An innovative method of cellulose acetate membrane based isolation of mitochondria and mtDNA extraction from the liver of *Duttaphrynus melanostictus* (Schneider, 1799)

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Mitochondria isolated by a non-conventional method of membrane filtration were used for mtDNA extraction. This technique allows trapping of mitochondria on cellulose acetate membrane. The procedure does not involve sophisticated instruments and can be performed out of laboratory conditions. The advantages of this procedure are discussed in this paper.

**Keywords**: Cellulose acetate membrane, *Duttaphrynus melanostictus*, mitochondria, mtDNA

**Introduction**

Mitochondria are semiautonomous eukaryotic cell organelle, essential for the functioning of several life processes. Apart from its importance in the respiration and yielding energy, it also plays a vital role in the process of aging and apoptosis. Distinctive feature of a mitochondrion is the presence of double stranded circular DNA molecule (mtDNA) and independent protein synthesis machinery, resembling the prokaryotic cells. Mutations in the mtDNA appear to be related to certain diseases, such as, diabetes, Alzheimer’s disease and muscle disorders in humans. Further, mitochondrion’s unique pattern of maternal inheritance makes it an ideal tool for the study of genetic variation¹ and phylogenetic relationships². Evolutionary biologists take the advantage of the uniqueness of the mtDNA to study the genetic relationship among the individuals of the same species and among closely related species and also predict the migratory patterns of specific population in historical times.

The first criterion to analyze the mtDNA of a species is to isolate the mitochondria with relative ease. Moreover, for population study, the mtDNA isolation from mitochondria must be hassle free and suitable for screening large samples in field conditions, requiring minimal technical expertise and also should be inexpensive in terms of chemicals, equipment and time. Early techniques for isolation of mitochondria and mtDNA involved ultracentrifugation of cell homogenate over cesium chloride gradients³, which was expensive and time consuming. More simplified procedures⁴,⁵ were evolved to isolate mitochondria but they too were laborious. Subsequently, improved versions of mitochondria and mtDNA isolation⁶,⁷ have been described but all the procedures are confined to laboratory-based conditions. Existing methods for mitochondria isolation require a well equipped laboratory with high speed centrifugation machines.

In the present study, we describe an innovative cellulose acetate filter based method for separating mitochondria from the liver of toad, *Duttaphrynus melanostictus* (Schneider, 1799). It is an ideal method for use in field conditions. Isolated mitochondria can either be immediately processed or stored for extraction of mtDNA later in the laboratory.

**Materials and Methods**

**Animal Model**

*D. melanostictus* that falls under the “least concerned” category of the World Conservation Union (IUCN) red list 2007⁸, was considered as the animal model for the present study. Adult, healthy individuals were captured from the wild and maintained in the laboratory under proper hygienic conditions and fed with house flies and chopped chicken liver. The animals were sacrificed following the guidelines and approval of the Animal Ethical Committee of the institute.
Isolation of Mitochondria following Standard Protocol

The liver was removed aseptically from anesthetized toad and washed with chilled lysis buffer-I (10 mM Tris-HCl; pH 7.8; 1 mM EDTA; pH 8.0; 0.32 M sucrose) twice to rinse off the blood. Approximately 0.8 to 1.2 g of liver was minced thoroughly followed by homogenization of the minced tissue in Teflon-coated glass homogenizer. The homogenized tissue was then centrifuged at 400 g for 15 min in table top cold centrifuge (Plasto Craft, Rota 4R-V/FA). The nuclear pellet and cell debris collected at the bottom of the tube was discarded. The supernatant was gently collected with the aid of a 2 mL syringe. The syringe was then fitted with a filter paper holder containing 0.45 µm cellulose acetate filter paper (Sartorius GmbH, Germany). By applying mechanical pressure the clear cell lysate was forced through the filter paper to trap the mitochondria in the cellulose acetate filter. The filter paper was resuspended in the same buffer and again centrifuged at 13000 g for 10 min at 4°C. The pellet containing mitochondria is ready for mtDNA isolation.

Isolation of Mitochondria by Membrane Filtration

Surgically removed liver was minced in lysis buffer-I and homogenized as described above. The homogenate was distributed in 2 mL plastic vials and centrifuged at 400 g for 15 min in a portable microcentrifuge (Spinwin, Tarson, India) to precipitate the nuclei and cell debris. The clear supernatant was gently collected with the aid of a 2 mL syringe. The syringe was then fitted with a filter paper holder containing 0.45 µm cellulose acetate filter paper. By applying mechanical pressure the clear cell lysate was forced through the filter paper to trap the mitochondria in the cellulose acetate filter. The filter paper was then removed from the holder and was either processed immediately for mtDNA isolation or preserved in an ordinary refrigerator for later isolation.

Isolation of mtDNA

a) To the mitochondrial pellet isolated by conventional method, 1 mL lysis buffer-II (10 mM Tris-HCl pH 8.0; 150 mM NaCl, 2 mM EDTA, 1% SDS & 100 µg proteinase K) was added and mixed gently until the pellet got dissolved and then incubated at 55°C for 60 min.

b) In case of mitochondria trapped in filter paper, the membrane was placed in 1 mL lysis buffer-II, rocked gently for 5 min and then incubated at 55°C for 60 min. After the incubation period, the membrane was squeezed to collect the buffer and then discarded.

After the completion of incubation period, similar procedure was followed to isolate mtDNA in both the cases. Equal volume of buffer-saturated phenol was added to the incubated solutions and mixed gently. The mixture was then centrifuged at 13000 g for 5 min. The upper aqueous layer was collected and the same process was repeated again. The upper aqueous layer was transferred in a fresh tube and equal volume of chloroform was added and mixed gently, followed by centrifugation at 13000× g for 10 min. The upper layer was again collected and the bottom layer was discarded. 200 µL of 5 M NaCl was added for every 500 µL of sample and 2.5 volumes of absolute alcohol to precipitate the mtDNA. If required, the final mixture was kept at ~20°C overnight for proper precipitation. The precipitate was centrifuged at 13000× g for 10 min and supernatant discarded. The pellet was then washed with 70% alcohol twice. The 70% alcohol was drained off and air dried till there were no visible droplets. Finally the pellet was suspended in 200 µL sterile double distilled water and preserved at 4°C until further use. The amount of mtDNA was quantified by spectrophotometry at 260 nm (Jasco, V-530, Japan).

Primers and PCR parameters

Several primers were designed using the software Primer3 from the mtDNA sequence available in the GenBank (Locus AY458592)9. For the present study, only 3 primers were used that gave specific single bands with mtDNA from D. melanostictus. The three primers: mtP1: Fw: 5′-ggtttcccattctttaaccatt-3′, Re: 5′-tcgtgtaggattgggctagg3′; mtP5: Fw: 5′-acccatccgcaataggttg-3′, Re: 5′-tcgtgtaggattgggctagg3′; mtP7: Fw: 5′-ttttgcagttgttctctct-3′, Re: 5′-agggaggccttctttctc-3′ were used to test the quality of mtDNA isolated from mitochondria separated by filtration. Genomic DNA contamination was assessed with D. melanostictus immunoglobulin M gene (IgM) specific primers10 (IgM-P4: Fw: 5′-tttacctgaggagaagatag-3′; Re: 5′-gtgctccatcttaaccct-3′). PCR was carried out following standard protocol in Primus25 peqlab with annealing temperature at 54°C. 100 bp ladders from Chromus Biotech, Bangalore, India were used for mol wt determination of the PCR product.

Results

Spectrophotometric Analysis of mtDNA

mtDNA extracted from the mitochondria, isolated by conventional method or through cellulose acetate
filter paper, gave similar absorption peak (Fig. 1). The quantity of mtDNA recovered too did not vary much as shown in Table 1. Protein contamination was judged by noting the absorption ratio at 260 and 280 nm. In all the cases, the ratio was above 1.7, which is considered good enough for conducting PCR experiments.

**PCR Results**

The PCR products using mtDNA from the conventional source are illustrated in Fig. 2. All the three primers gave a distinct single sharp band. The product size of primers mtP1 and mtP5 in lane 1 and 2, respectively, was slightly above 1 kb, while mtP7 depicted in lane 3 was almost 1kb. In lane 4, 100 bp DNA ladder was run to determine the mol wt of the PCR product. It may be noted that the same primers were used with genomic DNA isolated from blood to cross check their specificity. In all cases negative results were obtained.

An identical banding pattern was seen when the same primers were used with the mtDNA from mitochondria isolated by cellulose acetate paper as template (Fig. 3). The mtP1 and mtP5 produced bands that were more than 1kb, as seen in lane 1 and 3, while mtP7 generated bands which is nearly 1kb. Here, the lane 2 depicts the mol wt marker.

![Fig. 1](image1.png)

**Fig. 1**—UV spectrophotometric absorption profile of mtDNA isolated by conventional (-----) and the cellulose acetate filter method (-----). (In both the cases, the peak is at 260 nm indicating the presence of DNA)

<table>
<thead>
<tr>
<th>Weight of liver (g)</th>
<th>mtDNA extraction by conventional method</th>
<th>mtDNA extraction by membrane filtration method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absorption at 260 nm</td>
<td>0.224</td>
<td>0.215</td>
</tr>
<tr>
<td>Absorption at 280 nm</td>
<td>0.124</td>
<td>0.121</td>
</tr>
<tr>
<td>mtDNA (mg/mL)</td>
<td>11.2</td>
<td>10.75</td>
</tr>
<tr>
<td>260nm/280nm</td>
<td>1.81</td>
<td>1.78</td>
</tr>
</tbody>
</table>

![Fig. 2](image2.png)

**Fig. 2**—PCR amplified products of mtDNA isolated by the conventional procedure: Lanes 1, 2 & 3 represent products of specific primers mtP1, 5 & 7, respectively; & lane 4 represents 100 bp DNA ladder.

![Fig. 3](image3.png)

**Fig. 3**—PCR amplified products of the mtDNA isolated by the cellulose acetate membrane based method: Lanes 1, 3 & 4 represent products of specific primers mtP1, 5 & 7, respectively; & lane 2 represents 100 bp DNA ladder.
The same batch of mtDNA isolated by the above two procedures were subjected to PCR with *D. melanostictus* specific genomic DNA primer IgM-P4 and the results are shown in Fig. 4. A single band of approximately 470 bp was obtained from the genomic DNA (lane 1) but no bands were visible in lane 2 and 3, which were loaded with PCR product having mtDNA isolated by conventional method and by cellulose acetate paper as template, respectively, confirming that the mtDNA was free of genomic DNA contamination.

**Discussion**

mtDNA is a vital source of information for the evolutionary biologist for the study of population genetics and molecular phylogeny of eukaryotic organisms. To pursue such analytical study, isolation of mitochondria is a prerequisite step. Existing methodologies for isolation of mitochondria demands sophisticated instruments and a good laboratory setup. The present technique describes a relatively easy procedure for separating mitochondria from other cellular components by way of filtration that does not involve any sophisticated instruments and, thus, can also be performed outside the laboratory setup. In an experiment, the livers from two toads of the same batch were processed simultaneously adapting the conventional method and membrane filtration method. The data obtained from the two simultaneously performed experiments showed very little variation as shown in Table 1. As in conventional method, a small amount of lysosomes and peroxisomes were pelleted with mitochondria, membrane filtration technique could also trap some amount of lysosomes and peroxisomes on the membrane. But these contaminants did not come in the way of mtDNA isolation as they got removed during the process of phenol chloroform treatment. The PCR results (Figs 2 & 3) also exemplify that the filtration technique used to isolate mtDNA from mitochondria gave equally good bands and was absolutely free of genomic DNA contamination (Fig. 4). Therefore, adaptation of this new procedure in all probability will not affect downstream experiments like RFLP or sequencing of amplified segments. Moreover, the cellulose acetate filter paper containing mitochondria can be stored for some time before processing for mtDNA extraction. For storage, the filter paper can either be kept in a refrigerator or it can be kept dissolved in lysis buffer-II in an air tight container. But care needs to be taken to avoid fungal and microbial infections.

![Image](https://example.com/image.png)

**Fig. 4—**Assessment of genomic DNA contamination in mtDNA isolated by the conventional procedure and by cellulose acetate membrane based method by using *D. melanostictus* IgM gene specific primers: Lane 1, PCR product from genomic DNA showing positive result; lanes 2 & 3, PCR products of mtDNA from conventional source and cellulose membrane source, respectively showing negative results meaning both the mtDNA source are free of genomic DNA contamination; & lane 4, 100 bp DNA ladder.

The other advantage of this technique over the existing protocols for mitochondrial isolation is that the isolation-process can be done outside the laboratory setup. The only requirements are a homogenizer, microcentrifuge machine, a 2 mL syringe, cellulose acetate filter and a filter holder, all of which are easily portable and can be carried during field study. Thus, this method will particularly be useful for sampling large number of specimens in a relatively short span of time during field study.

**Acknowledgement**

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**References**

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