STEP-BY-STEP RSR PROTOCOL

I. Yeast cell-free extract preparation

Prepare translationally active Cell-Free Extract (CFE) from the *Saccharomyces cerevisiae* BY4741 strain following the cryo-grinding protocol described in detail in (Trainor et al. 2021). Aliquot CFE into RNase-free 1.7 mL microcentrifuge tubes (100 μL per tube) and determine RNA and protein concentrations. Store the aliquots at -80°C.

Note: In our experience, the typical RNA concentration in a CFE prepared with the protocol described in (Trainor et al. 2021) is ~4-6 μg/μL, and the protein concentration is ~12-16 μg/μL.

Note: CFE is prepared in buffer A: 20 mM Hepes-KOH (pH 7.4), 100 mM KOAc, 2 mM $Mg(OAc)_2$, 2 mM DTT.

II. Preparation of ribosomes and the ribosome-free extract for RSR assays

Note: Throughout the procedure, care should be taken to avoid sample contamination with RNases. Make sure all solutions are RNase-free. Use RNase-free tubes and filtered tips. Wear gloves and if necessary, clean the bench, pipets and tube racks with RNase AWAY (Thermo Scientific) or a similar surface decontaminant.

A. *Preparation of ribosomes from CFE by one-step precipitation*

1. Thaw an aliquot of CFE by warming the tube in your hand and periodically tapping it gently to mix the content.

2. As soon as the CFE is thawed, place the tube on ice. Transfer CFE into a pre-chilled thick-wall 1.5 mL polypropylene ultracentrifuge tube (Beckman, cat# 357448).

3. Place tubes in a TLA55 Beckman rotor and centrifuge at 180,000 × g (55,000 rpm) for 2 h at 4°C.

4. When the centrifugation is complete, collect the supernatant with a P200 pipet and place it into a 1.7 mL microcentrifuge tube. This is the translationally active, ribosome-free lysate fraction (S180) that will be used in the translation reactions.

Note: do not touch the pellet while collecting S180. Leave a small volume of S180 (~20-30 μL) above the pellet while collecting the S180.

Note: we do not recommend any freezing and thawing of the S180 fraction.

5. The remaining pellet in the tube (P180) is enriched with translationally active ribosomes. Quickly rinse the P180 with 500 μL of cold buffer A, remove the rinse solution at once.

Note: Do not touch the pellet with a pipet tip! The pellet is very sticky, and touching it results in losing the material.

6. To resuspend the P180, add 100 μL of buffer A, place the tube into an Eppendorf Thermomixer set at 21°C/1200 rpm. Incubate with shaking for 30 min.

7. Centrifuge the resulting P180 suspension at 21,000 × g for 15 min in a tabletop centrifuge at 4°C. Collect the supernatant and transfer it into a new 1.7 mL microcentrifuge tube, avoiding any pelleted fraction.

8. Measure the RNA concentration of the resuspended ribosomes spectrophotometrically. These ribosomes are ready to be used in translation reactions.

B. *Purification of ribosomes from CFE through a 20% glycerol cushion*

9. Add 500 μL of 20% glycerol prepared in buffer A into a 1.5 mL polypropylene ultracentrifuge tube from Beckman. This is the glycerol cushion.

10. Layer 100 μL of CFE onto the cushion.

11. Centrifuge CFE through the cushion at 180,000 × g (55,000 rpm) for 2 h at 4°C in a TLA55 Beckman rotor.

12. Discard the supernatant. To aspirate the supernatant, use either a P1000 pipet or a P200 tip attached to a vacuum source. In the latter approach, be careful to avoid touching the pellet.

13. Rinse the pellet (P180) with cold buffer A and proceed with pellet resuspension as described in steps 5-8.

C. *Purification of ribosomes from cells*

14. Collect yeast cells (a total of 60-70 OD_{600} units) by centrifugation at 2,200 × g for 3 min at 4°C. We use an Eppendorf centrifuge 5810R equipped with the A-4-62 rotor at 3,300 rpm.

15. Resuspend cell pellet in 10 mL of ice-cold buffer A supplemented with 200 μg/mL heparin. Pellet cells as in step 14, then resuspend the cells in 2 mL of buffer A/heparin, transfer cell suspension to a 15 mL RNase-free conical centrifugation tube, and pellet cells again.

16. Lyse cells in 1 mL buffer A/heparin using 425-600 μm glass beads by 10–12 cycles of 30-sec vortexing, each followed by a 30-sec incubation on ice.

17. Centrifuge lysate at 3,200 × g for 10 min at 4°C. We use an Eppendorf centrifuge 5810R (A-4-62 rotor) at 4,000 rpm.

18. Transfer the crude lysate supernatant into a 1.7 mL microcentrifuge tube and centrifuge at 21,000 × g for 15 min in a tabletop centrifuge at 4°C.

19. Collect the supernatant while avoiding the pelleted fraction.

20. Layer ~500 μL of the supernatant onto 500 μL of 20% glycerol cushion prepared in buffer A (see step 9).

21. Proceed as described in steps 11-13.

III. Generation of mRNA reporters for translation reactions

A. *Amplification of the reporter DNA sequence by PCR*

22. If the reporter gene is cloned in a plasmid containing the T7 promoter, design the forward primer to anneal upstream of the T7 sequence, and the reverse primer to anneal downstream of the reporter gene sequence. If the T7 promoter is not available, incorporate the T7 promoter sequence (5'-TAATACGACTCACTATAGGG-3') into the forward primer.

23. Perform the PCR reaction and check a small aliquot ($1/10^{th}$ of the reaction volume) on a 1% agarose gel. Use a molecular weight marker to determine the size of the PCR product. If you detect additional bands, repeat the PCR reaction with an increased primer annealing temperature to achieve a single band corresponding to the reporter gene's size.

24. Purify/concentrate the PCR product using a DNA clean-up spin column kit (ZYMO cat# D4004 or analogous). Follow the protocol provided with the kit. Elute the purified PCR product from the columns with RNase-free H_2O. Measure DNA concentration spectrophotometrically.

B. *Generation of m7G-capped mRNA*

25. Use 1 μg of the PCR product from step 24 for a transcription/capping reaction with the mMESSAGE mMACHINE™ T7 transcription kit. Follow the protocol provided with the kit.

26. Precipitate generated m7G-capped mRNA with LiCl supplied with the mMESSAGE mMACHINE™ T7 transcription kit. For precipitation, follow the protocol provided with the kit.

27. Resuspend RNA in RNase-free H_2O and measure the RNA concentration spectrophotometrically. Working on ice, aliquot RNA into RNase-free tubes and immediately freeze aliquots at -80°C.

Note: We do not recommend multiple freeze-thaw cycles of the RNA aliquots.

IV. RSR translation reactions

A. *Translation reaction with a reporter mRNA*

28. Prepare 10× Energy Mix (10×EM). For 1 mL of 10×EM: mix 200 μL of 1M HEPES-KOH pH 7.4, 100 μL of 100 mM ATP, 10 μL of 100 mM GTP, 400 μL of 500 mM creatine phosphate, 20 μL of 1M DTT and 270 μL of RNase-free H_2O. Aliquot 10×EM RNase-free tubes (30 μL per aliquot) and store at -80°C.

29. Prepare the 2.5× translation reaction master mix. Combine 45.8 μL of H_2O, 5 μL of 2M KOAc, 4 μL of 100 mM MgOAc, 20 μL of 10×EM from step 28, 2 μL of 1 mM amino acids, 2 μL of RiboLock (40 units/μL), 1.2 μL of creatine kinase.

30. Per one reaction (15 μL) of the RSR assay, mix 6 μL of 2.5× master mix from step 29, 1.5 μL of capped mRNA from step 27 (final amount of mRNA may range between 100-400 ng), 6 μL of S180 from step 4 and 1.5 μL of ribosomes (steps 8, 13 or 21).

31. *Optional:* For a control reaction with the complete CFE, mix 6 μL of 2.5× master mix from step 29, 1.5 μL of capped mRNA from step 27 and 7.5 μL of CFE described in section I.

32. Incubate reactions (RSR, step 30, or CFE, step 31) at 21°C for 60-180 min, depending on experimental goals.

B. *Translation reaction using endogenous S180-derived mRNAs*

33. Prepare the 2× translation reaction master mix. Combine 56.8 μL of H_2O, 5 μL of 2M KOAc, 4 μL of 100 mM MgOAc, 20 μL of 10×EM (step 28), 2 μL of 1 mM amino acids (-Met, -Cys), 2 μL of RiboLock (40 units/μL), 1.2 μL of creatine kinase, and 9 μL of EasyTag EXPRESS^{35}S Protein Labeling mix, [^{35}S], 11 mCi/mL (Perkin-Elmer).

34. Per one reaction (15 μL) of the RSR assay, mix 7.5 μL of 2× master mix from step 33; 6 μL of S180 from step 4 and 1.5 μL of ribosomes (step 8, 13, or 21).

35. Incubate reactions at 21°C for 5-60 min, depending on experimental goals.

References

Trainor BM, Komar AA, Pestov DG, Shcherbik N. 2021. Cell-free Translation: Preparation and Validation of Translation-competent Extract From Saccharomyces cerevisiae. *BioProtocols*. DOI: 10.21769/BioProtoc.4093. ***In press.***