Chapter 11

Mitochondria-Targeted RNA Import

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Abstract

The import of a modest number of nucleus-encoded RNAs into mitochondria has been reported in species ranging from yeast to human. With the advent of high-throughput RNA sequencing, additional nucleus-encoded mitochondrial RNAs are being identified. Confirming the mitochondrial localization of candidate RNAs of interest (e.g., small noncoding RNAs, miRNAs, tRNAs, and possibly lncRNAs and viral RNAs) and understanding their function within the mitochondrion is assisted by in vitro and in vivo import assay systems. Here we describe these two systems for studying mitochondrial RNA import, processing, and functions.

Key words Mitochondria, RNA import, Polynucleotide phosphorylase (PNPASE)

1 Introduction

Mitochondria import a range of nucleus-encoded RNAs, with different species having different substrate specificities [1]. Some of these RNAs are processed within mitochondria and have functions different from their cytosolic or nuclear counterparts [2]. Studying nucleus-encoded mitochondrial RNAs emphasizes a few general approaches. First, RNA localization inside mitochondria can be confirmed using organelle fractionation after the substrate RNA is imported in vitro. Second, the processing or cleavage of imported RNAs can be studied using northern blotting or in vitro import of radiolabeled RNA substrates of known length. Specific RNA processing sites and the addition of nucleotides can be identified using RNA ligation, followed by semiquantitative RT-PCR and sequencing, as described previously [3]. Finally, the functions of imported RNAs can be studied using approaches that are specific to the RNA species of interest. In addition to mechanistic and functional studies, mitochondrial RNA import pathway(s)

Volkmar Weissig and Marvin Edeas (eds.), *Mitochondrial Medicine: Volume I, Probing Mitochondrial Function*,
Methods in Molecular Biology, vol. 1264, DOI 10.1007/978-1-4939-2257-4_11,

and signal sequence(s) can be co-opted to import RNAs of interest into mitochondria to rescue defects caused by mtDNA mutations or to change the mitochondrial genome expression profile [4, 5]. Here, we describe two mitochondrial RNA import systems for these studies. One system is an in vitro import assay that uses in vitro-transcribed RNA substrates and either isolated yeast or mammalian mitochondria. A second, more challenging, and currently less efficient and less well-understood system is an in vivo import assay that utilizes an RNA import signal sequence in exogenously expressed DNA to target nucleus-encoded RNAs for import into mitochondria. The RNA substrates for in vitro import can be either radiolabeled or unlabeled and detected using autoradiography or RT-PCR, respectively. For yeast mitochondria, exogenous expression of the mammalian RNA import protein, polynucleotide phosphorylase (PNPASE), generates a system with enhanced import efficiency for substrate RNAs [6]. The efficiency of the in vivo import system varies markedly depending on the RNA to be imported; unfortunately, the precise rules for efficient RNA import in vivo have not been fully elucidated. In vivo, pre-mitochondrial processing of target RNA in the nucleus and/or cytosol and trafficking of RNA to the mitochondrion needs to be carefully considered, as these factors seem to greatly affect RNA import efficiency [5].

2 Materials

All solutions should be prepared using RNase-free water and the reagents used should be analytical grade. Unless indicated otherwise, all reagents should be prepared and stored at room temperature.

2.1 In Vitro Mitochondrial RNA Import

2.1.1 In Vitro Transcription

1. MEGAscript® SP6 Kit from Ambion (catalog number: AM1330) is used for in vitro transcription. The kit contains an enzyme mix, 10× reaction buffer, and solutions of ATP, CTP, GTP, and UTP (*see* **Note 1**).
2. α-P^{32}-labeled CTP, 6,000 Ci/mmol, 10 mCi/mL (*see* **Note 2**).

2.1.2 RNA Isolation

1. RNase-free water. Store at 4 °C.
2. Trizol reagent (Invitrogen). Store at 4 °C.
3. Chloroform.
4. 75 % Ethanol (RNAse-free). Store at 4 °C.
5. Isopropyl alcohol.

2.1.3 In Vitro RNA Import

1. 2× Import buffer for yeast mitochondria: 1.2 M sorbitol, 100 mM KCl, 100 mM HEPES, 20 mM $MgCl_2$, pH 7.1. Store at –20 °C.

2. 2× Import buffer for mammalian mitochondria: 0.45 M mannitol, 0.15 M sucrose, 20 mM HEPES, 50 mM KCl, 10 mM $MgCl_2$, pH 7.4. Store at –20 °C (*see* **Note 3**).
3. 100 mM ATP. Store at –20 °C.
4. 100 mM DTT (prepare fresh).
5. 500 mM NADH. Store at –20 °C.
6. 500 mM sodium succinate (prepare fresh).
7. 10 mg/mL RNase A. Store at –20 °C.
8. 10 mg/mL proteinase K. Store at –20 °C.
9. SDS buffer: 1 % SDS, 100 mM NaCl, 10 mM Tris–HCl, pH 7.4.

2.1.4 Urea-Polyacrylamide Gel Electrophoresis (Urea-PAGE)

1. 5× Tris-borate-EDTA (TBE) buffer.
2. 40 % Acrylamide/bisacrylamide (29:1) solution. Store at 4 °C.
3. Ammonium persulfate: 10 % solution in water (prepare fresh).
4. *N,N,N,N*-tetramethyl-ethylenediamine (TEMED).
5. Formamide loading buffer: 95 % deionized formamide, 5 mM EDTA, 0.025 % (w/v) SDS, 0.025 % (w/v) bromophenol blue. Store at –20 °C (*see* **Note 4**).

2.2 In Vivo Mitochondrial RNA Import

1. 2× HEPES-buffered saline (HBS): 50 mM HEPES, 280 mM NaCl, 1.5 mM Na_2HP0_4, pH 7.1.
2. 1 M $CaCl_2$.

2.2.1 Transient Transfection

2.2.2 Mitochondria Isolation

1. Mitoprep buffer: 0.225 M mannitol, 0.075 M sucrose, 20 mM HEPES, pH 7.4. Store at 4 °C.
2. 0.5 M EDTA. Store at 4 °C.
3. 0.2 M PMSF. Store at –20 °C.

2.2.3 RNase A Treatment, RNA Isolation, and DNase Treatment

1. 10 mg/mL digitonin (prepare fresh).
2. 10 mg/mL RNase A. Store at –20 °C.
3. SDS buffer: 1 % SDS, 100 mM NaCl, 10 mM Tris–Cl, pH 7.4.
4. RNase-free water. Store at 4 °C.
5. Trizol reagent (Invitrogen). Store at 4 °C.
6. Chloroform.
7. 75 % RNase-free ethanol. Store at 4 °C.
8. Isopropyl alcohol.
9. DNase I, RNase-free (Thermo Scientific).

2.2.4 RT-PCR

1. One-Step RT-PCR Kit (e.g., Promega).

3 Methods

Carry out all procedures at room temperature unless otherwise specified.

3.1 In Vitro Mitochondrial RNA Import

3.1.1 In Vitro Transcription

1. Prepare a DNA template that contains the SP6 polymerase promoter site for in vitro transcription.
2. Perform in vitro transcription using the MEGAscript® Kit from Ambion (*see* **Note 5**). Mix all of the components at room temperature in a 0.5 mL Eppendorf tube. The following is an example of a 20 μL reaction (*see* **Note 6**):

ATP	1.5 μL
CTP	1.0 μL
GTP	1.5 μL
UTP	1.5 μL
10× Reaction buffer	2.0 μL
α-P^{32}-CTP	7.5 μL
Linear template DNA[a]	0.1–1.0 μg
Enzyme mix	2.0 μL
Nuclease-free water	to 20 μL

[a]Use 0.1–0.2 μg PCR-product template or ~1 μg linearized plasmid

3. Pipette the mixture up and down or flick the tube gently. Then centrifuge the tube briefly to collect the reaction mixture at the bottom of the tube. Incubate at 37 °C for 2–4 h. If the transcripts are less than 500 ribonucleotides (nt), a longer incubation time (up to 16 h) may be advantageous.

3.1.2 RNA Isolation

1. After incubation, add 400 μL Trizol reagent and 170 μL chloroform to the mixture and vortex for 1 min.
2. Centrifuge the sample at 12,400 × *g* for 5 min at room temperature.
3. Transfer the upper aqueous phase into a fresh Eppendorf tube carefully without disturbing the interphase. Add 300 μL isopropanol to the aqueous solution and mix by pipetting or inverting the tube.
4. Centrifuge the sample at 20,000 × *g* for 10 min at 4 °C (*see* **Note 7**).
5. Carefully remove the supernatant. Add 600 μL ice-cold 75 % ethanol to the tube and invert the tube ten times.
6. Centrifuge at 20,000 × *g* for 2 min at 4 °C. Remove the supernatant (*see* **Note 8**).

7. Dry the RNA pellet at 37 °C and resuspend the RNA in RNase-free water (*see* **Note 9**). The RNA can be used fresh or stored at −80 °C for later use.

3.1.3 In Vitro RNA Import Assay

For import into mammalian mitochondria, always use freshly isolated mitochondria. For import into yeast mitochondria, freshly isolated mitochondria or mitochondria that were previously isolated, flash-frozen, and stored at −80 °C can be used. For import, finding a good negative control RNA that is not imported is required, and it is recommended that at least two different concentrations of test and control RNA is used in each experiment.

1. Freshly prepare 500 mM sodium succinate and 100 mM DTT. Thaw the 2× RNA import buffer, 500 mM NADH, and 100 mM ATP at room temperature.
2. Assemble the import reaction at room temperature. The following amounts are for a single 200 µL reaction in a 1.5 mL Eppendorf tube:

   Yeast mitochondria import reaction:

2× Import buffer (yeast)	98 µL
RNase-free water	62 µL
100 mM ATP	10 µL
500 mM NADH	2.0 µL
100 mM DTT	4.0 µL
Mitochondria (100 µg)	10 µL
5 pmol RNA	10 µL

   Mammalian mitochondria import reaction:

2× Import buffer (mammals)	98 µL
RNase-free water	58 µL
100 mM ATP	10 µL
500 mM sodium succinate	6.0 µL
100 mM DTT	4.0 µL
Mitochondria (100 µg)	10 µL
5 pmol RNA	10 µL

3. Add together everything in the import reaction except the RNA and incubate at 30 °C for 5 min.
4. Add RNA to the import reaction and mix gently. Incubate at 30 °C for 10 min to import RNA (*see* **Note 10**).

5. After import, add 1 μL of 10 mg/mL RNase A to the import reaction. Incubate at 30 °C for 20 min (*see* **Note 11**).
6. Transfer the mixture to a new tube and incubate at 30 °C for an additional 10 min (*see* **Note 12**).
7. Centrifuge the mitochondria into a pellet at 11,000 × *g* for 4 min at room temperature. Proceed immediately to **step 2** in the next section.

3.1.4 RNA Purification

1. Add proteinase K to 1× SDS buffer at a final concentration of 25 μg/mL and heat the SDS buffer to 95 °C.
2. Add 100 μL of SDS buffer from the previous step to the mitochondrial pellet and incubate at 80 °C for 1 min.
3. Immediately isolate RNA according to the purification steps detailed in Subheading 3.2, above, and dry the RNA pellet.

3.1.5 5 % Urea-PAGE

1. Prepare a 5 % urea-polyacrylamide gel before starting the import assay and pre-run the gel for 30 min at 300 V.
2. Dissolve mitochondrial RNA in 30 μL of formamide loading buffer and incubate at 95 °C for 10 min.
3. Immediately load RNA samples onto the urea-polyacrylamide gel. Electrophorese at 300 V until the dye front reaches the bottom of the gel (*see* **Note 13**).

When unlabeled RNA is used, the imported RNA can be detected by semiquantitative RT-PCR. Since many more steps are required, it may potentially introduce additional inefficiencies and/or may result in increased RNA degradation. Also, it is important to note that if the RNA is processed upon import, the primers that detect the RNA precursor form may not detect the processed RNA version.

3.1.6 Film Exposure

1. Following electrophoresis, remove the gel and place it onto a Whatman filter paper and dry it on a vacuum gel dryer.
2. Expose the gel for 1–7 days on X-ray film (*see* **Note 14**).

3.2 In Vivo Mitochondrial RNA Import

The system described here is for adherent mammalian cells (e.g., HEK293, HeLa, mouse embryonic fibroblasts) only. Clone the RNA-encoding gene into a desired mammalian vector. Noncoding RNA promoters or mRNA promoters have both been used successfully. Since semiquantitative RT-PCR is used for detection, it is essential to design primers that specifically amplify the imported RNA and distinguish it from endogenous sequences. In vivo RNA import is generally more complicated than in vitro RNA import with many parameters that affect efficiency still to be defined and with RNA localization and processing issues to consider, so a mitochondrial RNA import signal appended to an RNA of interest alone rarely results in successful mitochondrial import without further expression vector modifications.

3.2.1 Mammalian Tissue Culture Cell Transformation

1. Split a fairly confluent (70–90 %) culture between 1:3 and 1:4 into 15 cm plates (*see* **Note 15**).
2. When the cells are 70 % confluent, transfect the cells with control and RNA import constructs of interest (the protocol below is one example method of many; *see* **Note 16**).
   (a) Replace cell media with fresh DMEM + 10 % FBS and supplements.
   (b) In a sterile tube, mix 100 μg of DNA with 128 μL 1 M $CaCl_2$, 500 μL 2× HBS, and enough ddH_2O to make up a total volume of 1 mL (*see* **Note 17**). Incubate the mixture at room temperature for 20 min.
   (c) Carefully add the mixture to the plates in a dropwise fashion. Mix by rocking back and forth.
   (d) Incubate the transfected cells in a 5 % CO_2 incubator for 48 h (*see* **Note 18**).

3.2.2 Mitochondria Isolation

1. Collect cells by centrifuging at 2,000 × *g* for 2 min at room temperature. Remove the supernatant. Wash the cells with 1 mL 1× PBS, pH 7.4.
2. Resuspend the cells in 1.5 mL mitoprep buffer with 1 mM EDTA and 0.5 mM PMSF and transfer to a 5 mL glass/Teflon homogenizer. Perform 30 dounce strokes (*see* **Note 19**).
3. Transfer homogenate into a 1.5 mL Eppendorf tube and centrifuge at 800 × *g* for 5 min at 4 °C. Transfer the supernatant to a new Eppendorf tube.
4. Resuspend the pellet in 1.5 mL mitoprep buffer. Perform 20 dounce strokes. Transfer homogenate into a 1.5 mL Eppendorf tube and centrifuge at 800 × *g* for 5 min at 4 °C. Transfer the supernatant to a new Eppendorf tube. Take out 25 μL from each supernatant and keep it on ice as a measure of assay input.
5. Spin down the supernatants from the first and second dounce at 800 × *g* for 5 min at 4 °C.
6. Transfer the supernatants to new Eppendorf tubes and centrifuge at 11,000 × *g* for 5 min at 4 °C. Combine the pellets and wash with 1 mL mitoprep buffer. Centrifuge at 11,000 × *g* for 5 min at 4 °C, remove the supernatant, and resuspend the pellet in 30 μL mitoprep buffer.

3.2.3 RNase A Treatment, RNA Isolation, and DNase I Treatment

1. RNase A treatment.
   (a) Add 200 μg fresh mitochondria into 200 μL mitoprep buffer.
   (b) Add 1 μL 10 mg/mL digitonin to the mitochondria. Vortex gently at a low speed (*see* **Note 20**).

(c) Add 0.5 μL RNase A to the mixture (*see* **Note 21**). Incubate the mixture at 27 °C for 20 min. After incubation, centrifuge at 11,000 × *g* for 5 min. Remove as much supernatant as possible.

2. Trizol RNA isolation.

(a) Resuspend the pellet from the previous step in 100 μL SDS buffer with 25 μg/mL proteinase K preheated to 95 °C. Quickly put the tubes in an 80 °C heat block for 5 min (*see* **Note 22**).

(b) Add 400 μL Trizol reagent and 170 μL chloroform to the lysate. Purify the RNA as indicated in Subheading 3.2, above, of the in vitro import protocol (*see* **Note 23**).

3. DNase I treatment.
Resuspend the nucleic acid mixture in 30 μL DNase buffer with 0.5 unit of DNase I. Incubate at 37 °C for 30 min. Transfer the tubes to 65 °C and incubate for 10 min to inactivate DNase I.

3.2.4 RT-PCR

Use 1 μL sample as template for a 20 μL reaction. Specific primers are used for the one-step RT-PCR reaction (*see* **Note 24**).

4 Notes

1. For in vitro transcription, different RNA polymerase promoters, such as SP6, T7, or T3, can be used.
2. The radiolabel can be on any of the four ribonucleosides, but it must be labeled at the α position. Do not use β- or γ-labeled ribonucleoside triphosphates. Make sure to use caution when working with radioactive materials and follow institutional handling, use, and waste management protocols.
3. Many different buffers are used for mammalian mitochondria isolation. However, we recommend the use of a mannitol-sucrose buffer for the isolation procedure, so the mitochondria are maintained in the same conditions as the import buffer.
4. Heating the RNA sample with formamide loading buffer denatures the RNA and nucleases.
5. It is essential to pick the kit for the specific promoter. It is not necessary to use a kit, and homemade transcription systems can be used instead. However, we have observed that the commercial kits generally have a higher yield and increased full-length RNA products.
6. Different genes can have very different yields even with the same promoter and the same kit, so it is important to adjust the volume and reaction time as needed.

7. In most cases, a pale white RNA pellet will form at the bottom of the tube.
8. Remove as much supernatant as possible. Otherwise the drying would take much longer.
9. Do not dry the RNA at temperatures higher than 37 °C. Drying at a higher temperature makes it more difficult for the pellet to dissolve in water. For unlabeled RNA that requires semiquantitative RT-PCR for detection, it is necessary to perform the DNase treatment before proceeding to the next step.
10. In general, RNA import is quick. 10 min is normally sufficient, but the import reaction time can be adjusted up to 20 min.
11. RNase A is chosen because it does not require a strict buffer condition, so a buffer exchange step is not required. It is important to handle RNase A carefully, so it will not contaminate the samples in later steps. Hold the stock tube with only one hand and change gloves right after adding the enzyme. Alternatively, 25 μg/mL of S7 nuclease may be used in a 200 μL reaction containing 50 mM Tris–Cl, pH 8.0, and 5 mM $CaCl_2$.
12. This step ensures thorough RNase A treatment because some RNA may stick on the upper wall of the tube and not mix with the enzyme.
13. The percentage of the gel should be decided according to the length of the precursor and mature RNA species of interest. For RNA lengths between 200 and 500 nt, a 5 % urea-polyacrylamide gel is sufficient. It is not recommended to run the dye front off of the gel. This will cause the buffer to become radioactive.
14. Often, the import efficiency is not high, so a long exposure time is needed.
15. This method works best for adherent cells. The transfection efficiency varies among cell lines.
16. A control construct should encode RNA that is not imported into mitochondria. An import construct encodes the RNA of interest or RNA with an import signal and other sequences that are required for import.
17. The amount of the construct used should be adjusted so that the control and the import RNAs are expressed at similar levels, if possible.
18. The incubation time after transfection should be determined according to the experimental need.
19. It is important to avoid bubbles. Perform dounce strokes slowly.
20. This step permeabilizes the mitochondrial outer membrane.
21. Do not add too much RNase A because it cannot be easily inactivated even by added SDS and heat.

22. SDS buffer should be preheated to 95 °C to enhance the inactivation of RNase A. In addition, put the sample in 80 °C quickly to prevent RNA degradation.
23. It is important to dry the RNA at 37 °C. Do not over-dry the pellets.
24. The import efficiency in general can be low, so ~35 PCR cycles may be needed.

Acknowledgments

Supported by NIH grants F31 NS076228 (E.S.), T32 CA009120 (M.N.), R01 GM073981 (C.M.K., M.A.T.), R01 GM61721 (C.M.K.), R01 MH085683 (C.M.K.), P01 GM081621 (M.A.T.), R01 CA156674 (M.A.T.), R01 CA90571 (M.A.T.), and the Whitcome Pre-doctoral training program (E.S.)

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