGCCGTCTTCTGCTTGidT 3′

>5′ adaptor (26.71)
5′ GUUCAGAGUUCUACAGUCCGACGAUC 3′

>RT primer/5′ PCR primer (18.206)
5′ CAAGCAGAAGACGGCATA 3′

>3′ PCR primer (44.45)
5′ AATGATACGGCGACCACCGACAGGTTCAGAGTTCTACAGTCCGA 3′

>Sequencing primer used on Solexa flow cell (underlined in final contruct)
5′ CGACAGGTTCAGAGTTCTACAGTCCGACGATC 3′

Final construct:

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5′ AATGATACGGCGACCACCGACAGGTTCAGAGTTCTACAGTCCGACGATCNNNNNNNNNNNNNNTCGTATGCCGTCTTCTGCTTG 3′
3′ TTACTATGCCGCTGGTGGCTGTCCAAGTCTCAAGATGTCAGGCTGCTAGNNNNNNNNNNNNNNAGCATACGGCAGAAGACGAAC 5′
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For formulation of gels, loading dyes and preparation of the 3′ adenylated adaptor, please see the Bartel lab Solexa Library Protocol.

Section 5: Miscellaneous procedures

5.1 Gel purification

To each sample RNA:

1. Add 2x loading dye
2. Heat to 90 °C for 5 min
3. Run on a 10% denaturing polyacrylamide gel until dye front about ~1 inch from bottom
4. Cut out gel piece containing RNA of desired size range
5. Elute RNA from the gel piece by adding 440 µl 0.3 M NaCl, and rotating the tube at 4 °C overnight
6. (Next day) Remove supernatant (~400 µl) and add 1 ml 100% ethanol (optional: add GlycoBlue to help visualize RNA pellet after precipitation
7. Precipitate at –20 °C (for at least 2 hr)
8. After precipitation, pellet RNA by spinning at 4 °C, 25 min
9. Remove all ethanol, air-dry pellet for 5 min and resuspend pellet in H_2O

5.2 Phenol/chloroform extraction

To each sample RNA:

1. Add equal volume of phenol (at appropriate pH), vortex for 1 min
2. Centrifuge at 4 °C for 10 min
3. Remove aqueous phase carefully to fresh tube
4. Add equal volume of chloroform, vortex for 1 min
5. Centrifuge at 4 °C for 10 min
6. Remove aqueous phase carefully to fresh tube
7. Add 0.1x vol NaOAc (3 M, pH 5.2), 2.5x 100% EtOH, GlycoBlue
8. Precipitate at –20 °C (for at least 2 hr)
9. After precipitation, pellet RNA by spinning at 4 °C, 25 min
10. Remove all ethanol, air-dry pellet for 5 min and resuspend pellet in H_2O