

Chapter 2

Isolation of Mitochondria from Liver and Extraction of Total RNA and Protein: Analyses of MicroRNA and Protein Expressions

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Abstract

Several studies have indicated the presence of microRNAs (miRNAs) within mitochondria, although the origin, as well as the biological function of these mitochondrially located miRNAs is largely unknown. The identification and significance of this subcellular localization is gaining increasing relevance to the pathogenesis of certain disease states. Here we describe the isolation of highly purified mitochondria from rat liver by differential centrifugation, followed by RNase A treatment to eliminate contaminating RNA. The coupled extraction of total RNA and protein is a more efficient design for allowing the downstream evaluation of miRNA and protein expression in mitochondria.

Key words Differential centrifugation, miRNA, Mitochondria, Protein, TRIzol

1 Introduction

Mitochondria are central organelles in the regulation of cellular homeostasis, playing a pivotal role in energy metabolism and cell viability, with their dysfunction or dysregulation being associated with multiple diseases. Human mitochondria harbor a compact circular genome of 16,569 bp in length, encoding 13 protein subunits of the electron transport chain, and a number of noncoding RNAs. Replication and transcription of mitochondrial DNA (mtDNA) are initiated from the small noncoding region known as the D loop, and regulated by proteins encoded in the nuclear genome, which are posttranslationally imported into the mitochondria [1]. In addition, transcription and translation of mtDNA, and mitochondrial transcript processing are regulated by several types of noncoding RNAs, either encoded in the mitochondrial genome, or in the nuclear genome following translocation to the mitochondria [2]. Interestingly, mitochondrial RNAs are transcribed from both strands as long polycistronic precursor transcripts, which undergo

processing and final release of both noncoding and coding RNAs, including tRNAs, rRNAs, and mRNAs.

In the last two decades, microRNAs (miRNAs) have emerged as a class of noncoding RNAs processed from endogenous transcripts, functioning as master regulators and fine-tuners of the genome, via posttranscriptional mechanisms [3]. miRNA gene-silencing occurs via miRNA-mRNA binding, typically with incomplete albeit high sequence complementarity between the 3'-untranslated region target sites of mRNA transcripts and the miRNA seed sequence located at position 2–7 of its 5'-end [4]. This allows miRNAs to regulate the expression of multiple target genes and signaling pathways involved in the regulation of key cellular processes including cell growth, proliferation, differentiation, and apoptosis [5] as well as mitochondrial function [2]. Consequently, deregulation of a given miRNA can lead to malfunction of pivotal cellular mechanisms, contributing to disease onset and/or progression. Therefore, it is not surprising that miRNA modulation is increasingly demonstrated as a relevant therapeutic strategy in human disease [3, 6–9].

Since their discovery, miRNAs have been identified in multiple biological systems. Currently, almost 2,600 mature human miRNAs have been identified (mirbase.org), and predicted to regulate at least 60 % of protein-coding genes. Recent studies, including one by our group have demonstrated the presence of mature miRNAs not only in the cytoplasm, where target gene silencing of the mRNA transcript occurs, but also in other cellular compartments, including the nucleus [10], nucleolus [11, 12] and mitochondria [13–16]. In addition to mature miRNAs, precursor miRNAs have also been found in mitochondria [15]. However, it is not known whether mitochondrial localized miRNAs are encoded in the mitochondrial genome or in the nuclear genome and later imported into the mitochondria. Nevertheless, and regardless of their provenance from the nuclear or mitochondrial genome, the biological and pathophysiological implications of this unexpected finding are incompletely explored. Based on recent studies, it is conceivable that mitochondria-localized miRNAs may putatively function as posttranscriptional regulators, or fine-tuners, of the mitochondrial genome. This is illustrated by the mitochondrial translocation of the nuclear encoded miR-181c, which was shown to regulate the expression of the mitochondria-encoded cytochrome c oxidase subunit 1 (mt-cox1) in rat cardiac myocytes [17].

The first evidence that RNAi components may localize to the mitochondria was provided almost a decade ago, by the demonstration that human Ago2 interacts with mitochondrial tRNA^{met} [18]. Interestingly, miRNAs were later identified in mouse liver mitochondria by small RNA sequencing [19]. Aside from this unexpected finding, it was proposed that the presence of miRNAs in mouse liver mitochondria may arise from cytosolic contamination

of the isolated mitochondria. Subsequently, we have overcome this technical limitation by treating isolated and purified rat liver mitochondria with RNase A, to ensure that mitochondria are devoid of contaminant RNAs from the cytosol or other cellular organelles and compartments. Coupling this extremely relevant purification step to the detection of mature miRNAs by microarray followed by northern blot confirmation, we have clearly demonstrated that mature miRNAs are indeed present in highly purified mitochondria [13]. Since then, additional reports using RNase treatment and microarray methodologies, confirmed the detection of miRNAs in purified mitochondria isolated from mouse liver [14], and from human cervical adenocarcinoma (HeLa) cells [16].

The identification of miRNAs in mitochondria and the exploration of their biological role in this subcellular organelle have been gaining increasing relevance. Mitochondria may be easily isolated and purified from multiple organs or cultured cells. The methodologies described in this chapter allow the straightforward isolation of highly purified mitochondria, and the coupled extraction of total RNA and protein, allowing downstream evaluation and comparison of mitochondrial miRNA and protein expression, in the same sample.

2 Materials

Prepare all solutions using ultrapure water (prepared by purifying deionized water to attain a sensitivity of 18 M Ω cm at 25 °C) and analytical grade reagents. Prepare and store all reagents at 4 °C (unless otherwise indicated). Diligently follow all waste disposal regulations when disposing waste materials.

2.1 Mitochondrial Isolation: Materials and Reagents

1. *Speed controlled mechanical drill*: Tri-R model K41 skill drill (Tri-R Instruments).
2. *Tissue grinder*: Glass mortar with radially serrated PTFE pestle (Fischer Scientific).
3. *Ultra-spin buffer*: 0.25 M Sucrose, 1 mM Ethylene glycol-bis (2-aminoethylether)- N,N,N',N'-tetraacetic acid (EGTA). Add 150 ml of water to a graduated cylinder. Weigh 21.39 g sucrose and 95.1 mg EGTA, and transfer to the graduated cylinder. Mix and adjust pH to 7.4 with KOH. Complete volume with water to 250 ml (*see Note 1*).
4. *PERCOLL* (Colloidal PVP coated silica for cell separation) (#P1644, Sigma-Aldrich).
5. *Homogenate buffer*: 70 mM sucrose, 220 mM mannitol, 1 mM EGTA, 10 mM 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid, N-(2-Hydroxyethyl)piperazine- N'-(2-ethanesulfonic acid) (HEPES), pH 7.4. Add 700 ml of water to a glass beaker.

Weigh 23.6 g sucrose, 40.8 g mannitol, 380.4 mg EGTA, and 2.38 g HEPES and transfer to the cylinder. Mix and adjust pH to 7.4 with KOH. Adjust volume to 1 l.

6. *Mitochondria wash buffer 1*: 0.1 M KCl, 5 mM 3-[N-Morpholino]propanesulfonic acid (MOPS), 1 mM EGTA. Add 800 ml of water to a glass beaker. Weigh 7.46 g KCl, 1.05 g MOPS, and 0.380 mg EGTA, and transfer to the glass beaker. Mix and adjust pH to 7.4 with KOH. Adjust volume to 1 l.
7. *Mitochondria wash buffer 2*: 0.1 M KCl, 5 mM MOPS. Add 800 ml of water to a glass beaker. Weigh 7.46 g KCl, and 1.05 g MOPS, and transfer to the glass beaker. Mix and adjust pH to 7.4 with KOH. Adjust volume to 1 l.
8. *Mitochondria suspension buffer*: 125 mM Sucrose, 50 mM KCl, 5 mM HEPES, 2 mM KH_2PO_4 . Add 70 ml of water to a glass beaker. Weigh 4.28 g Sucrose, 372.8 mg KCl, 119.15 mg HEPES, and 27 mg KH_2PO_4 and transfer to the glass beaker. Mix and adjust volume to 100 ml.
9. *EDTA-free Complete[®]-Mini protease inhibitor cocktail* (#04693159001, Roche Applied Science).
10. *Chelex[®] 100 Resin* (#143-2832, Bio-Rad Laboratories, CA, USA).
11. *RNAse A*, 20 mg/ml in 50 mM Tris-HCl (pH 8.0), 10 mM EDTA (#12091-039, Invitrogen).

2.2 Total RNA

Isolation: Materials and Reagents

1. *NanoDrop* (Thermo Scientific).
2. *TRIzol[®]* (#15596-026, Life Technologies).
3. *Chloroform*.
4. *Isopropanol*.
5. *UltraPure[™] DNase/RNase-Free Distilled Water* (#10977-023, Invitrogen).
6. *75 % (vol/vol) Ethanol*. Add 15 ml of Ethanol and 5 ml of UltraPure[™] DNase/RNase-Free Distilled Water in a sterile tube, and mix well. Store at room temperature.

2.3 Total Protein

Isolation: Materials and Reagents

1. *Compact Ultrasonic Device*: model UP100H—100 W, ultrasonic frequency 30 kHz (Hielscher Ultrasonics GmbH, Teltow, Germany).
2. *Tris-Hcl 1 M, pH 8.0* (#T2694, Sigma-Aldrich).
3. *Protein wash buffer*: 0.3 M guanidine hydrochloride in 95 % ethanol. Add 142.5 ml of Ethanol and 7.5 ml of water to a graduated cylinder to prepare 150 ml of 95 % ethanol. Add 80 ml of 95 % ethanol to a beaker. Weigh 2.87 g of guanidine hydrochloride and transfer to the beaker. Bring the volume to

100 ml using 95 % ethanol, and mix well. Store at room temperature.

4. *1 % (wt/vol) Sodium dodecyl sulfate (SDS)*: Add 80 ml of water to a graduated cylinder. Weigh 1 g of SDS and transfer to the cylinder. Mix well and bring the volume to 100 ml. Store at room temperature.
5. *8 M Urea in Tris-HCl 1 M, pH 8.0*: Add 80 ml of water to a graduated cylinder. Weigh 48.05 g of Urea and transfer to the cylinder. Mix well and bring the volume to 100 ml. Store at room temperature.
6. *Protein resuspension buffer*: 1:1 (vol/vol) of 1 % SDS and 8 M urea in Tris-HCl 1 M, pH 8.0. Add 25 ml of 1 % SDS solution and 25 ml of 8 M urea in Tris-HCl 1 M, pH 8.0 to a glass flask, as 50 ml of Protein Resuspension Buffer. Mix well and store at room temperature.

3 Methods

3.1 Mitochondria Isolation

The flowchart in Fig. 1 depicts the protocol followed after preparing the buffers and obtaining liver fragments.

1. Prepare two self-generating gradients (ultra-spin buffer:Percoll (75:25); vol/vol) by adding 15 ml of ultra-spin buffer and 5 ml of PERCOLL to each ultracentrifuge tube. Mix well and centrifuge the tubes at $43,000 \times g$ for 30 min, at 4 °C. Carefully store the gradients at 4 °C until use (*see Note 2*).
2. Prepare homogenate buffer containing EDTA-free Complete®-Mini protease inhibitor cocktail, by adding 12 tablets to 120 ml of homogenate buffer. Mix to dissolution and place on ice (*see Note 3*).
3. Prepare 1 % (wt/vol) Chelex-100-treated mitochondria wash buffer 2. Add 1.5 g of Chelex to 150 ml of mitochondria wash buffer 2. Mix well by vigorous shaking by hand and incubate for 10 min, at room temperature (*see Note 4*). Place on ice until use.
4. Prepare 1 % (wt/vol) Chelex-100-treated mitochondria suspension buffer. Add 100 mg of Chelex to 10 ml of suspension buffer. Mix well by vigorous shaking by hand, and incubate for 10 min at room temperature (*see Note 4*). Place on ice until use.
5. Fill three glass petri dishes with 50 ml of ice-cold homogenate buffer, and place them on ice, together with an additional empty glass plate.
6. Sacrifice an adult male 175–200 g Sprague-Dawley or Wistar rat by exsanguination under CO₂ anesthesia.

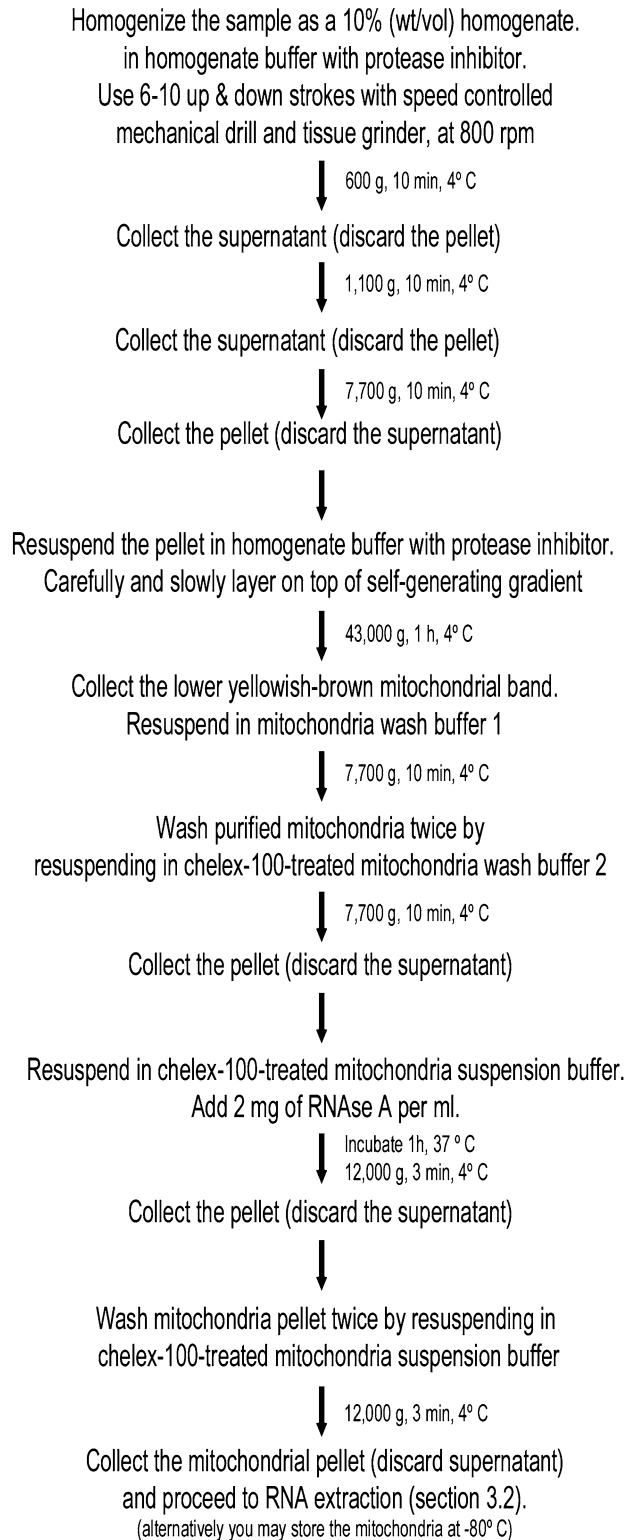


Fig. 1 Flowchart of mitochondrial isolation. Includes all steps following the preparation of buffers and collecting the liver sample for processing

7. Remove the rat liver, and using tweezers, rinse the liver in the three petri dishes containing ice-cold homogenate buffer (prepared in **step 5**), and keep the rat liver on ice in the final rinse solution (*see Note 5*).
8. Using the ice-cold empty glass plate and a laboratory scale, weigh approximately 10 g of rat liver, and mince the sample into small fragments using scissors (*see Note 6*).
9. Homogenize the sample as a 10 % (wt/vol) homogenate. Homogenize the 10 g of minced liver in 100 ml ice-cold homogenate buffer containing EDTA-free Complete®-Mini protease inhibitor cocktail (prepared in **step 2**), using 6–10 complete up and down strokes with a speed controlled mechanical drill and tissue grinder, at 800 rpm. Place the homogenate on ice.
10. Centrifuge the homogenate at $600\times g$ for 10 min, at 4 °C. Collect the supernatant and discard the pellet (*see Note 7*).
11. Centrifuge the supernatant at $1,100\times g$ for 10 min, at 4 °C. Collect the supernatant and discard the pellet (*see Note 8*).
12. Centrifuge the supernatant at $7,700\times g$ for 10 min, at 4 °C. Discard the supernatant and keep the pellet (*see Note 9*).
13. Resuspend the pellet in 4 ml of homogenate buffer containing EDTA-free Complete®-Mini protease inhibitor cocktail (prepared in **step 2**), to obtain a crude mitochondrial extract.
14. Carefully, and slowly, layer 2 ml of the crude mitochondrial extract to each of the 20 ml of self-generating gradients (prepared in **step 1**). To purify the mitochondria, centrifuge at $43,000\times g$ for 1 h, at 4 °C.
15. Carefully collect the lower yellowish-brown mitochondrial band and resuspend each in 30 ml of mitochondrial wash buffer 1.
16. Centrifuge at $7,700\times g$ for 10 min, at 4 °C. Discard the supernatant and keep the purified mitochondrial pellet.
17. Perform two sequential washes of the purified mitochondria, by resuspending the mitochondrial pellet in 30 ml of Chelex-100-treated mitochondrial wash buffer 2 (prepared in **step 3**) (*see Note 10*), followed by centrifugation at $7,700\times g$ for 10 min, at 4 °C, discarding the supernatant and keeping the pellet.
18. Resuspend and pool the purified mitochondrial samples in 1–2 ml of Chelex-100-treated mitochondrial suspension buffer (prepared in **step 4**).
19. Perform RNase treatment by adding 2 mg of RNase A per ml of purified mitochondrial suspension, and incubate at 37 °C for 1 h (*see Note 11*).

20. Centrifuge the mitochondrial suspension at $12,000\times g$ for 3 min, at 4 °C. Discard supernatant and maintain the purified mitochondrial pellet on ice.
21. Wash the mitochondrial pellet two times by resuspending in Chelex-100-treated mitochondrial suspension buffer (prepared in **step 4**) (*see Note 10*), and centrifuging at $12,000\times g$ for 3 min, at 4 °C.
22. Discard the supernatant and place the purified mitochondria on ice. Proceed directly to total RNA extraction (go to Subheading 3.2) (*see Note 12*).

3.2 Total RNA Extraction from Isolated Mitochondria

The flowchart in Fig. 2 depicts the protocol followed.

1. Add TRIzol® to the purified mitochondria pellet, and pipette up and down to homogenize the sample (*see Note 13*). Incubate for 5 min at room temperature.
2. Add 0.2 ml of Chloroform per ml of TRIzol® reagent used for sample homogenization, and vigorously shake, by hand, for 15 s, followed by a 3-min incubation at room temperature.
3. Centrifuge at $12,000\times g$ for 15 min, at 4 °C to allow phase separation, and collect the upper aqueous phase into a new tube (*see Note 14*). The interphase and lower phenol–chloroform phase may be stored at 4 °C, or at –80 °C for prolonged storage, and subsequent total protein extraction (Subheading 3.3) (*see Note 15*).
4. Precipitate the RNA by adding 0.5 ml of Isopropanol per ml of TRIzol® reagent used for sample homogenization to the aqueous phase containing the RNA. Shake by hand for 15 s and incubate at room temperature for 10 min (*see Note 16*).
5. Centrifuge at $12,000\times g$ for 10 min, at 4 °C, and discard the supernatant.
6. Wash the RNA pellet by adding 1 ml of 75 % Ethanol per ml of TRIzol® reagent used for sample homogenization, and mix the tube by inversion $\times 10$.
7. Centrifuge at $7,500\times g$ for 5 min, at 4 °C, and discard the supernatant.
8. Air-dry the RNA pellet for 5 min (*see Note 17*).
9. Resuspend the RNA in UltraPure™ DNase/RNase-Free Distilled Water and quantitate the RNA using NanoDrop 1000 (Thermo Scientific) (*see Note 18*).
10. Proceed to downstream applications (*see Note 19*) or store the RNA at –80 °C.

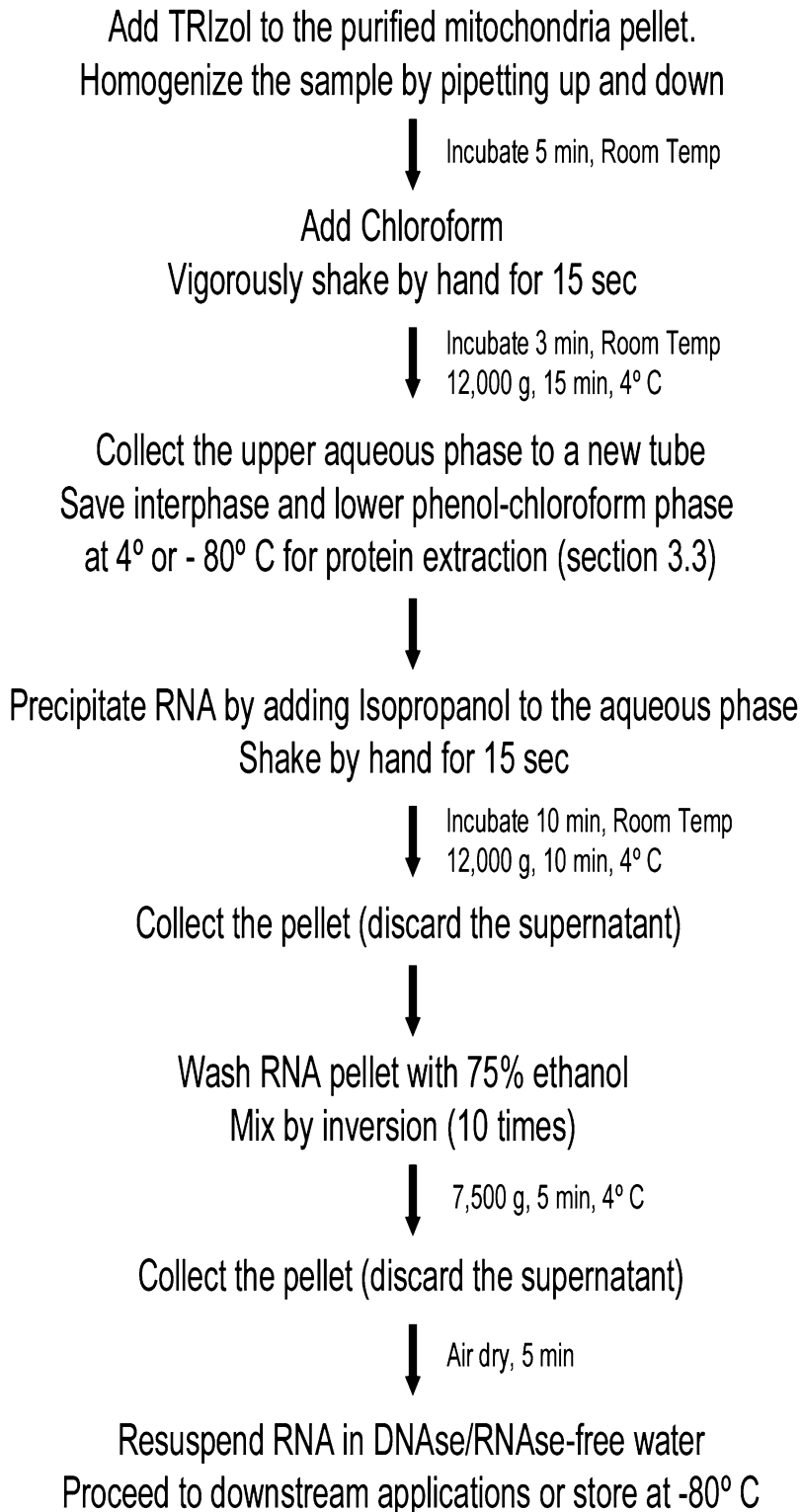


Fig. 2 Flowchart of total RNA extraction from isolated mitochondria

3.3 Total Protein Extraction from TRIzol®-Treated Isolated Mitochondria

The flowchart in Fig. 3 depicts the protocol followed.

1. Start with the interphase and lower phenol–chloroform phase obtained after RNA isolation (**step 3** of Subheading 3.2), and stored either at 4 °C or at –80 °C. If necessary, thaw the samples at room temperature, and centrifuge at 12,000×*g* for 15 min, at 4 °C. Discard the remaining upper aqueous phase.
2. Add 0.3 ml of 100 % Ethanol per ml of TRIzol® reagent used for sample homogenization to precipitate DNA. Mix by inversion and incubate for 3 min at room temperature.
3. Centrifuge at 2,000×*g* for 5 min, at 4 °C. Transfer the phenol–ethanol supernatant to a new tube for protein isolation (*see Note 20*).
4. Add 1.5 ml of Isopropanol per ml of TRIzol® reagent used for sample homogenization. Mix by inversion and incubate for 10 min, at room temperature.
5. Centrifuge at 12,000×*g* for 10 min, at 4 °C to precipitate protein, and discard the supernatant.
6. Perform three sequential washes of the protein pellets by adding 2 ml of protein wash buffer per ml of TRIzol® reagent used for sample homogenization, vigorously shaking the tubes by hand, incubating at room temperature for 20 min, and centrifuging at 7,500×*g* for 5 min, at 4 °C (*see Note 21*).
7. After the third wash and spin, add 2 ml of 100 % ethanol per ml of TRIzol® reagent, incubate at room temperature for 20 min, and centrifuge at 7,500×*g* for 5 min at 4 °C (*see Note 21*). Discard the supernatant and maintain the protein pellet on ice (*see Note 22*).
8. Solubilize the protein pellet by adding protein resuspension buffer, followed by five cycles of 15 s sonication and 30 s ice incubation, using a compact ultrasonic device with amplitude adjusted to 80 % and pulse to 90 %.
9. Centrifuge at 3,200×*g* for 10 min at 4 °C, to sediment insoluble material. Discard the pellet, and collect the supernatant containing the solubilized proteins to a fresh tube and store at –80 °C for downstream applications.

4 Notes

1. Sucrose solutions are easily contaminated, even when stored at 4 °C. Use the solution within 1 week. Ideally, prepare just before use.
2. The gradients should always be prepared prior to use, and carefully handled to avoid mixing and discontinuities that may alter the efficiency of the separations.

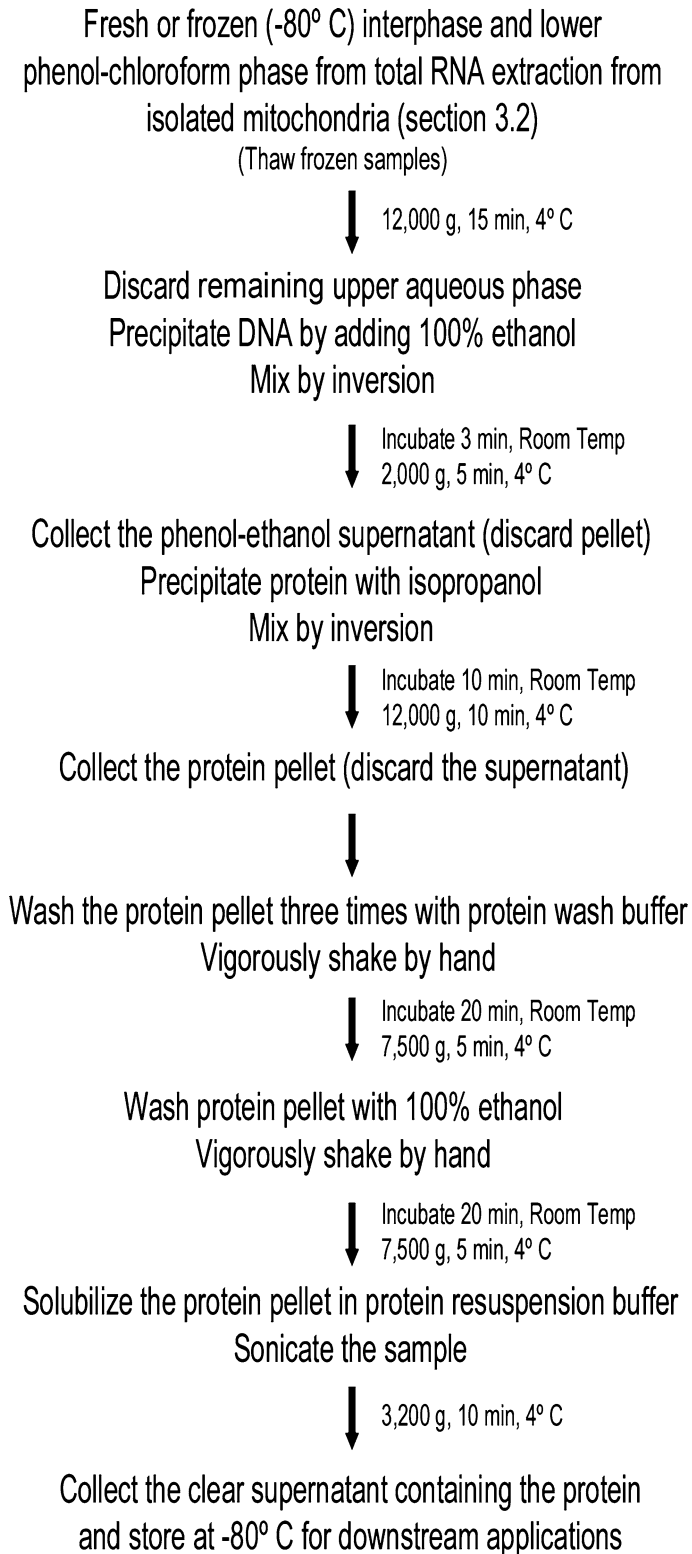


Fig. 3 Flowchart of total protein extraction from TRIzol®-treated isolated mitochondria

3. EDTA-free Complete®-Mini protease inhibitor cocktail should be added before use, and the buffer kept on ice. Always prepare fresh, discarding remainder of the solution, to ensure maximal protease inhibition.
4. Treatment of buffers with chelating resin Chelex-100 should be performed before use. Discard the remainder of Chelex-100 treated buffers, to ensure optimal clearing of the buffers.
5. Rinsing in three consecutive changes of homogenate buffer eliminates blood and other contaminants, which may be located on the outer surface of the liver during animal sacrifice and organ collection. After the final rinse of the liver in ice-cold homogenate buffer, the buffer should be clear and devoid of blood and macroscopic debris. If necessary, perform additional rinses in similar fashion.
6. Additional liver fragments may be stored for subsequent analysis (e.g., protein and/or RNA extraction from whole liver), by snap-freezing the samples in liquid nitrogen, followed by storage at -80°C .
7. This step clears the sample from insoluble tissue, incompletely lysed cells and other debris.
8. This step further separates the sample from insoluble tissue, incompletely lysed cells and other debris.
9. This step precipitates mitochondria and other cellular organelles.
10. The Chelex-100 resin precipitates by gravity. Following the 10 min incubation at room temperature, the Chelex-100 resin deposits at the bottom of the solution. Pipette the buffer without disturbing the Chelex-100 resin deposit.
11. RNase treatment of isolated mitochondria eliminates RNAs in solution and those located on the outer surface of the mitochondria, ensuring the absence of non-mitochondrial contaminating RNAs in the sample. Optionally, before and/or after RNase A treatment, you may take a sample of purified mitochondria to evaluate mitochondria morphology and purity by transmission electron microscopy and western blot, as previously described [13, 20].
12. You may optionally snap-freeze the purified mitochondria in liquid nitrogen, followed by storage at -80°C .
13. Isolation of total RNA using TRIzol® allows the isolation of microRNAs, suitable for multiple downstream applications [6–9, 21–23]. Alternatively, samples may be homogenized in TRIzol® reagent using a motor-driven Bio-vortexer (No1083; Biospec Products, Bartlesfield, OK) and disposable RNase/DNase free sterile pestles (Thermo Fisher Scientific, Inc., Chicago, IL) [8].

14. The sample mixture separates into an upper colorless aqueous phase, an interphase, and a lower red phenol–chloroform phase. The RNA containing the microRNAs is exclusively in the upper colorless aqueous phase.
15. Phenol–chloroform phases may be stored at 4 °C overnight or at –80 °C for long-term storage, of at least 2 years [24].
16. Alternatively, you may incubate the samples at –20 °C overnight to increase RNA precipitation, or as a stop point in the protocol.
17. Do not allow the RNA pellet to completely dry as this reduces its solubility.
18. Alternatively, you can quantitate the RNA by diluting the sample in UltraPure™ DNase/RNase-Free Distilled Water and determining its absorbance at 260 and 280 nm. RNA concentration may be determined using the formula: $A_{260} \times \text{dilution} \times 40 = \mu\text{g of RNA per ml}$.
19. Downstream applications may involve, among others, evaluation of microRNA expression by microarray analysis [6, 13, 22, 23], northern blotting [21], or Taqman real-time RT-PCR [7–9].
20. This step precipitates DNA, with the proteins remaining in the phenol–ethanol supernatant.
21. It is not necessary to resuspend the protein pellet. Vigorously shake by hand and incubate at room temperature. Following centrifugation, completely remove each buffer solution to maximize wash efficacy.
22. Alternatively, the protein pellets may be snap-frozen in liquid nitrogen and stored at –80 °C until resuspension.

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Mitochondrial Regulation

Methods and Protocols

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