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Protocol

Schizosaccharomyces pombe Polysome Profile Analysis and RNA Purification

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Polysome profile analysis is widely used by investigators studying the mechanism and regulation of translation. The method described here uses high-velocity centrifugation of whole cell extracts on linear sucrose gradients 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. After centrifugation, the gradient is fractionated and RNA (and/or protein) is prepared from each fraction for subsequent analysis of individual species using northern or western blots. The entire RNA population in each fraction can be analyzed by hybridization to microarrays or by high-throughput RNA sequencing, and the proteins present can be identified by mass spectrometry analysis.

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MATERIALS

It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous material used in this protocol.

RECIPES: Please see the end of this protocol for recipes indicated by <R>. Additional recipes can be found online at http://cshprotocols.cshlp.org/site/recipes.

Reagents

Cycloheximide (100 mg/mL in H_2O) (optional; see Step 1)
Ethanol (85% [v/v] in water) (optional; see Step 18)
Ethanol (100%)
Ethylenediaminetetraacetic acid (EDTA) (500 mM) (optional; see Step 4.ii)
Guanidinium hydrochloride (8 M in double-distilled H_2O) (optional; see Step 16.iv)
Isopropanol
Polyribosomal lysis buffer <R> (for nonpuromycin–treated isolation procedures)
Polyribosomal lysis buffer with puromycin <R> (for puromycin-treated isolation procedures only)
Puromycin dihydrochloride (1 mM) (optional; see Step 4.i)

Puromycin is a tRNA mimic that causes polypeptide chain termination.

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From the Fission Yeast collection, edited by Iain M. Hagan, Antony M. Carr, Agnes Grallert, and Paul Nurse.

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Cite this protocol as *Cold Spring Harb Protoc*; doi:10.1101/pdb.prot091637

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Cold Spring Harbor Laboratory Press

RNase I (Ambion) (optional; see Step 4.vi)
RNase A (optional; see Step 4.iv)
RNase T1 (Ambion) (optional; see Step 4.iv)
RNeasy kit (QIAGEN)
S. pombe cells ($0.5–1.0 \times 10^7$ cells/mL)
Sucrose gradient solutions (10%, 50%, and 55% [w/v], prepared in polyribosomal lysis buffer)
Water, RNase-free

Equipment

Beads, zirconia (0.5 mm diameter) (Biospec; Sigma-Aldrich)
Centrifuge, benchtop, equipped with swing-out rotor for 50-mL tubes
FastPrep device (MP Biomedicals)
Gradient fractionator (Brendel or ISCO)
Gradient maker (e.g., Biocomp or Sigma-Aldrich)
Microcentrifuge
Microcentrifuge tubes (flip-top, 1.5-mL)
Microcentrifuge tubes (screw-cap, 1.5-mL)
Microscope, phase contrast/bright field, equipped with 40× objective and 10× eyepieces
Needle (18-gauge)
Spectrophotometer (NanoDrop)
Tubes, glass (to fit the fraction collector)
Tubes (screw-cap, 50-mL) (Beckman)
Tubes, ultracentrifuge, polyallomer
Ultracentrifuge (e.g., Beckman Optima X), equipped with Sw40Ti or Sw41Ti rotors

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METHOD

1. Harvest $0.3–0.7 \times 10^9$ mid-log phase cells by pouring the culture directly into 50-mL screw-cap centrifuge tubes on ice containing cycloheximide at a final concentration of 100 µg/mL. Centrifuge for 4–5 min at 670–1000*g* or 3 min at 2000*g*. Discard the culture fluid by decanting carefully.

   Alternatively, add cycloheximide to the culture 5 min before harvesting to allow time for the inhibitor to work. In this case, pellet cells by centrifugation immediately (i.e., without additional added cycloheximide).

   For studies in which polysome disruption involves treatment with puromycin (see Step 4.i), omit the cycloheximide treatment from Step 1, and use polyribosomal lysis buffer with puromycin throughout the protocol in place of the standard polyribosomal lysis buffer.

2. Wash cells with 0.9 mL of polyribosomal lysis buffer to remove residual media. Resuspend the pellet in 200 µL of ice-cold polyribosomal lysis buffer.

3. Transfer the cell suspension to prechilled 1.5-mL screw-cap microcentrifuge tube containing ~600 µL of zirconia beads. Lyse in a FastPrep device at level 6.0 for 18 sec (shorter times down to 13 sec can also be used).

   Check an aliquot of the suspension using a microscope to ensure that at least 50%–70% of the cells have been lysed. If >30%–50% cells remain intact, repeat the FastPrep step.

4. (Optional) Disrupt polysomes as necessary using one of the following methods:

   ### *To induce polypeptide chain termination*

   i. Treat lysate with 1 mM puromycin for 15 min.

      Alternatively, add 1 mM puromycin to the cells for 1–10 min before disruption.

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To dissociate ribosomal subunits

ii. Add 25 µL of the 500 mM EDTA stock solution to 500 µL cell lysate, that is, to a final concentration of 25 mM.

iii. Incubate for 15 min at room temperature.

To cleave RNA between individual ribosomes (Method 1)

iv. Add RNase A and RNase T1 to final concentrations of 25 and 1000 U/mL, respectively, to the cell lysate.

v. Incubate for 15 min at room temperature.

To cleave RNA between individual ribosomes (Method 2)

The efficacy of this procedure can vary with the batch of RNase and/or strain of yeast under investigation.

vi. Add 750 U of RNase I to a cell lysate suspension with an OD_{260} of 40 units.

vii. Incubate for 30 min at room temperature.

5. After lysis (and any subsequent dissociative treatments), poke a hole in the bottom of the screw-cap microcentrifuge tube using an 18-gauge needle.
6. Insert the perforated tube inside a 1.5-mL flip-top microcentrifuge tube. Separate the liquid from the beads in the upper tube by centrifugation at 5000*g* for 5 min at 4°C. Reserve the liquid collected in the flip-top tube.
7. Add 250–350 µL of polyribosomal lysis buffer to the beads in the perforated tube. Repeat Step 6. Combine the flowthroughs from Steps 6 to 7.
8. Clear the lysate by centrifugation at 20,000*g* for 15 min at 4°C.
9. Add 2 µL of the lysate to 98 µL of dH_2O. Measure the OD_{260} of the diluted solution in a NanoDrop spectrophotometer.
10. Prepare 11-mL linear (10%–50%) sucrose gradients.

    Sucrose gradients can be stored in a cold room for up to 4 h at 4°C, with or without lysate layered on top. Do not store in a refrigerator; vibrations from the motor can disturb the gradient. See Pospísek and Valásek (2013) for details on gradient separation.

11. Dilute aliquots corresponding to 20–25 OD_{260} units to 350 µL with lysis buffer (with or without the protease inhibitor cocktail, heparin and RNasin). Layer onto the sucrose gradients.

    When comparing polysome profiles from different cells, it is important to load the same number of OD units.

12. Centrifuge the sucrose gradients at 4°C in a Beckman SW40Ti rotor at 39,000 rpm (~277,000*g*) for 150 min or a Beckman SW41Ti rotor at 35,000 rpm (~246,000*g*) for 160 min.

    It is important to precool the centrifuge and all rotor parts.

13. Remove gradients carefully to avoid disturbing the separation.
14. Prewash the tubing through which the fractionated samples will pass with ethanol, then with water, and finally with sucrose solution.
15. Fractionate each tube by upward displacement with 55% sucrose using a gradient fractionator:

    The precise manipulations will vary depending on the exact combination of fractionator, UV monitor, and fraction collector. Some fractionators require a warming-up period.

Using a Brendel fractionator

i. Set the flow rate at 800 µL/min.

Using an ISCO fractionator

ii. Use a pump speed of 50%, 10× (~1.25 mL/min).

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16. From each gradient, collect 12–13 fractions of 800–900 µL each:

For protein isolation

i. Collect fractions into empty glass tubes.

For RNA isolation (Method 1)

ii. Collect fractions into tubes prefilled with 1.9–2.1 mL 100% isopropanol.
The final isopropanol concentration should be ~70%.

iii. Mix each sample with the isopropanol. Precipitate overnight at −20°C.

For RNA isolation (Method 2)

iv. Collect fractions into tubes prefilled with 2 mL of 8 M guanidinium hydrochloride.
The final concentration after collection should be 5.5 M.

v. Add 1 volume of 100% ethanol. Mix by inversion. Precipitate overnight at −20°C.

17. After RNA precipitation (i.e., Step 16.iii or Step 16.v), centrifuge samples for 20 min at >10,000*g* at 4°C. Discard the supernatant.
Alternatively, centrifuge at 3000g for 90 min.

18. (Optional) Wash pellets with 1 mL of 85% ethanol (see Protocol: **Preparation of Total RNA from Fission Yeast** [Bähler and Wise 2016] for tips to avoid losing the pellet).

19. Air-dry the RNA pellets for 20 min. Resuspend in 100 µL of RNase-free water.

20. Purify RNA using a QIAGEN RNeasy kit according to the manufacturer's instructions.

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RELATED INFORMATION

For additional information on the use of polysome profile analysis to study the mechanisms and regulation of translation, see Rallis et al. (2014). Procedures for RNA analysis by microarray hybridization are described in Lackner et al. (2012).

RECIPES

Polysomal Lysis Buffer

Tris-HCl (pH 7.5)	20 mM
KCl	50 mM
$MgCl_2$	10 mM
Cycloheximide	100 µg/mL
Dithiothreitol (DTT)	1 mM
cOmplete protease inhibitor cocktail tablets, EDTA-free (Roche)	2 tablets/100 mL culture
Heparin	0.2 mg/mL
RNasin (Promega)	1 µL/mL
Phenylmethylsulfonyl fluoride (PMSF)	1 mM

Combine the first three ingredients. (If desired, a 10× stock solution containing 200 mM Tris-HCl, 500 mM KCl, and 100 mM $MgCl_2$ can be prepared and stored on a long-term basis.) Just before use, add the cycloheximide and DTT, as well as the protease inhibitor tablets, heparin, and RNasin (the latter three are optional but highly recommended). For processing meiotic cells, include PMSF.

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Polysomal Lysis Buffer with Puromycin

Tris-HCl (pH 7.5)	20 mM
KCl	500 mM
$MgCl_2$	2 mM
Puromycin	1 mM
Dithiothreitol (DTT)	1 mM
cOmplete protease inhibitor cocktail tablets, EDTA-free (Roche)	2 tablets/100 mL culture
Heparin	0.2 mg/mL
RNasin (Promega)	1 µL/mL
Phenylmethylsulfonyl fluoride (PMSF)	1 mM

Combine the first three ingredients. Just before use, add the puromycin and DTT, as well as the protease inhibitor tablets, heparin, and RNasin (the latter three are optional but highly recommended). For processing meiotic cells, include PMSF.

ACKNOWLEDGMENTS

D.A.W. was supported by the National Institutes of Health USA, J.B. was supported by the Wellcome Trust UK, and J.A.W. was supported by the National Science Foundation USA. This protocol is an amalgamation of procedures followed in the laboratories of Dieter Wolf and Jürg Bähler. The authors thank Charalampos Rallis (University College London, UK) for helpful comments.

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