Chapter 6

Scalable Isolation of Mammalian Mitochondria for Nucleic Acid and Nucleoid Analysis

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Abstract

Isolation of mitochondria from cultured cells and animal tissues for analysis of nucleic acids and bona fide mitochondrial nucleic acid binding proteins and enzymes is complicated by contamination with cellular nucleic acids and their adherent proteins. Protocols presented here allow for quick isolation of mitochondria from a small number of cells and for preparation of highly purified mitochondria from a larger number of cells using nuclease treatment and high salt washing of mitochondria to reduce contamination. We further describe a method for the isolation of mitochondrial DNA–protein complexes known as nucleoids from these highly purified mitochondria using a combination of glycerol gradient sedimentation followed by isopycnic centrifugation in a non-ionic iodixanol gradient.

Key words Mitochondria, mtDNA nucleoids, Mitochondrial RNA

1 Introduction

Research on mammalian mitochondrial biogenesis is dependent on the purification of mitochondria in high yield with minimal contamination by non-mitochondrial nucleic acids and proteins. The early efforts to purify mitochondria have been reviewed by Ernster and Schatz [1]. Most modern procedures are based on that of Hogeboom, Schneider, and Palade [2], who used isotonic sucrose solutions to stabilize mitochondria and differential centrifugation to purify them. Mannitol-sucrose-HEPES buffer (MSH) containing EDTA is used in our protocols since mannitol is not metabolized or imported into mitochondria. HEPES is used since it has a better buffering capacity than Tris in the pH 7.5 range and does not interfere with amino group-specific reagents used in some chemical modification procedures. Bovine serum albumin is typically included in isolation buffers to help stabilize mitochondria and to help control proteolysis, but can be omitted when desired for proteomic analyses.

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Investigators have modified these procedures to selectively remove the contaminants of greatest concern to their research. For example, in membrane research it is most important to remove microsomal contaminants derived from the plasma membrane or endoplasmic reticulum, capitalizing on the greater density and faster sedimentation of mitochondria. Preparation of mitochondrial ribosomes required multiple wash steps to remove cytosolic ribosomes [2, 3]. In nucleic acid research, the greatest concern is contamination with non-mitochondrial nucleic acids. Removal of these contaminants by treatment of mitochondria with nucleases has been used routinely [4].

Numerous publications on mitochondrial DNA (mtDNA) have used hypotonic cell disruption and differential sedimentation, often with sucrose density gradients, to purify mitochondria. Based on extensive experience with such procedures [5], we have identified a number of problems requiring adjustments, as follows: (1) Hypotonic lysis can result in mitochondrial swelling, often with membrane damage or rupture; (2) Mechanical disruption of small quantities of cells and/or of cells with sparse cytoplasm may be inefficient; (3) Nuclear disruption and the release of chromosomes from mitotic cells leads to artifactual adherence of nuclear DNA on the surface of mitochondria; (4) This procedure does not remove cytoplasmic RNA and ribosomes that bind to the mitochondrial outer membrane, due to either physiological association of ribosomes or to artifactual causes [6].

Here, we provide protocols that we have found to be effective for rapid preparation of mitochondria for nucleic acid analysis in a wide variety of settings. The basic approach employs treatment of cells with a low concentration of digitonin to permit facile cell lysis under isotonic conditions, basically as described [7]. Following removal of nuclei by two or more successive sedimentation steps, mitochondria are pelleted and washed with buffer containing 1 M KCl. Figure 1 shows that mitochondria prepared **without** a high salt wash contain abundant nuclear DNA (nuDNA) contaminants. Treatment of mitochondria with DNase I degrades this contaminating nuDNA, as noted by Higuchi and Linn [8] but leaves oligonucleosome-size fragments associated with mitochondria. Importantly, these contaminating fragments may not be retained on agarose gels, giving a false impression that the nuDNA has been removed. In our view, the failure to appreciate this has led to great confusion over the incidence of damaged residues in mtDNA, since many publications have failed to remove nuDNA effectively. In the larger scale Method 2 we suggest incubating mitochondria during nuclease treatment in a buffer that supports respiration and enzyme activity, adapted from [9]. Our proteomic analyses have shown such nuclease-treated mitochondria still contain considerable amounts of nuclear histones and cytoplasmic ribosomal proteins (data not shown). Washing mitochondria with mannitol-sucrose buffer

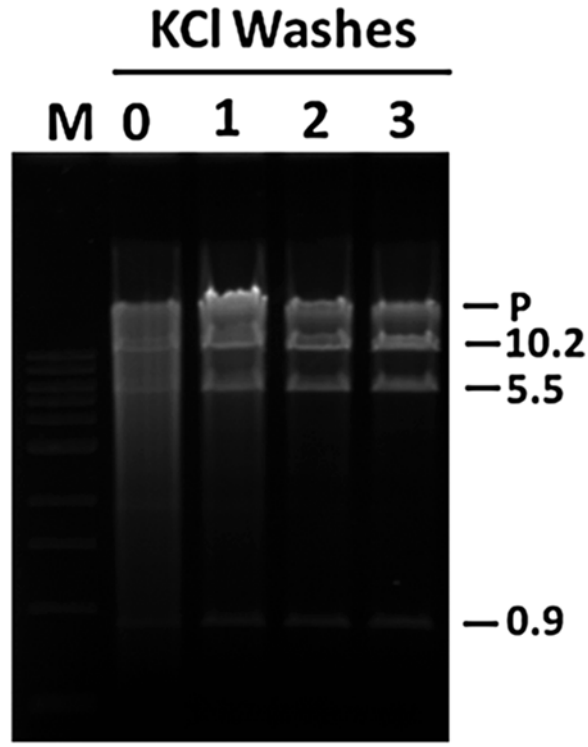

Fig. 1 HindIII digest of purified HeLa mitochondrial DNA shows the removal of nuclear DNA contaminants with successive 1 M KCl washes, without nuclease treatment. ***Lane M*** contains a 1 kB ladder of DNA mobility markers and other lanes are designated by the number of 1 M KCl washes employed. The sizes of mtDNA fragments are shown in kB, along with a partial digest product (***P***)

containing 1 M KCl removes a substantial fraction of nuDNA and cytoplasmic rRNA. Figure 2 shows that this treatment also minimizes contamination by a cytoplasmic ribosomal protein. Several important caveats apply here. First, it is generally easier to prepare highly purified mitochondria from some tissue sources than from cultured cells. Liver mitochondria, for example, are easy to prepare since they occupy 16 % of the volume of hepatocytes and nuDNA contamination is reduced since the mitotic index is very low. Second, mitochondria are highly dynamic organelles that engage with other organelles, such as endoplasmic reticulum, interact with cytoplasmic enzymes under normal physiological conditions, and host cytoplasmic ribosomes participating in co-translational import of certain mitochondrial proteins. Therefore, it is impossible to prepare "pure" mitochondria. In cases where cell fractionation is used as a criterion to show that a particular protein is an authentic component of mitochondria, it is necessary to show that it is increasingly enriched as mitochondria are more highly purified. This concept has been adapted in high-throughput proteomic studies as protein correlation profiling [10] or subtractive proteomics [11].

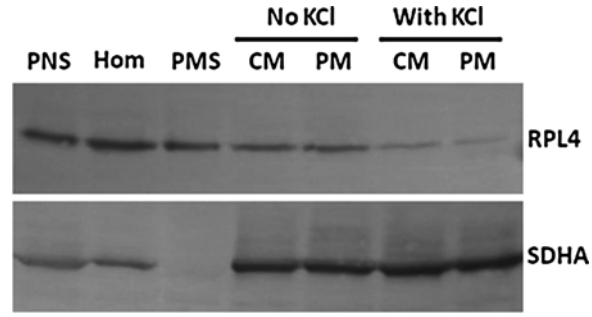

Fig. 2 HeLa cells fractionated either without or with one wash using buffer containing 1 M KCl, producing crude mitochondria (***CM***) which were then further purified on a 1.7 M/1 M sucrose step gradient (PM). 10 μg of protein were loaded in each lane of a 12 % SDS polyacrylamide gel. Proteins were immunoblotted for RPL4, a cytosolic ribosomal protein, or SDHA, a mitochondrial protein subunit of Complex II. ***PNS*** post-nuclear supernatant, ***Hom*** whole-cell homogenate, ***PMS*** post-mitochondrial supernatant

In this article, we present three protocols to prepare mitochondria and mtDNA nucleoids. Method 1 permits rapid preparation of mitochondria from small quantities (e.g., one to three 10 cm tissue culture dishes) from several samples handled in parallel. Method 2 is a scaled-up version that employs nuclease treatment and additional sucrose gradient sedimentation to obtain more highly purified mitochondria. Examples of the application of these methods are found in [12–14]. We have used these methods for a variety of cultured cells (HeLa, A549, H358, normal human fibroblasts, mouse 3T3) as well as mouse liver and Xenopus oocytes.

Method 3 uses mitochondria prepared with Method 2 for preparation of mtDNA nucleoids. This consists of a procedure for lysis of mitochondria with the non-ionic detergent Triton X-100 and a two-dimensional centrifugation protocol to prepare nucleoids based on their large size and buoyant density. In the first dimension, a 15–40 % glycerol gradient is used to separate large nucleoids from smaller complexes, including mitoribosomes. We had previously used sedimentation for 1.5 h to separate slowly and rapidly sedimenting nucleoids [15]. In that study, we reported that the rapidly sedimenting nucleoids are more closely associated with cytoskeletal proteins. In Method 2, we employ a more lengthy 4 h centrifugation step to permit both forms of nucleoids to sediment to a pad containing 20 % glycerol/30 % iodixanol in order to facilitate their rapid collection and further analysis using isopycnic centrifugation in a non-ionic iodixanol gradient. This second separation takes advantage of the density of nucleoids with their associated proteins and, as an equilibrium method, is not strictly time-sensitive. We routinely prepare and lyse mitochondria and complete the glycerol gradient in a single day without exposing material

to freezing and thawing. The iodixanol gradient is then run overnight for 14–16 h. We reported that most proteins shown to function in mtDNA replication and transcription remain associated with the mtDNA through both centrifugation steps, including TFAM, mtSSB, mtDNA polymerase γ, mtRNA polymerase, and proteins involved in mtRNA processing and early stages in ribosome assembly [14]. This final fraction also contains mitofilin and other components of the MICOS complex [16] as well as cytoskeletal proteins. MICOS complexes and cytoskeletal structures are much more abundant than nucleoids, so that it is difficult to determine if the apparent association of these proteins with nucleoids is biologically significant. Others have reported a role for actin and myosin in mtDNA maintenance [17]. Hopefully, the methods presented here will help to advance understanding of mtDNA nucleoid dynamics.

2 Materials

1. Trypsin–EDTA and Trizol.
2. 10 % (wt/vol) digitonin in DMSO (20 % inert); store 200 μL aliquots at 4 °C.
3. Mannitol.
4. 1 M 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) pH 8.0, dissolved in water, autoclaved; store at room temperature.
5. 0.25 M ethylenediaminetetraacetic acid (EDTA)–NaOH pH 8.0, dissolved in water, autoclaved; store at room temperature.
6. 0.5 M (wt/vol) dithiothreithol (DTT), dissolved in water, filter sterilized; store 1 mL aliquots at −20 °C to avoid oxidation.
7. 4 mg/mL radioimmunoassay grade bovine serum albumin (BSA) (US Biochemical) in H_2O; sterilized by filtration through a 0.22 μm membrane; store at 4 °C.
8. 3 M KCl in H_2O; autoclaved; store at room temperature.
9. 200 mM phenylmethanesulfonylfluoride (PMSF), dissolved in isopropanol; store at room temperature.
10. 5 mg/mL leupeptin in H_2O; store 500 μL aliquots at −20 °C.
11. 1 mM pepstatin A dissolved in methanol; store at −20 °C.
12. 0.4 % trypan blue in PBS.
13. Glutamic acid, sodium malate, adenosine diphosphate, sucrose, and ultrapure glycerol.

14. 2 mg/mL DNase I Type II; dissolved in 10 mM Tris, pH 7.5, 50 mM NaCl, 10 mM $MgCl_2$, 2 mM DTT, 40 % glycerol; store at –20 °C.
15. 1 mg/mL RNase A (Type IA) in 10 mM NaAcetate, 50 mM HEPES, pH 8.0, 40 % glycerol; store at –20 °C.
16. 10 % Triton X-100.
17. Turbo DNase (*Serratia marcesens* nuclease; either Benzonase (EMD) or Turbo DNAse (Accelagen)); store at –20 °C.
18. Iodixanol 60 % solution.
19. SYBR Green I and II dyes.

2.1 Equipment

1. Inverted light microscope (10× and 40× objectives) with microscope slides and cover glass.
2. Hemocytometer or alternate cell counting device.
3. Refrigerated swinging bucket centrifuge.
4. Super-speed refrigerated centrifuge with swinging bucket rotor and polycarbonate tubes.
5. Ultracentrifuge and polyallomer tubes.
6. 1 and 3 mL syringes with 25-G needle.
7. Gradient forming device.

2.2 Reagent Stock Preparation

NOTE Prepare all reagents prior to starting the procedure to avoid delays.

For all buffer solutions, labile ingredients such as 2 mM DTT, 0.2 mM PMSF, 5 μg/mL leupeptin and 1 μM pepstatin A are added from stock solutions shortly before use.

1. 2.5× MSH Buffer (2.5× Mannitol-Sucrose-Hepes, pH 8.0-EDTA): 525 mM mannitol, 175 mM sucrose, 50 mM Hepes, pH 8.0, 5 mM EDTA. Stock solution filter sterilized and stored at 4 °C.
2. 2.5× MSH/$MgCl_2$: 2.5× MSH Buffer with 5 mM $MgCl_2$ substituted for EDTA.
3. Mitochondrial Isolation Buffer (MIB): 1× MSH, 0.2 mg/mL BSA, 2 mM DTT, 0.2 mM PMSF, 5 μg/mL leupeptin, 1 μM pepstatin A.
4. Potassium-containing MIB (KIB): MIB supplemented with 1 M KCl.
5. MIB/Mg: MIB supplemented with 10 mM $MgCl_2$.
6. Sucrose Gradient Buffers: 1.7 M or 1 M sucrose, 20 mM HEPES, pH 8.0, 2 mM EDTA. Stock solutions filter sterilized and stored at 4 °C. Supplement with 2 mM DTT, 0.2 mM PMSF, 5 μg/mL leupeptin, 1 μM pepstatin A just before use.

7. 1× Nuclease Buffer (5 mL): 2 mL 2.5× MSH/Mg, 1.25 mL 4× Substrate mix (see below), 0.1 mL 3 M KCl (60 mM final), 50 µL 1 M $MgCl_2$ (10 mM final), 10 µL 500 mM K_2HPO_4 (1 mM final) and 1.59 mL water. Supplement with 2 mM DTT, 0.2 mM PMSF, 5 µg/mL Leupeptin, 1 µM Pepstatin A just before use.
8. 4× Substrate mix (25 mL): 4 mM ADP, 20 mM glutamic acid, 20 mM Na_2-malate, 1 mg/mL BSA. Dissolve powders in about 20 mL of water, neutralize the organic acids with 200 µL 5 M NaOH, add sterile-filtered BSA in solution and adjust to 25 mL. If BSA is exposed to the acids without neutralization, a precipitate develops. Filter sterilize and store frozen aliquots of 1.25 mL.
9. 5× Mitochondrial Lysis buffer: 100 mM HEPES, pH 8.0, 100 mM NaCl, 10 mM EDTA, 10 mM DTT, 0.2 mM PMSF, 5 µg/mL Leupeptin, 1 µM Pepstatin A.
10. Glycerol Gradient Buffers (12 ml each).

15 %	40 %
2.25 mL 80 % glycerol	6 mL 80 % glycerol
2.4 mL 5× lysis buffer	2.4 mL 5× lysis buffer
0.24 mL 10 % TX-100	0.24 mL 10 % TX-100
7.11 mL H_2O	3.36 mL H_2O

11. 20 % Glycerol/30 % iodixanol Pad: 500 µL of 1:1 mix of 40 % glycerol gradient buffer and 60 % Iodixanol.
12. Iodixanol Gradient Buffers (12 mL each).

20 %	40 %
4 mL 60 % iodixanol	8 mL 60 % iodixanol
2.4 mL 5× lysis buffer	2.4 mL 5× lysis buffer
0.24 mL 10 % TX-100	0.24 mL 10 % TX-100
5.36 mL H_2O	1.36 mL H_2O

13. TE buffer: 10 mM Tris–HCl, pH 7.5, 1 mM EDTA.

3 Methods

3.1 Method 1. Small-Scale Mitochondrial Preparation with KCl Wash (See Note 1*)*

Note on scale: This protocol is based on packed cell volume (PCV) under 200 µL from 1 to 3 10-cm plates. The emphasis on rapid purification and the ability to handle multiple samples in parallel with no sucrose gradient step provides slightly lower purity than Method 2. Brief RNase treatment replaces DNase treatment as in Method 2. With KCl wash, cytoplasmic RNA contamination is minimal.

1. Trypsinize cells and stop trypsin with complete medium as usually performed in tissue culture. Centrifuge the cell suspension at 600×*g*, 5 min, rinse with PBS and repellet cells. All steps should be on ice or 4 °C unless otherwise indicated.
2. Resuspend cells in 1 mL MIB. Transfer to a microfuge tube.

   Add 3 μL 10 % digitonin in DMSO. Gently invert the suspension several times, then check cell permeability with trypan blue by mixing 3 μL of cells with 3 μL Trypan blue stock. >90 % of cells should be stained with trypan blue (*see* **Note 2**).
3. Add MIB to 1.5 mL to decrease the digitonin concentration; repellet cells this time at 900×*g*, 5 min in adapters in a swinging bucket rotor.
4. Remove the supernatant, resuspend the pellet in 0.8 mL MIB. Homogenize by passage through a 25 G needle fitted to a 1 mL syringe. About ten "firm" passages with minimal frothing should suffice. Confirm cell breakage under the microscope and add more passages as needed (*see* **Note 5**).
5. Add 0.8 mL MIB and centrifuge at 1,200×*g*, 5 min to pellet nuclei and unbroken cells.
6. Withdraw the supernatant to a new tube, avoiding the pellet, and repeat the nuclear sedimentation.

   The final supernatant is referred to as the "post-nuclear supernatant," PNS. Microscopic examination should reveal no nuclei. If nuclei are present, repeat the centrifugation.
7. Pellet mitochondria by centrifuging in a refrigerated microfuge at 15,000×*g*, 10 min.
8. (a) *For protein only; if cytoplasmic RNA is not an issue:*
   Resuspend the mitochondrial pellet in 1 mL KIB; repellet mitochondria as above.
   (b) *If RNAse treatment is desired:*
   Resuspend the mitochondrial pellet with 400 μL MIB/Mg supplemented with 4 μL 1 mg/mL RNase A and 0.5 μL Turbonuclease. Incubate at room temperature for 15 min. Add 1 mL KIB, mix and repellet mitochondria as above.
9. Resuspend mitochondrial pellet with 500 μL MIB and repellet. If samples are for analysis of mitochondrial proteins, use MIB lacking BSA.
10. Process the mitochondrial pellet for RNA using Trizol if desired; for protein analysis, either store the pellet frozen or resuspend in sample loading buffer.

3.2 Method 2. Medium/Large-Scale Mitochondrial Preparation with Nuclease Treatment and Sucrose Gradient

1. *Preparation of buffers.* The reagents in this protocol are scaled for 2×10^8 cells, which can be cultured from twelve 150 mm × 25 mm tissue-culture plates.

   Prepare 30 mL MIB, 5 mL KIB, 1 mL Nuclease Buffer, 5 mL 1× MSH Buffer and 5 mL 0.5× MSH Buffer. All buffers should be cooled on ice and all steps performed at 4 °C unless otherwise indicated.

2. *Collect cells.* If working with adherent cells, harvest cells by scraping or with trypsin–EDTA, then neutralize the trypsin with media containing serum. If working with suspension cells, omit the trypsin step. Count the cells with a hemocytometer. Collect the mammalian culture cells into a suitable centrifuge tube and pellet the cells by centrifugation at 500 × *g* for 5 min.

3. Remove the media and resuspend the cells in 5 mL PBS. Pellet the cells again, then resuspend the cells in 3 mL MIB and transfer to a 17 × 100 mm tube (*see* **Note 3**).

4. *Digitonin treatment.* Add 10 % digitonin until all of the cells are permeable to trypan blue staining. Initially add 12 μL digitonin to the cells. Mix 3 μL cell suspension with 3 μL trypan blue, and check for complete permeabilization by microscopy. If necessary, add additional small increments of 2–4 μL 10 % digitonin until >90 % of cells are stained (*see* **Note 2**).

5. Add 7 mL of MIB to dilute the concentration of digitonin in the cell suspension. Centrifuge the cells at 1,200 × *g* for 5 min (*see* **Note 4**).

6. *Cell disruption.* Resuspend the cells in 3 mL MIB and pass through a 25-G needle 8–10 times. Inspect a sample volume for lysis using a microscope (*see* **Note 5**).

7. *Pellet nuclei.* Add 5 mL of MIB to the lysate to increase the volume of lysate and to reduce the amount of mitochondria trapped in the nuclear pellet. Centrifuge the lysate at 900 × *g* for 5 min. Collect the supernatant (post-nuclear supernatant, PNS) into a new 15 mL conical tube by pipetting, not by decanting, to avoid nuclear contamination. Repeat the centrifugation, withdraw the supernatant to a fresh tube and check a small sample under the microscope (*see* **Note 6**).

8. *Pellet mitochondria.* Transfer the post-nuclear supernatant to a tube compatible with an HB-6 rotor or equivalent. To obtain crude mitochondria, centrifuge at 16,000 × *g* for 15 min. Remove the supernatant (post-mitochondrial supernatant).

9. *KCl wash.* Resuspend the crude mitochondrial pellet in 3 mL KIB and centrifuge in the HB-6 rotor as before (*see* **Note 7**).

10. *Nuclease treatment.* Discard the supernatant. Resuspend the high-salt-washed mitochondria in 1 mL Nuclease Buffer, transfer to a microfuge tube and add 20 μL of 2 mg/mL

DNaseI and 1 μL Turbonuclease. Incubate in a 37 °C water bath for 20 min. RNase may be included if removal of cytoplasmic RNA is desired. Add 100 μL 0.25 M EDTA to stop DNase activity.

11. *Sucrose gradient centrifugation*. While nuclease treating the mitochondria, prepare a sucrose step gradient. Add 3 mL of 1.7 M sucrose buffer to the bottom of an SW-41 tube (14×89 mm) and carefully layer 6 mL of 1 M sucrose buffer on top. Formation of this sucrose step gradient should be performed by using 5 mL serological pipettes and an electronic pipetter on the slowest speed. Carefully layer the mitochondria on top of the sucrose step gradient. Centrifuge in a Beckman ultracentrifuge with an SW-41 swinging bucket rotor at 77,000×*g* (25,000 rpm) for 25 min (*see* **Note 8**).
12. *Collect mitochondria*. Mitochondria will form a light brown layer at the 1.7/1 M sucrose interface. Carefully aspirate the upper solution which contains some residual nuclease. Collect the purified mitochondria from the sucrose interface and dilute it with 2–3 volumes of 0.5× MSH to reduce the sucrose concentration sufficiently (*see* **Note 9**). This suspension can be distributed into microfuge tubes. Pellet the mitochondria at 10,000×*g* for 10 min and then remove the supernatant. Mitochondria may be frozen at –80 °C at this point or directly taken into further steps for analysis (*see* **Note 10**).

3.3 Method 3. Two-Dimensional mtDNA Nucleoid Preparation

1. *Mitochondrial lysis*. Remove the supernatant from the final mitochondrial pellet and add 200 μL 5× lysis buffer and an appropriate volume of H_2O to adjust the sample to a volume of 800 μL. Add 200 μL 10 % Triton X-100 to final 2 % concentration. Incubate on ice with gentle mixing for 15 min. Pellet insoluble debris in a microfuge tube at 5,000×*g* for 5 min.
2. *First dimension separation on a glycerol gradient*. Carefully layer the cleared lysate on a pre-formed 10 mL 15–40 % glycerol gradient in lysis buffer over a 500 μL pad of 20 % glycerol/30 % iodixanol in an SW41 centrifuge tube (*see* **Note 11**). The gradient can be generated using a gradient-forming device or by layering several zones of solutions containing progressively lower glycerol concentrations. Centrifuge for 4 h at 151,000×*g* (35,000 rpm in an SW41 rotor).
3. *Collect fractions*. The 4 h glycerol gradient is designed to permit nucleoids to sediment to the pad. The glycerol gradient is fractionated by withdrawing 14×750 μL samples successively from the top of the gradient (*see* **Note 12**).
4. *Detect mtDNA*. Nucleic acid is detected in gradient fractions using SYBR Green II staining in microtiter dishes (*see* **Note 13**). Mix 3 μL of fraction with 100 μL of 1:10,000 SYBR Green II diluted in TE buffer. Fluorescence is detected using a

Typhoon scanner (GE Healthcare) or a fluorescent plate reader. Most free proteins and tRNA are found in the top 3–4 fractions. Ribosomal subunits and many mRNAs are in the center of the gradient and can be recovered for analysis as desired.

5. *Second dimension purification of nucleoids on an iodixanol gradient.* Fractions containing nucleoids (generally fraction #1 at the dense pad) are layered on a preformed 10 mL 20–40 % Optiprep gradient in 1× lysis buffer in an SW41 tube. Samples are centrifuged overnight (14–16 h) at 22,000 rpm (60,000 × *g*). Fractions of 700 μL are collected from the tube bottom. Nucleoids are generally in fraction #5, but this can be confirmed by SYBR Green I staining as above.

4 Notes

1. The small-scale protocol is generally comparable to some commercial kits for mitochondrial isolation, but is much more economical and allows for greater control over the ratio of detergent to the number of cells.
2. The amount of digitonin added is critical since an excess amount will solubilize the mitochondrial outer membrane. It is recommended to start with a conservative amount as indicated and adjust the volume added in steps. After a batch of reagent is adequately tested with a given cell line, it is often possible to target the appropriate amount on the first addition. The amount of digitonin required for permeabilization will depend on the total number of cells being processed due to its stoichiometric binding to membranes.
3. The suggested tube permits the 3 mL syringe to reach the bottom. It is essential that all of the solution is passed through the syringe needle at each stroke.
4. A higher centrifugal force is used when pelleting cells from the higher density mannitol-sucrose solution.
5. Use of a 40× objective is advised to distinguish cells from nuclei. The nuclei of lysed cells appear as rounded structures, generally with clean margins, whereas nuclei of intact cells will be surrounded by other organelles (endoplasmic reticulum, Golgi apparatus, and mitochondria) and the plasma membrane. If necessary, increase the number of passages through the syringe to increase the percentage of cell lysis. Cell lines differ in the extent of association of other organelles, especially endoplasmic reticulum, to nuclei.
6. If nuclei can still be seen in the second post-nuclear supernatant, a third centrifugation will help reduce contamination.

Also note that swinging bucket rotors are preferred when available to avoid streaking of pelleted material along the wall of a fixed angle centrifuge.

7. It is important to AVOID exposing the cell lysate to high salt until after the nuclei have been removed by centrifugation to limit the tendency of organelles to aggregate and to avoid salt extraction of non-histone nuclear proteins.
8. When alternative combinations of centrifuge and rotor are used, alter the angular velocity to conserve the centrifugal force. Conversion calculators are available on-line, such as https://www.beckmancoulter.com/wsrportal/wsr/research-and-discovery/products-and-services/centrifugation/rotors/index.htm?t=3.
9. The mitochondria may form a layer that can impede the sedimentation of other dense organelles, so that overloading is undesirable. The mitochondrial layer may also have some tendency to adhere to the wall of the centrifuge tube, requiring an effort to dislodge it. We prefer to use a sterile Pasteur pipette to collect this layer.
10. We have found the sucrose gradient and one re-pelleting step is sufficient to physically remove nucleases. If there is evidence of degradation of mitochondrial nucleic acids, an additional resuspension in MSH and sedimentation may be performed.
11. The continuous exposure to non-ionic detergent during sedimentation assures that membrane proteins are removed.
12. We generally number fractions from the bottom. Nucleoids sediment to the pad in fraction 1. This is an imprecise fractionation, but this is a preparative procedure intended to separate free ribosomes from nucleoids. These conditions do not optimally separate large and small ribosomal subunits, for example.
13. SYBR Green I and II are marketed as selective for DNA and RNA, respectively, but both dyes have some ability to detect both forms of nucleic acid.

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