

Purification of Mitochondrial Ribosomes with the Translocase Oxa1L from HEK Cells

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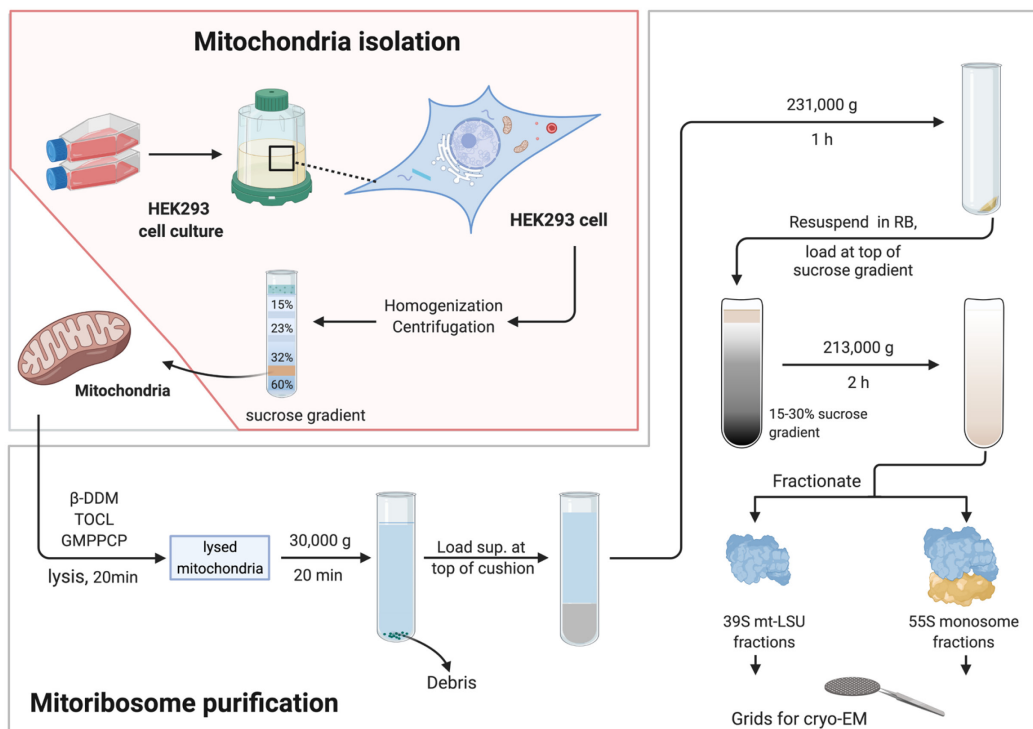
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[Abstract] Mitochondrial ribosomes (mitoribosomes) perform protein synthesis inside mitochondria, the organelles responsible for energy conversion and adenosine triphosphate (ATP) production in eukaryotic cells. To investigate their functions and structures, large-scale purification of intact mitoribosomes from mitochondria-rich animal tissues or HEK cells have been developed. However, the fast purification of mitoribosomes anchored to the mitochondrial inner membrane in complex with the Oxa1L translocase remains particularly challenging. Herein, we present a protocol recently developed and modified in our lab that provides details for the efficient isolation of intact mitoribosomes with its translocase Oxa1L. We combined the cell culture of PDE12^{-/-} or wild-type HEK293 cell lines with the isolation of mitochondria and the purification steps used for the biochemical and structural studies of mitoribosomes and Oxa1L.

Graphic abstract:



Schematic procedure for the purification of mitoribosomes from HEK cells. The protocol described herein includes two main sections: 1) isolation of mitochondria from HEK cells; and 2) purification of mitoribosome-Oxa1L from mitochondria. RB: Resuspension Buffer (see Recipes) (Created with [BioRender.com](https://www.biorender.com)).

Keywords: Mitochondria, Ribosome purification, Mitoribosome, Oxa1L, Biochemistry, Cryo-EM

[Background] Human mitochondria possess their own genome and house all the components necessary for transcription, RNA maturation, and translation of the encoded genetic information (Dennerlein *et al.*, 2017). The mitoribosome, one of the most important machineries within mitochondria, is responsible for the translation of essential mitochondrial mRNAs encoding components of oxidative phosphorylation (OXPHOS) complexes (Ott *et al.*, 2016). Since all proteins translated by the human mitoribosome are membrane proteins, knowledge of the mechanism of co-translational membrane protein insertion is central to understanding mitoribosome translation (Hildenbeutel *et al.*, 2012; Stiller *et al.*, 2016). In human mitochondria, the oxidase assembly 1-like (Oxa1L) translocase plays a central role in the membrane insertion of mitochondrially encoded products (Haque *et al.*, 2010; Stiller *et al.*, 2016); however, due to the unique features of mitochondria, little is known regarding the molecular and quality control mechanisms underlying mitochondrial translation.

Owing to the structures obtained by single-particle electron cryo-microscopy (cryo-EM), opportunities now arise to comprehensively study the molecular mechanisms underlying the translational action and quality control of the human mitoribosome (Rathore, 2020). Although the existence of quality control in mitochondria has been predicted based on analogy with bacterial and eukaryotic cytosols (Ayyub *et al.*, 2020), biochemical or structural evidence of ribosome-associated quality control in mitochondria remain elusive. We reasoned that any attempt to induce translational stalling may trap mitoribosomes in various stages of the translation cycle during the act of nascent chain insertion into the inner mitochondrial membrane, and generate intermediates suitable for structural analysis by cryo-EM. To this end, we purified mitoribosomes from a genetically engineered human cell line lacking the 2'-5' phosphodiesterase exonuclease 12 (PDE12). PDE12 facilitates the maturation of mt-tRNA; therefore, a PDE12 knockout can lead to aberrant polyadenylation of the 3' ends of mt-tRNAs and, consequently, mitoribosome stalling (Rorbach *et al.*, 2011; Pearce *et al.*, 2017). As expected, we observed a substantial proportion of stalled mitoribosomes and discovered a mitoribosome-associated quality control pathway (mtRQC) to rescue the elongational stalling ribosomes (Desai *et al.*, 2020). Our report of the first mitoribosome-associated quality control pathway provides novel insights into the regulation of mitochondrial translation. Since the cell needs to detect and respond rapidly even to subtle changes in translation rates, quality control is intimately coupled to elongation. During purification, we included GMPPCP, a non-hydrolysable GTP analog, to prevent dissociation of GTPases from mitoribosomes, and successfully captured five additional structures of virtually every elongating ribosomal state. Purifying the mitoribosome in complex with its translocase Oxa1L was challenging since membrane proteins are unstable and tend to dissociate during complicated isolation steps. Accordingly, we added

n-dodecyl β -D-maltoside (β -DDM) along with cardiolipin to solubilize mitoribosomes anchored to the mitochondrial inner membrane via the Oxa1L translocase. We also trapped Oxa1L on all our active and stalled ribosomes, giving us a first glimpse at the co-translational insertion of mitochondrial inner membrane proteins.

This protocol was modified from prior methods developed in our lab (Amunts *et al.*, 2015; Brown *et al.*, 2017) and by others (Greber *et al.*, 2014 and 2015; Aibara *et al.*, 2018), as well as in our current work (Desai *et al.*, 2020). The method described here can be used to obtain mitoribosomes in complex with Oxa1L, which has advantages such as the applicability for mitochondria isolation from large volumes of suspension cell culture, the use of common media and buffers, ease of handling, and absence of additional affinity purification steps. In our previous publication and experiments, we observed a good reproducibility between HEK293 cell batches using this methodology.

Materials and Reagents

1. T75/T175 flasks (Thermo Scientific™)
2. 1 L/2 L Tissue Culture-treated roller bottles (Corning™)
3. 50 ml/250 ml/5 L polycarbonate Erlenmeyer flasks (Corning™)
4. Teflon/glass Dounce homogenizers (Cambridge Glassblowing Limited)
5. Miracloth (Merck Millipore Calbiochem, catalog number: 475855)
6. SW40 tubes for mitochondria gradient (Beckman, catalog number: 344060)
7. Polycarbonate bottle with cap assembly for type 70Ti rotor, for cushion (Beckman, catalog number: 355618)
8. TLS-55 tubes for mitoribosome gradient (Beckman, catalog number: 347356)
9. Dulbecco's Modified Eagle Medium (Gibco™, Life technologies)
10. Tetracycline-free fetal bovine serum (Gibco™, Life technologies)
11. Freestyle 293 Expression medium (Gibco™, Life technologies)
12. Dnase (RNA-free) (HT Biotechnology, N401a)
13. n-Dodecyl β -D-maltoside (Anatrace, catalog number: D310)
14. 18:1 Cardiolipin (TOCL; Avanti, catalog number: 710335C)
15. β , γ -Methyleneguanosine 5'-triphosphate sodium salt (GMPPCP; Sigma, catalog number: M3509)
16. Protease inhibitor (cOmplete™, Roche)
17. Rabbit anti-mS27 (Abcam, catalog number: ab67157)
18. Mouse anti-OXA1L (Abcam, catalog number: ab88975)
19. Goat anti-mouse Alexa Fluor Plus 800 (ThermoFisher, catalog number: A32730)
20. Goat anti-rabbit Alexa Fluor Plus 680 (ThermoFisher, catalog number: A32734)
21. MIB Buffer (Mitochondria isolation buffer, see Recipes)
22. SM4 Buffer (Sucrose/mannitol buffer, see Recipes)
23. MIBSM Buffer (MIB+SM4: Experimental buffer, see Recipes)

24. SEM Buffer (Sucrose EDTA MOPS buffer, see Recipes)
25. 60%, 32%, 23%, and 15% Sucrose Gradient Buffer (see Recipes)
26. Lysis Buffer (see Recipes)
27. Sucrose Cushion Buffer (see Recipes)
28. Resuspension Buffer (see Recipes)
29. 15-30% Linear Sucrose Gradient Buffer (see Recipes)

Equipment

1. Automated cell counter (Countess[®] II FL)
2. Avanti-J26 XP centrifuge (Beckman Coulter)
3. High speed centrifuge (Beckman Coulter, model: Avanti J-26XP)
4. Ultracentrifuge (Beckman Coulter, model: Optima L-80 XP)
5. Tabletop ultracentrifuge (Beckman Coulter, model: Optima[™] MAX)
6. Centrifuge (Eppendorf, model: 5430 R)
7. NanoDrop[®] 2000 (Thermo)
8. Odyssey[®] CLx Infrared Imaging System (LI-COR Biosciences)
9. Vitrobot Mark IV (Thermo Fisher Scientific)
10. Titan Krios microscope (Thermo Fisher Scientific FEI)

Procedure

A. Cell Culture

1. Maintain PDE12^{-/-} HEK293 cells in T75 flasks in DMEM supplemented with 10% FBS, at 37°C and 5% CO₂. Cells should be ready for transfer to a T175 flask in ~2-3 days.
2. Scale up by splitting every 2-3 days until the target cell mass is reached. At least 8 flasks with 30 ml cells per flask is recommended as a starting point for adapting to suspension culture.
3. At 90% confluence, detach cells with 0.05% trypsin-EDTA for 10-15 min at 37°C, and monitor the cell rounding. Centrifuge at 200 × g to remove trypsin, then resuspend the pelleted cells 2-3 times in 10-20 ml Freestyle medium with 1% FBS to wash out old media.
4. Resuspend the detached cells in 30-50 ml Freestyle media with 1% FBS, adjust cell density with media to around 0.5 × 10⁶ cells/ml, and grow at 37°C, 8% CO₂, 120 × g.
5. Scale up by diluting and splitting with pre-warmed fresh Freestyle media with 1% FBS. Count cells using an automated cell counter and adjust cell concentration to 0.5 × 10⁶ cells/ml.
6. Observe and count the cells every other day, and proceed to split/dilute the cells if the cell density is above 1.5 × 10⁶ cells/ml.
7. The final cell density can range from 0.8 to 1.5 × 10⁶ cells/ml, and the isolation of mitochondria requires at least 10 L as a starting point (Please see *Notes* for starting volume of wild-type).

8. Harvest cells by centrifugation at $1,000 \times g$ for 7 min at 4°C . Decant the supernatant carefully and resuspend the pelleted cells quickly in pre-cooled PBS to wash out the media.
9. Centrifuge the resuspended cells at $1,200 \times g$ for 10 min at 4°C . Decant the supernatant carefully and weigh the pellet. Continue to isolate the mitochondria from harvested cells, or snap-freeze the cells in liquid nitrogen and store at -80°C .

B. Mitochondria isolation

1. Pre-cool the Teflon/glass Dounce homogenizer O/N prior to use.
2. Resuspend the pellet (e.g., 10 g) in 60 ml MIB buffer.
3. Allow cells to swell by gently stirring in a 4°C cold room for 15 min.
4. Add 20 ml SM4 buffer (1/3 of MIB buffer volume).
5. Dounce homogenize the samples (> 60 up-and-down passes).
6. Centrifuge the homogenized sample at $800 \times g$ for 15 min at 4°C . Collect supernatant (Supernatant-1) through a Miracloth (or other cheese cloth with a typical pore size of $\sim 22\text{-}25 \mu\text{m}$). Keep the pellet.
7. Resuspend the pellet in 20 ml MIBSM (3:1 MIB:SM) buffer, homogenize manually (~ 15 up-and-down passes), then centrifuge at 4°C , $800 \times g$ for 15 min.
8. Collect supernatant (Supernatant-2) through a Miracloth and combine with Supernatant-1 from B-6. Centrifuge at $1,000 \times g$ for 15 min at 4°C . Collect supernatant.
9. Centrifuge the supernatant from the previous step at $10,000 \times g$ for 15 min at 4°C .
10. Keep pellet, but carefully wash out loose pellet without disturbing the tight portion.
11. Resuspend in 10 ml MIBSM buffer, add 100 U RNase-free DNase I per 10 g cells, rotate on a roller in a cold room for 20 min.
12. Centrifuge at $10,000 \times g$ for 15 min at 4°C .
13. Resuspend the pellet in ~ 2 ml SEM buffer and homogenize gently with a small Dounce homogenizer. Perform no more than five up-and-down passes.
14. Prepare a 15-23-32-60% sucrose gradient in SW40 tubes. Place 1.5 ml of the 60% sucrose stock buffer into the Beckman Ultra-Clear centrifuge tube. Carefully overlay 4.5 ml of the 32% sucrose stock buffer. Repeat with 1.5 ml of the 23% sucrose stock buffer and then with 1.5 ml of the 15% sucrose stock buffer.
15. Load resuspended crude mitochondrial sample from Step B13 on top of the 15% sucrose in the density gradient prepared in Step B14. Centrifuge in an SW40 rotor at $28,000 \times g$ for 1 h at 4°C .
16. The intact mitochondria form a brown band at the 60%-32% sucrose interface. Carefully remove the sucrose from the top until reaching the mitochondrial band, then take the brown band with a pipette at the interface of 32% and 60% sucrose.
17. Snap-freeze the purified mitochondria in liquid nitrogen and store at -80°C .

C. Mitoribosome-Oxa1L purification

1. Defrost the frozen mitochondria on ice.

2. Add 2 volumes of lysis buffer to mitochondria (e.g., Add 8 ml lysis buffer to 4 ml mitochondria). Mix immediately by inverting the tube several times.
3. Homogenize with a small Teflon/glass Dounce homogenizer to assist lysis.
4. Rotate on a roller in a cold room for 20 min to complete the lysis.
5. Centrifuge the lysed material (approximately 9 ml) at $30,000 \times g$ for 20 min at 4°C to remove the insoluble material. Decant the supernatant carefully from the pellet and discard the pellet.
6. Prepare the sucrose cushion in Ti70 (tube vol approx. 24 ml): underlay 7 ml 1 M (34%, w/v) Sucrose Cushion Buffer at the bottom of the tube.
7. Layer approximately 17 ml lysed mitochondrial sample on the sucrose cushions carefully, resulting in a lysate:cushion ratio of 2.5:1.
8. Centrifuge the sample at $\sim 231,550 \times g$ for 60 min at 4°C .
9. Discard the supernatant and rinse the tubes sequentially with resuspension buffer to remove residual sucrose (without disturbing the pellet).
10. Resuspend pellets in a total of 100 μl resuspension buffer.
11. Measure mitoribosome absorption at A_{260} .
12. Load the entire sample onto a linear 15-30% sucrose gradient tube. Centrifuge in a TLS-55 rotor at $213,626 \times g$ for 60-90 min at 4°C .
13. Fractionate the gradient, determine the optical density at A_{260} , and pool the fraction corresponding to the nucleic acid peak together. The typical $A_{260}:A_{280}$ ratio of the peak is >1.6 .
14. Exchange the buffer if necessary and dilute the sample with at least 5-10 volumes of final buffer; concentration or dialysis are recommended. Calculate the final concentration using a NanoDrop and the conversion: $1 A_{260} = 0.1 \text{ mg/ml}$.
15. Use the purified mitoribosome sample for subsequent experiments or snap-freeze in resuspension buffer and store at -80°C .

Data analysis

The mitoribosome-Oxa1L purification protocol described here employs PDE12^{-/-} HEK293 cells, but is applicable to wild-type or other HEK293-derived cells. The high-quality purified sample enables biochemical and structural analysis, which can be applied to study human mitoribosomal translation. Representative results for structural studies are presented as figures below; these analyses are just briefly described to work as quality controls for the sample preparation in this protocol.

Following subsequent centrifugation steps, the intact mitochondria are separated on the sucrose gradient (Figure 1); special care is needed to collect the brown band at the 60%-32% interface with minimal contamination from the surrounding buffer. Preparation of high-quality intact mitochondria can be extrapolated to purify or study other mitochondrial macromolecules.

Mitochondria that have been successfully isolated are lysed, and mitoribosome-Oxa1L are finally purified with a sucrose cushion followed by a continuous linear 15-30% sucrose gradient. The sucrose gradient can then be fractionated, either automatically or manually, by carefully taking

fractions with a pipette or punching the tube bottom with a 21 G needle and collecting the drops. Two major mitoribosomal populations are identified in the gradient: the 55S monosome and the large 39S subunit, as illustrated in Figure 2. An additional peak located closer to the bottom may appear in preparations contaminated with 80S cytoplasmic ribosomes. Oxa1L was present in our samples, as confirmed by western blotting (Figure 2B) and cryo-EM maps (Figure 3). To investigate nascent chain translocation, we performed focused classification with signal subtraction (FCwSS) on the mitoribosomal exit tunnel. For further information or other *in silico* processing, please see Figure S3, S12A in our published paper (Desai *et al.*, 2020).

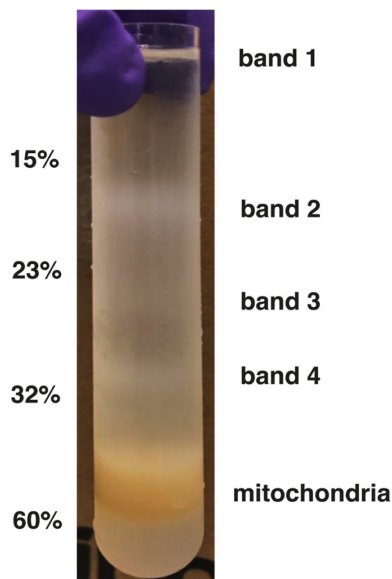


Figure 1. Isolation of mitochondria on a sucrose gradient. Mitochondria were separated on a discontinuous sucrose gradient. The purified mitochondria are found at the 32%/60% interface.

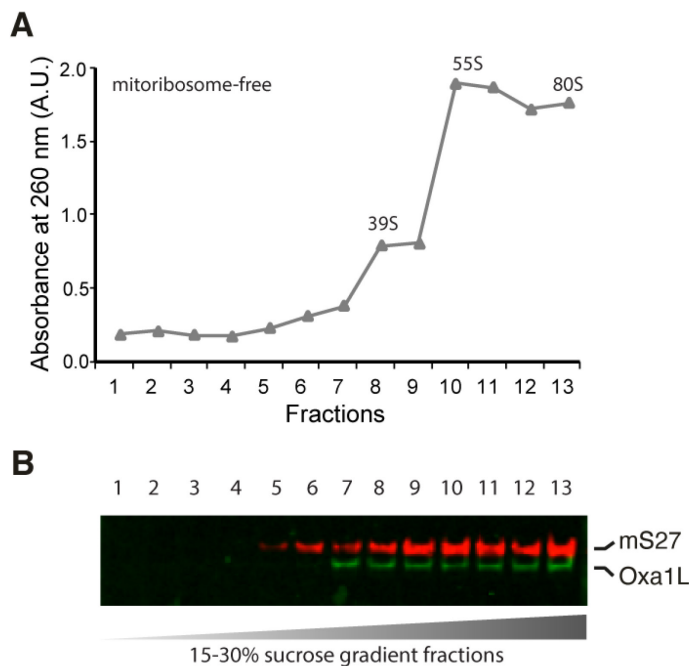


Figure 2. Purification of mitoribosomes with Oxa1L on a sucrose gradient. A. Absorbance at 260 nm of the sucrose gradient fractions. B. Western blotting of mitoribosomal protein mS27 and OXA1L in fractions, showing that Oxa1L co-pellets with mitoribosomes in our preparation.

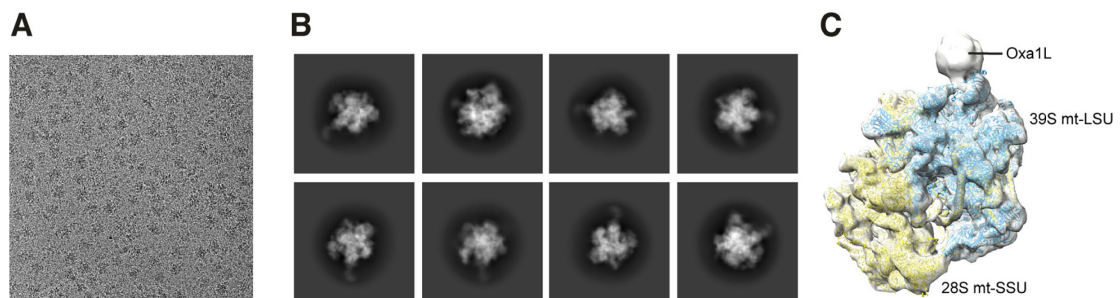


Figure 3. cryo-EM micrograph, 2D classes, and 3D initial reconstruction. A. A micrograph of the mitoribosome-Oxa1L sample. B. Representative processing data (2D classes). C. 3D reconstruction of the mitoribosome-Oxa1L complex.

Notes

1. Notes for cell culture:

The cell viability should be maintained at >90%, and for the final culture that is harvested >95%. The cell culture procedure is specifically for PDE12^{-/-} cells used in this manuscript.

For wild-type cells, all steps are the same except two:

- Grow cells in FreeStyle media + 5% FBS in suspension culture. Split when the cell density reaches 2×10^6 cells/ml (compared with A3-4).
- Harvest cells at a concentration of $\sim 3-4 \times 10^6$ cells/ml.

Once you have 10-20 g cell pellets (from 10-15 L PDE12^{-/-} HEK cells or 2 L wild-type

HEK293 cells) as a starting point, there are no differences in the following steps for mitochondria isolation and mitoribosome purification.

2. Notes for mitochondria isolation:
 - a. It is important to work quickly and keep everything on ice throughout the procedure.
 - b. Besides a Dounce homogenizer in Step B5; alternatively, you could use nitrogen cavitation at a pressure of 500 psi for 20 min (Aibara *et al.*, 2018). Pre-cool the nitrogen cavitation chamber O/N at 4°C.
3. Notes for mitoribosome-Oxa purification:
 - a. You can always start with a smaller volume of cells and subsequently prepare a smaller volume of buffers to keep the ratio the same.
 - b. The small sucrose gradient with the TLS-55 rotor in this protocol allows for the rapid purification of mitoribosome-Oxa1L; however, there is normally an overlap between 55S monosome and 39S large subunit peaks. Separation of the 55S and 39S populations is not necessary for cryo-EM studies, given that further processing will be performed. If a better separation is needed in other situations, larger tubes (*e.g.*, SW40) and a longer running time (*e.g.*, 16 h) are recommended.
 - c. In our dataset, among all the mitoribosome samples, the proportion of complexes of mitoribosome with Oxa1L is around 12%.

Recipes

Buffers for mitochondria isolation

1. Mitochondrial isolation buffer (MIB)
 - 50 mM HEPES-KOH, pH 7.4
 - 10 mM KCl
 - 1.5 mM MgCl₂
 - 1 mM EDTA
 - 1 mM EGTA
 - 1 mM DTT
 - Protease inhibitors (1 tablet/50 ml)
2. Sucrose/mannitol buffer (SM4)
 - 280 mM sucrose
 - 840 mM mannitol
 - 50 mM HEPES-KOH, pH 7.5
 - 10 mM KCl
 - 1.5 mM MgCl₂
 - 1 mM EDTA
 - 1 mM EGTA
 - 1m M DTT

- Protease inhibitors (1 tablet/50 ml)
3. Experimental buffer (MIBSM)
3 volumes MIB mix with 1 volume SM4 (e.g., 120 ml MIB buffer + 40 ml SM4 buffer)
 4. Sucrose-EDTA MOPS (SEM) buffer
250 mM sucrose
1 mM EDTA
20 mM MOPS-KOH (alternative: HEPES-KOH, pH 7.4)
 5. Sucrose gradient (SG) buffer
20 mM HEPES-KOH, pH 7.4
1 mM EDTA and 60%/32%/23% and 15% sucrose, respectively (make separate stock solutions of 4 different sucrose concentrations for the preparation of the stepwise sucrose gradient).

Buffers for mitoribosome purification

1. Lysis buffer
25 mM HEPES-KOH, pH 7.4
150 mM KCl
50 mM MgOAc
1.5% β -DDM
0.15 mg/ml Cardiolipin
500 mM GMPPCP
2 M DTT
Protease inhibitors (1 tablet/50 ml)
2. Sucrose cushion (SC)
1 M sucrose (34% w/v)
20 mM HEPES-KOH, pH 7.4
100 mM KCl
20 mM MgOAc
0.6% β -DDM
0.06 mg/ml Cardiolipin
250 mM GMPPCP
2 mM DTT
3. Resuspension buffer (RB)
20 mM HEPES-KOH, pH 7.4
100 mM KCl
5 mM MgOAc
0.15% β -DDM
0.015 mg/ml Cardiolipin
250 mM GMPPCP
2 mM DTT

4. 15-30% linear sucrose gradients
15-30% linear sucrose in 20 mM HEPES-KOH, pH 7.4
100 mM KCl
5 mM MgOAc
0.05% β -DDM
0.005 mg/ml Cardiolipin
250 mM GMPPCP
2 mM DTT

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Competing interests

The authors declare no competing financial or non-financial interests.

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