Protocol 1. Total RNA preparation

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ABSTRACT

This method is widely used by fission yeast researchers to prepare RNA for analysis of individual species (e.g., Helmlinger et al. 2008) or for genome-wide studies (e.g., Bitton et al. 2011; Wilhelm et al. 2008). Treatment with hot phenol breaks open the cells and begins to strip away bound proteins from the RNA. Deproteinization is completed by multiple extractions with chloroform/isoamyl alcohol and separation of the aqueous and organic phases using Phase Lock Gel, an inert material that acts as a physical barrier between them. The final step is concentration of the RNA by ethanol precipitation. The protocol can be used to prepare RNA from several cultures grown in parallel but it is important not to process too many samples at once as delays can be detrimental to RNA quality.

Materials

Reagents:
- Cells grown in YES or other appropriate media (25-50 mL at 0.5 –1 x 10^7 cells/mL)
- Chloroform
- Ethanol
- ddH_2O (double-distilled)
- Isoamyl alcohol
- Phenol (water-saturated, acidic-equilibrated, e.g. Sigma P1944)
- Sodium acetate (NaOAc) (3 M pH 5.2)
- TES buffer <R>
- Agarose
- TBE Agarose Gel running buffer

Equipment:
- Bench top centrifuge with swing-out rotor for 50 mL tubes
- Dry ice/ethanol bath
- Microcentrifuge
- Microcentrifuge tubes
- Phase Lock Gel tubes (Eppendorf)

Vortex Mixer
Water bath or heat block (65°C)
Agarose Gel electrophoresis tank and casting sets to generate gels
Note: Due to the widespread use of RNAse in small-scale DNA preparations, some labs use electrophoresis tanks that are specifically dedicated to running RNA gels to avoid degradation.

Method

1. Prepare the work area by cleaning racks, pipettes and work surfaces, e.g. using RNase-ZAP wipes (Ambion or Sigma). Prepare all solutions including gels and gel buffers using RNAse-free technique and wear gloves throughout the procedure.
2. Transfer cells to 50 mL screw-cap tubes and harvest by centrifugation for 5 min at 1,000 x g or 3 min at 2,000 x g and discard the culture fluid by carefully decanting. Snap-freeze the cell pellet by placing the tube in a dry ice/ethanol bath. Cells may be stored at -80°C prior to extracting RNA.
3. Thaw cells by placing the tube on ice (5 min). Add 1 mL of pre-chilled ddH_2O to resuspend the cells (adjust volume accordingly if using more or less than the specified number of cells). Transfer the cell suspension to a 2 mL microfuge tube and spin for 10 sec at 20,000 x g; remove the supernatant (SN) and discard.
4. To the cell pellet add 750 µL of TES buffer, resuspend the cells with a pipette, immediately add 750 µL of acidic phenol (stored at 4°C), vortex vigorously, and incubate in a 65°C water bath or heat block. Treat the next sample in the same way.
5. Incubate all samples at 65°C for 1 hr, vortexing for 10 sec every 10 min.

   Note: A similar protocol for budding yeast (Rio et al. 2011, Chapter 2, Protocol 7, p. 38-40) uses a shorter (10 min) incubation at high temperature, which is recommended if problems with degradation are encountered (see step 15).
6. Place the samples on ice for 1 min, vortex for 20 sec, and spin in a micro-centrifuge for 15 min at 20,000 x g at 4°C.

   Note: Some labs use room temperature as the phenol should prevent RNA degradation.
7. Pre-spin the appropriate number of yellow Phase Lock tubes for 10 sec to return the gel to the bottom and add 700 µL of acidic phenol-chloroform to each.
8. Take 700 µL of the aqueous phase from step 6 and add to the tubes from step 7, thoroughly mix by inverting (do not vortex as this will fragment the gel), and centrifuge for 5

min at 20,000 g at 4°C.

9. Add 700 µL of chloroform:isoamyl alcohol (24:1) to the appropriate number of new 2 mL microfuge tubes.
10. Take 700 µL of the aqueous (top) phase from step 8 and add to the tubes from step 9, thoroughly mix by inverting (not vortexing), and centrifuge for 5 min at 20,000 g at 4°C.
11. To the appropriate number of standard 2 mL microfuge tubes, add 1.5 mL of 100% EtOH (4°C) and 50 µL of 3 M NaAc pH 5.2.
12. Transfer 500 µL of the aqueous phase from step 10 to the tubes from step 11, vortex for 10 sec. The samples can be precipitated at -20°C overnight (or at -70°C in a dry ice-ethanol bath for 30 min).
13. Spin in a micro-centrifuge for 10 min at 20,000 g at RT. Discard SUPERNATANT, add 500 µL 70% EtOH (V/V, diluted with water & stored at 4°C), flick the tube gently with a finger but don't vortex, then spin for 1 min using the same tube orientation to avoid dislodging the pellet. Aspirate most of the SUPERNATANT, spin for 5 sec, and remove the remaining liquid with a pipette. Air dry for 5 min at RT.
14. Add 100 µL of ddH_2O (adjust volume to pellet size), and incubate for 1 min at 65°C (or 10 min at RT). Dissolve the pellet by first pipetting up and down (~30x) until no particles are left, then gently vortex for 10 sec.
15. To assess the integrity of the RNA, run 2 µL of the sample on a 1% agarose gel under RNAse-free conditions. An even better option if available is to use a Bioanalyzer, which can generate a "virtual gel" from the electropherogram. If the preparation is free of degradation, the two large ribosomal RNAs will form two single sharp bands, whereas a partially degraded sample will show decreased intensity of the large subunit band and a ladder of more rapidly migrating bands below the major bands (for an example, see Rio et al. 2011, Chapter 2, Protocol 12, Fig. 2-7). On a stained agarose gel, the degradation products may appear as a smear rather than discrete bands. In addition to assessing integrity, such gels can also be used to normalize the amounts of RNA in different preps.
16. To assess the purity of the RNA, use a spectrophotometer to determine A_{260}, A_{270} and A_{280}. If the sample is uncontaminated, the A_{260}:A_{280} ratio should be between 1.8 and 2.2. An $A_{270} \geq A_{260}$ may indicate phenol contamination (see Rio et al. 2011, Chapter 1, p. 8).
17. The yield of RNA from this protocol is generally ~200 µg but cells grown under some conditions may yield less RNA. Quantitate the yield of RNA using a standard or nanoscale

spectrophotomeer (see Rio et al. 2011, Chapter 2, Protocol 13, p. 67-68). Adjust different samples to be analyzed in parallel to the same concentration, generally 1-2 µg/µL, by adding ddH_2O.

Reagents:

TES buffer: 10 mM Tris, pH 7.5
10 mM EDTA, pH 8.0
0.5% SDS

Additions and variations:

- To eliminate RNAse activity, some labs treat glassware with diethyl pyrocarbonate (DEPC) and/or use DEPC-treated water to make solutions. Others avoid this practice, as residual DEPC not destroyed by autoclaving can react with adenines in the RNA. Do NOT treat buffers containing Tris with DEPC.
- To avoid stress responses during harvesting, cells may be collected by filtration rather than centrifugation (Chapter 2) and the filter disc can be snap-frozen in liquid nitrogen (step 2).
- Some labs use standard Eppendorf microfuge tubes rather than Phase Lock tubes to separate the organic and aqueous phases (step 6).
- Some labs add an additional purification step using Qiagen RNeasy columns, but it should be noted that this introduces a bias towards large (> 200 nt) RNAs (see Rio et al. 2011, Chapter 2, Protocol 4, p. 30-32 for additional information about RNA purification kits). If you elect to include Qiagen purificaton, postpone analysis of purity and integrity until after it is completed. After quantitation and adjusting concentration, proceed as follows:

18. Use 100 µg of each RNA preparation for Qiagen purification. Measure the volume of the remaining RNA, add 1/10 volume of 3 M NaOAc (pH 5.2) and 3 volumes of 100% EtOH, mix by inversion, and store at -70□C as a backup.
19. Purify 100 µg of each of your RNAs over an RNeasy mini-spin column as described in the RNeasy Mini Handbook supplied by the manufacturer. Elute twice with 30 □L RNase-free water. Hold on ice.

20. Determine the yield, integrity and purity of the RNA as described above (steps 15-17) and concentrate by ethanol precipitation if necessary.

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Protocol 2. Polysome profile analysis and RNA purification

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ABSTRACT

Polysome profile analysis is widely used by investigators studying the mechanism and regulation of translation. The method described below uses high-velocity centrifugation of whole cell extracts on linear sucrose gradients (Pospisek and Valasek 2013) to separate 40S and 60S ribosomal subunits from 80S monosomes and polysomes. Cycloheximide is included in the lysis buffer to "freeze" polysomes by blocking translation elongation. After centrifugation, the gradient is fractionated and RNA (and/or protein) is prepared from each fraction for subsequent analysis of individual species on blots, or the entire population for identification by hybridization to microarrays or by high throughput RNA sequencing.

Materials

Reagents: Cells (100 mL at 0.5 – 1 x 10^7 cells/mL)
Cycloheximide
Protease inhibitors (2 tablets "Complete, EDTA-free, Roche" for 100 mL culture)
Zirconia beads (Biospec)
Polysomal lysis buffer <R>

Equipment: FastPrep device (MP Biomedicals)
Bench top centrifuge with swing-out rotor for 50 mL tubes
Microcentrifuge
Nanodrop spectrophotometer
Phase contrast/bright field microscope with 40 x objective and 10 x eyepieces

Method

1. Harvest 0.5 – 1 x 10^9 mid-log phase cells by decanting the culture directly into 50 mL screw cap centrifuge tubes on ice containing cycloheximide to a final concentration of 100 □g/mL.
   Note: Some protocols advise adding cycloheximide 5 min before harvesting to allow time for the inhibitor to work, in which case cells can be pelleted by centrifugation immediately upon harvesting.

2. For lysis, cells are suspended in 200 µL of ice-cold polysomal lysis buffer. Protease and RNAse inhibitors are optional but highly recommended.
   Note: In one variation of the protocol, cells are washed in 0.9 mL of polysomal lysis buffer.
3. Cell suspensions are transferred to a pre-chilled 1.5-mL screw cap microfuge tube containing approximately 600 µL of 0.5 mm Zirconia beads (Biospec) and lysed in a FastPrep device at level 6.0 for 18 seconds (shorter times down to 13 sec may also be used).
   Note (optional but recommended): An aliquot can be checked using a microscope to ensure that at least 50-70% of the cells have been lysed.
4. After lysis, a hole is poked in the bottom of the tube and the lysate is separated from the beads by centrifugation at 5,000 x g for 5 min at 4°C, collecting the liquid into a 1.5 mL microfuge tube.
5. The flow through is combined with 250-350 µL of lysis buffer (depending on desired final concentration and the density of cells at the time of harvest).
6. The lysate is cleared by one centrifugation step at 20,000 x g for 15 min at 4°C.
7. 2 µL of the lysate is added to 98 µL of dH_2O and the OD_{260} is measured in a Nanodrop spectrophotometer.
8. Aliquots corresponding to 20 OD_{260} units (diluted to 350 µL with lysis buffer with or without the protease inhibitor cocktail and RNasin) are layered carefully onto an 11 mL linear sucrose gradient (linear 10 to 50% w/v; prepared using polysomal lysis buffer).
   Note: Sucrose gradients can be stored for up to 4 hours at 4°C, with or without lysate layered on top.
9. The sucrose gradients are centrifuged in a Beckman SW 40Ti rotor at 39,000 rpm (~277,000 x g) for 150 min at 4°C.
   Note: for a Beckman SW-41Ti rotor, use 35,000 rpm (~246,000 x g) *for 160 min.*
10. Remove gradients carefully to avoid disturbing and fractionate each one by upward displacement with 55% (w/v) sucrose, prepared using polysomal lysis buffer, using a gradient fractionator (Brendel or ISCO). The precise manipulations will vary depending on the exact combination of fractionator, UV monitor and fraction collector. For the Brendel machine, the flow rate should be set to 800 µL per min; for the ISCO machine, use a pump speed of 50%, 10x (~1.25 mL/ min).
11. Collect 12-13 fractions of 800-900 µL from each gradient. For protein isolation, fractionate into empty glass tubes. For RNA isolation, tubes should be pre-filled with

100% isopropanol to give a final concentration of 70%, mix and store overnight at -20°C. [Variation: pre-fill tubes with 2 mL of 8 M guanidinium-HCl (final concentration = 5.5 M); add 1 volume of 100% ethanol, mix by inversion and precipitate overnight at -20°C.]

12. After precipitation, centrifuge for 20 min at >10,000 x g at 4°C and discard the supernatant.

    Note: Alternatively, centrifuge at 3,000 x g for 90 min.

13. Optional: Wash pellets with 1 mL 85% EtOH (V/V, diluted with water; see Protocol 1 for tips to avoid losing the pellet).
14. Air-dry RNA pellets for 20 min, then re-suspend in 100 µL of RNase-free water and proceed with RNA purification according to the instructions provided with the Qiagen RNeasy kit (see Protocol 1 for details).

Reagents:

Polysomal Lysis Buffer*	20 mM Tris-HCl (pH 7.5) 50 mM KCl 10 mM $MgCl_2$
Just before use, add	1 mM DTT (final concentration) 100 µg/mL cycloheximide (freshly prepared)
Optional	0.2 mg/mL heparin (to combat RNAse activity; store at -20 □C) RNAsin (Promega; 1 µL/mL according to manufacturer's instructions) Protease inhibitor cocktail (Roche; 1 tablet "Complete, EDTA-free")

For meiotic cells, add 1 mM PMSF (from a 10 mM stock; general protease inhibitor)

Note: A 10x salt solution containing 200 mM Tris-HCl, 500 mM KCl and 100 mM $MgCl_2$ can be prepared and stored on a long-term basis if desired.

<u>Treatment with agents that disrupt polysomes:</u>

Puromycin:

Puromycin is a tRNA mimic that causes polypeptide chain termination.

1. Treat cells growing at 32□C with 1 mM puromycyin for 15 min.
2. Include 1 mM puromycin and eliminate cycloheximide from the polysome lysis buffer while increasing KCl to 500 mM and decreasing $MgCl_2$ to 2 mM.

RNAse:

RNase treatment cleaves RNA between individual ribosomes.

Add RNAse after disrupting cells (25 µL/500 µL cells) and incubate for 15 min at RT.

Do not add cycloheximide

EDTA:

EDTA treatment dissociates ribosomal subunits.

Add EDTA to 25 mM (from a 500 mM stock) after disrupting cells (25 µL/500 µL cells) and incubate for 15 min at RT.

Do not add cycloheximide

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Protocol 3. 4-thiouridine labeling to analyze mRNA turnover

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ABSTRACT

In general, mRNA half-lives have been measured after inhibition of transcription to allow decay of the pre-existing population. The protocol presented here is a more recently developed strategy in which mRNA turnover is analyzed by measuring the decline in levels of newly synthesized RNA labeled with 4-thiouridine (4sU) during a brief pulse. After RNA extraction, the 4sU is biotinylated and the labeled species are purified using streptavidin beads. Finally, DNA microarrays can be used to compare this population with total RNA, allowing half-lives to be calculated.

Materials

Reagents:
Cells (50 - 100 mL at 0.5 – 0.7 x 10^7 cells/mL)
0.5 M EDTA
EZ-Link® Biotin-HPDP (Thermo 21341) <R>
Isopropanol
5 M NaCl
MPG buffer <R>
1 M Tris, pH 7.6
4-thiouridine (Sigma-Aldrich # T4509-100MG; 100 X stock) <R>
Dynabeads® M-280 Streptavidin (Invitrogen catalog # 112-05D)
Yeast tRNA (10 mg/mL)
100 mM dithiothreitol (DTT) or β-mercaptoethanol
RNAqueous columns (Ambion # 1931)
RNAqueous kit (Ambion)
Superscript Direct Plus cDNA labeling system (Invitrogen)

Equipment:
Bench top centrifuge with swing-out rotor for 50 mL tubes.
Microcentrifuge
Nanodrop spectrophotometer
Magnetic rack for 1.5 mL tubes

Method

In vivo labeling of wild-type cells

1. Grow *ura*$^+$ cells to mid-log phase in EMM or other appropriate media
   Note: For wild-type cells, growth is carried out at 32°C but another temperature may be more appropriate for conditional mutants*.*
2. Add 4-thiouridine (4sU) to a final concentration of 75 µg/mL.
3. Incubate culture at 32°C for the duration of the pulse (30-40 min).
   Note: Similar results were obtained with shorter pulses (Amorim et al. 2010; Hasan et al. 2014).
4. Centrifuge cells for 3 min at 2,000 x g; discard the supernatant and freeze the pellet at -80°C.

RNA extraction

Prepare total RNA from the 4sU labeled culture according to Protocol 1, scaling up or down as necessary. Use two separate tubes to extract RNA from 5 x 10^8 cells. The yield of RNA from 2.5 x 10^8 cells should be 300 – 400 µg. Stop after step 17 and freeze the RNA at -80°C*.*

RNA biotinylation

The protocol below is for 200 µg of total RNA, which should yield ~2 µg of biotinylated RNA. 100 µg should be sufficient to allow accurate half-life determination, for example in a time course experiment.

1. Prepare the following reaction:

	Volume (µL)
Biotin-HPDP 1mg/mL in DMSO	400
1 M Tris pH7.6	20
0.5 M EDTA	4
200 µg RNA in water	x
ddH_2O	2 mL final

2. Incubate at room temperature for 90 min.
3. During the incubation, purify total RNA to be used as the reference and prepare the streptavidin beads as described below.
4. Transfer the biotinylation reaction to phase-lock tubes (500 µL into each).

5. Add 500 µL of chloroform:isoamyl alcohol (24:1) and mix by inversion.
6. Spin for 15 min in a microcentrifuge at 20,000 x g at RT.
7. Transfer the upper phases (400 µL from each tube) to 2 mL Eppendorf tubes (800 µL in each).
   Note: Avoid taking the interphase; sometimes the intermediate phase in the phase-lock tubes may not separate well, possibly due to the DMSO in the reaction.
8. Add 1 volume of isopropanol and 1/10 volume of 5 M NaCl.
9. Incubate at -80°C for 30 min (can be left overnight).
   Note: This is a good point to stop if you do not want to complete the entire purification in one day.
10. Centrifuge for 15 min at 25,000 g at RT.
11. Discard supernatant, add 500 µL 75% Ethanol and spin for 1 min. Aspirate most of the supernatant, spin for 5 sec, and remove remaining liquid with a pipette. Air- dry the pellet for 2 min at RT.
12. Resuspend in 20 µl of water (each tube), pool into a single tube and proceed to the purification step (see below).

Total RNA purification

During the biotinylation reaction, further purify the total thiouridine-labeled RNA as in steps 18-20 of Protocol 1 for use as a reference. Briefly, pass 200 µg of the RNA preparation through a Qiagen RNeasy column. Elute in 30 µL of water and use the same water for a second elution. Quantify using a nanodrop spectrophotometer and prepare aliquots containing 20 µg of RNA in 12 µL to use in labeling reactions.

Preparation of streptavidin beads

Prepare ~3.5 mL of MPG buffer for the beads and ~3.5 mL for each sample

1. Use 100 µL beads/sample.
2. Wash 3x with 500 µL MPG buffer at room temperature (all washes are quick).
3. Re-suspend in 400 µL MPG buffer.
4. Add 20 µL tRNA to block the beads.
5. Incubate at RT (with rocking) for at least 30 min.
6. Wash 3x with 300 µL MPG buffer.
7. Resuspend in 160 µL MPG buffer.

Purification of biotinylated RNA

1. Heat RNA at 65°C for 5 min before cooling to RT for 1 min.
2. Add 160 µL of streptavidin beads equilibrated in MPG buffer as described above to 40 µL of biotinylated RNA.
3. Incubate at RT for 40 min with rocking.
4. Place tube in magnetic rack and remove the buffer once all the beads have been attracted to the side. Add 500 µL MPG preheated to 65°C (added hot, then incubated at RT for 5 min with rocking).
5. Repeat step 3 twice more.
   Note: If there are problems with bead retrieval, the best strategy is to find a stronger magnet. Alternatively, tubes can be centrifuged for 3 sec before placing in the rack.
6. Wash 3x with 500 µl MPG at RT (5 min with rocking).
7. Elute RNA from beads by reducing the linker with 5% β-mercaptoethanol (BMETH). Resuspend the beads in 50 µL of BMETH and incubate at RT for 5 min.
   Note: 100 mM DTT can be used in place of 5% BMETH but the latter is preferred.
8. Recover the supernatant.
9. Repeat steps 6 and 7.
10. Pool the supernatants from steps 8 and 9.
11. Further purify RNA from the pooled supernatants using RNAqueous micro columns.
12. Add 5 volumes of RNAqueous lysis buffer (500 µL) to the sample, mix and add 2.5 volumes of ethanol (250 µL).
13. Purify as described in the RNAqueous manufacturer's protocol. Load mixture onto columns in two rounds (the volume is too large to fit into the column all at once).
14. Wash 3 times with 180 µL of wash solution I, then twice with solution 2/3 . In between washes, centrifuge at 20,000 x g for 1 min.
15. Switch column to a new eppendorf tube and centrifuge for 1 min at 20,000 x g to dry the column.
16. Elute twice with 8 µL of RNase free water at 85 °C in the same microfuge tube. For elution, incubate the column and microfuge tube at 85°C for 1 min. Use 1 µL to quantify the RNA in a spectrophotometer.
17. Use the remainder of the RNA (should be ~12 µL) for half-life analysis by microarray analysis.

Half-life determination:

Fission yeast mRNA half-lives can be determined by analyzing data from 4sU labeling experiments using equations developed for studies in other organisms (Dolken et al 2008). Briefly, microarray analysis is used to produce an unnormalized measure of the relative abundance of a given 4sU-labeled (nascent) transcript compared with its abundance in total mRNA. See Amorim et al. (2010) and Hasan et al. (2014) for additional details.

Reagents: EZ-Link® Biotin-HPDP

4-thiourudine Prepare a 7.5 mg/mL solution (100X stock) in ddH_2O*; can be stored at -20°C but generally better to prepare fresh*

MPG buffer:
100 mM Tris pH7.6
1 M NaCl
10 mM EDTA
Prepare using Ambion nuclease free H_2O.

4-thiouridine:
Prepare a 1 mg/mL solution in DMSO (store at 4°C and avoid excessive freezing and thawing).

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