Isolation of high-quality, highly enriched mitochondrial DNA from mouse tissues

Marita A. Isokallio,James B. Stewart

Abstract

One remarkable issue in mitochondrial DNA sequencing and variant detection is heavy nuclear DNA contamination persistent throughout the commonly used mitochondria enrichment protocols including differential and gradient centrifugations [1,2]. The chromosomes contain nuclear mitochondrial sequences [3], which might cause false positive or negative results in variant detection. This problem is often overcome by enrichment of the mtDNA by long-range PCR [4], however, such methods are prone to early-cycle PCR errors. Here, we improved existing mtDNA isolation protocols (e.g. Kennedy et al. (2013) [11]) and describe a detailed step-by-step guide to obtain high-quality, highly enriched mtDNA suitable for sequencing and low-frequency variant detection as well as for other sensitive applications.

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Guidelines

INTRODUCTION

Although a cell harbors several thousands of mtDNA molecules, only <1 % of a total genomic DNA extraction is mtDNA. Thus, experiments focusing on mtDNA analysis often include an mtDNA enrichment step. Traditionally mitochondria are enriched from the cellular debris with differential or gradient centrifugation methods [1,2] or by amplification-based enrichment of the mtDNA [4,5]. The former methods will only partially enrich mtDNA, but samples still contain high nuclear DNA contamination levels [6]. Amplification-based methods suffer from errors caused by DNA polymerases or unintended amplification of nuclear sequences of mitochondrial origin (NuMTs) [7].

In the mouse genome, 172 chunks ranging from 33 bp to 4.7 kbp in length with 66–100 % identity were identified [8]. Others estimated that >95 % of the mouse mtDNA genome is present in the nuclear chromosomes [9]. NuMTs may be present or absent in different individuals and mtDNA is constantly being transferred to the nucleus, thus, NuMT content varies between individuals [7,8]. All these characteristics of NuMTs complicate the design of primers for mtDNA enrichment, and thus, might affect amplification- or blotting-based mtDNA studies [4,10]. Furthermore, ever increasing deep sequencing approaches to detect mtDNA mutations are especially prone for false results if a large amount of nuclear DNA is present in the sample. As NuMTs may be fully homologous to true mtDNA sequences, results would be biased towards wild-type reads [8]. On the other hand, polymorphic NuMTs would be observed as false-positive mutations which are impossible to distinguish from true mtDNA mutations [8].

Computational approaches [10] or fast plasmid preparations in combination with PCR-amplification [12] have been suggested as solutions to avoid NuMT reads in mtDNA mutation detection or to highly enrich mtDNA. Computational approaches, however, are very challenging to implement, whereas fast

DNA extraction procedures and amplification step may decrease the quality of the DNA and expose it to damage. DNA damage may also appear as false-positive mutations in sequencing applications, as DNA polymerase, for example, might bypass the damaged site by incorporating a wrong nucleotide or damage might increase the polymerase jumping increasing the amount of chimeric DNA molecules [14]. Furthermore, artefacts caused by oxidative damage to the DNA, often observed as G>T/C>A mutations, are a remarkable issue in reliable variant detection [15,16]. Hence, we reasoned that mtDNA analyses, especially variant detection by deep sequencing, should begin with high-quality, highly enriched mtDNA sample. Here, we describe a step-by-step protocol to extract mtDNA from mouse tissues suitable for highly-sensitive mtDNA experiments. The extremely high enrichment of mtDNA from nuclear DNA contamination is based on gentle tissue homogenization, extensive DNase I treatment of the isolated, intact mitochondria (modified from the protocol described by Kennedy et al. [2013] [11]) and DNA extraction by chloroform:isoamyl alcohol and ethanol precipitation.

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Before start

The protocol here is presented for a single tissue sample, but it can be scaled up for simultaneous preparation of several samples. However, significantly prolonged incubation times at any step due to increased amount of samples should be avoided.

The protocol has been mainly optimized for soft tissues, such as liver and brain (as in [11]). However, it also works for heart, although more extensive yet gentle homogenization is required. The mtDNA yield from heart is also much lower than from the bigger tissues and nDNA contamination levels are often higher.

REAGENT SETUP

CRITICAL: Reagent storage is at room temperature unless mentioned otherwise.

STOCK SOLUTIONS

• **Acetic acid, 2 M** Combine 11.48 ml glacial acetic acid with 88.52 ml distilled H_2O to obtain 100 ml 2 M acetic acid solution.

• **DNase I, 5 mg/ml in 5 mM $CaCl_2$** Dissolve DNase I according to the weight into suitable volume of 5 mM $CaCl_2$ (diluted from 1 M $CaCl_2$ stock solution, e.g. 7.5 µl 1 M $CaCl_2$ in 1.5 ml distilled H_2O) to obtain 5 mg/ml DNase I solution. Dispense into aliquots and store at -20 °C.

CRITICAL: DNase I activity might decrease if the storage period exceeds several months.

• **dNTPs, 1.25 mM each** Combine 250 µl of each dNTP (100 mM stocks) and add 19 ml distilled H_2O to obtain a solution containing 1.25 mM each dNTP. Dispense into aliquots and store at -20 °C.

• **EDTA, 0.5 M, pH 8.0** Dissolve 18.1 g EDTA into 80 ml distilled H_2O. Adjust the pH to 8.0 with NaOH and bring the volume to 100 ml with distilled H_2O.

• **EtOH, 70-%** Measure 35 ml of absolute EtOH and 15 ml distilled H_2O separately and combine to obtain ca. 48 ml of 70-% EtOH.

CRITICAL: 70-% EtOH is hygroscopic i.e. it will evaporate and absorb water over time, which will lower the concentration. Thus, always use rather freshly prepared 70-% EtOH solution.

• **NaOH, 15 M** Dissolve 600 g NaOH into 600 ml distilled H_2O. Bring the volume to 1 liter with distilled H_2O.

CAUTION! The reaction is exothermic. The solution can be kept on ice during preparation.

• **Potassium acetate, 6 M** Dissolve 58.92 g potassium acetate into 40 ml distilled H_2O. Adjust the pH to 7.5 with 2 M acetic acid. Bring the volume to 100 ml with H_2O.

CRITICAL: Long-term storage is recommended at -20 °C.

• **Proteinase K, 10 mg/ml** Dissolve 100 mg Proteinase K into 10 ml of distilled H_2O. Dispense into aliquots and store at -20 °C.

• **RNase A (free of DNase), 9.09 mg/ml** Dissolve RNase A at a concentration of 10 mg/ml into 10 mM sodium acetate pH 5.2 (e.g. dissolve 100 mg of RNase A into 1 ml of distilled H_2O, and dilute 100 µl of that RNase A solution with 890 µl of distilled H_2O and 10 µl of 1 M sodium acetate). Incubate the aliquot tubes in a heat block at 100 °C for 15 min. Allow the tubes to slowly cool down to room temperature. Adjust the pH by adding 100 µl (i.e. 0.1 volumes) of 1 M Tris (pH 7.4) to obtain 9.09 mg/ml RNase A solution. Dispense into aliquots and store at -20 °C.

CRITICAL: RNase A precipitates if the concentrated solution is boiled at neutral pH.

• **SDS, 10-%** Dissolve 10 g SDS into 80 ml distilled H_2O. Bring the volume to 100 ml with distilled H_2O.

• **Sodium acetate, 1 M, pH 5.2** Dissolve 24.61 g sodium acetate into 50 ml distilled H_2O. Adjust the pH to 5.2 with glacial acetic acid. Allow the solution to cool down to room temperature and adjust the pH again to 5.2 with glacial acetic acid. Bring the volume to 100 ml with distilled H_2O.

• **Tris, 1 M, pH 7.4** Dissolve 12.11 g Tris into 80 ml distilled H_2O. Adjust the pH to 7.4 with HCl. Allow the solution to cool down to room temperature and adjust the pH again with HCl. Bring the volume to 100 ml with distilled H_2O.

• **Tris, 1 M, pH 8.0** Prepare as Tris, 1 M, pH 7.4, but adjust the pH to 8.0.

BUFFERS

• **Lysis buffer: 20 mM Tris, 150 mM NaCl, 20 mM EDTA, 1 % SDS, pH 8.75** Dissolve 0.877 g NaCl into 60 ml distilled H_2O. Add 2 ml Tris (1 M, pH 8.0), 4 ml EDTA (0.5 M, pH 8.0) and 10 ml SDS (10-%). Adjust the pH to 8.75 with NaOH at room temperature. Bring the volume to 100 ml with distilled H_2O.

CRITICAL: High concentrations of NaCl and SDS precipitate.

CRITICAL: Tris pH is temperature dependent: pH 8.75 at room temperature corresponds to pH 7.8 at the usage temperature of 56 °C.

• **Mitochondria isolation buffer (MIB), 2x concentrate: 640 mM sucrose, 40 mM Tris, 2 mM EGTA, pH 7.2** Dissolve 219 g sucrose, 4.58 g Tris, 0.76 g EGTA into 950 ml distilled H_2O. Adjust the pH to 7.2 with HCl and bring the solution to 1 liter with distilled H_2O. Aliquot and store the 2x concentrate MIB at -20 ℃ until use.

CRITICAL: Tris pH is temperature dependent: pH 7.2 at room temperature corresponds to pH ca. 7.8

at the usage temperature of 0-4 ℃.

CRITICAL: MIB 2x concentrate solution can be stored frozen for very long periods of time (>12 months).

• **Mitochondria isolation buffer (MIB02): 320 mM sucrose, 20 mM Tris, 1 mM EGTA, 0.2 % BSA, pH 7.2** Thaw 2x concentrate MIB and dilute it with equal volume of distilled H_2O to obtain 1x MIB solution. Add 0.2 % (w/v) BSA to obtain MIB02 solution.

CRITICAL: Store thawed MIB at 4 ℃ only short periods of time (1-2 days).

CRITICAL: BSA-containing MIB solution should always be prepared freshly on the day of usage and stored on ice or at 4 ℃.

• **Mitochondria isolation buffer (MIB1): 320 mM sucrose, 20 mM Tris, 1 mM EGTA, 1 % BSA, pH 7.2** Thaw 2x concentrate MIB and dilute it with equal volume of distilled H_2O to obtain 1x MIB solution. Add 1 % (w/v) BSA to obtain MIB1 solution.

CRITICAL: Store thawed MIB at 4 ℃ only short periods of time (1-2 days).

CRITICAL: BSA-containing MIB solution should always be prepared freshly on the day of usage and stored on ice or at 4 ℃.

• **Mito-DNase buffer base: 300 mM sucrose, 10 mM MgCl~2~, 20 mM Tris, pH 7.5** Dissolve 10.27 g sucrose, 0.203 g $MgCl_2$ and 0.243 g Tris into 90 ml distilled H_2O. Adjust the pH to 7.5 with HCl and bring the solution to 100 ml with distilled H_2O. Aliquot and store the Mito-DNase buffer base at -20 ℃ until use.

CRITICAL: Tris pH is temperature dependent: pH 7.5 at room temperature corresponds to pH ca. 7.2 and 8.1 at the usage temperatures of 37 and 0-4 ℃, respectively.

• **Mito-DNase buffer: Mito-DNase buffer base, 0.15 % (w/v) BSA, 0.03 mg/ml DNase I, 0.02 mg/ml RNase A** Thaw Mito-DNase buffer base and add 0.15 % (w/v) BSA. Just before use, add 0.03 and 0.02 mg/ml DNase I and RNase A, respectively.

CRITICAL: BSA-containing Mito-DNase solution should always be prepared freshly on the day of usage and stored on ice or at 4 ℃.

EQUIPMENT

• 1.5-ml microcentrifuge tubes

• 250-µl low-bind, wide-orifice pipette tips (VWR, cat. no. 613-0370)

• 50-ml polypropylene Falcon tubes

• Gel electrophoresis system (e.g. Bio-Rad Sub-Cell(TM) GT Cell)

- Gel imaging system (e.g. Syngene U:Genius)
- Heat block for 1.5 ml microcentrifuge tubes (e.g. Grant QB-H2)
- Motor-driven glass/Teflon Potter Elvehjem homogenizer (e.g. Sartorius Potter S)
- PCR tubes (e.g. VWR® 8-well tube strips with bubble cap)
- PCR thermocycler (e.g. The Applied Biosystems® Veriti® 96-Well Thermal Cycler)
- Qubit® Assay tubes (Thermo Fisher Scientific, cat.no. Q32856)
- Qubit® 3.0 Fluorometer (Thermo Fisher Scientific, cat.no. Q33216)
- Refrigerated centrifuge for 1.5 ml microcentrifuge tubes (e.g. Eppendorf 5417R)
- Refrigerated centrifuge for 50-ml Falcon tubes, swing- and fixed rotors (e.g. Eppendorf 5804R)

Materials

37-% Hydrochloric acid 1.00317.1000 by Merck Millipore
Acetic acid, glacial 1.01830.2500 by Merck Millipore
Bovine serum albumin, essentially fatty-acid free A6003 by Sigma Aldrich
Calcium chloride 1.02378.0500 by Merck Millipore
Chloroform:isoamylalcohol 24:1 X205 by Amresco
Deoxyribonuclease I from bovine pancreas, type IV D5025 by Sigma Aldrich
dNTP Set, illustra Solution, 100 mM each dNTP 28-4065-52 by Ge Healthcare
Elution buffer (5 mM Tris/HCl, pH 8.5) 740917.1 by Macherey and Nagel
✓ Ethanol, absolute 99.8% 10342652 by Contributed by users
Ethidium bromide 10 mg/ml E1510 by Sigma Aldrich
Ethylenediaminetetraacetic acid disodium salt dihydrate ED2SS by Sigma Aldrich
Ethylene-bis(oxyethylenenitrilo)tetraacetic acid E3889 by Sigma Aldrich
GeneRuler 1 kb DNA Ladder SM0311 by Thermo Fisher Scientific
GeneRuler 100 bp DNA Ladder SM0241 by Thermo Fisher Scientific
GoTaq® Flexi DNA Polymerase M8291 by Promega
Magnesium chloride hexahydrate M2670 by Sigma Aldrich
Lambda DNA/HindIII Marker SM0101 by Thermo Fisher Scientific
Potassium acetate 1.04820.1000 by Merck Millipore
Proteinase K, recombinant, PCR grade 3115836001 by Sigma Aldrich
Qubit® dsDNA HS Assay kit Q32854 by Thermo Fisher Scientific
RNase A 740505 by Macherey and Nagel
Sodium acetate 1.06268.1000 by Merck Millipore
Sodium dodecyl sulfate, pellets CN30.2 by Carl Roth

Sodium hydroxide 1.06498.5000 by Merck Millipore

Sucrose S0389 by Sigma Aldrich

Tris(hydroxymethyl)aminomethane 1.08382.2500 by Merck Millipore

Ultrapure(TM) Agarose 16500100 by Thermo Fisher Scientific

Ultrapure(TM) TBE buffer, 10x 15581-0280 by Thermo Fisher Scientific

Primer: PolgA_F CTTCGGAAGAGCAGTCGGGTG by Sigma Aldrich

Primer: PolgA_R GGGCTGCAAAGACTCCGAAGG by Sigma Aldrich

Protocol

Tissue collection and homogenization (1-1.5 hour)

Step 1.

Sacrifice a mouse and collect 500-800 mg of liver tissue or a full brain (ca. 430 mg). Rinse the tissue in ice cold PBS. Store the tissue in a 50-ml Falcon tube in 5-10 ml ice cold PBS until homogenization.

NOTES

Marita A. Isokallio 01 Feb 2018

Use only freshly collected tissue for mitochondria isolation.

Tissue collection and homogenization (1-1.5 hour)

Step 2.

Homogenize the tissue with a teflon pestle by 5 strokes with 200 rpm. (Experimental: Homogenization of heart tissue requires harsher conditions.)

NOTES

Marita A. Isokallio 01 Feb 2018

Avoid breakage of the nucleus or mitochondria by too rigorous homogenization.

Tissue collection and homogenization (1-1.5 hour)

Step 3.

Remove PBS and transfer the tissue into glass homogenizer tube with 15 ml ice cold MIB1.

Tissue collection and homogenization (1-1.5 hour)

Step 4.

Transfer the homogenate back to the 50-ml Falcon tube and add 15 ml ice cold MIB1

Mitochondria isolation (~1 hour)

Step 5.

Remove cell debris by centrifugation at 800 g 4 ℃ for 10 min.

TEMPERATURE

4 °C Additional info: 800g

Mitochondria isolation (~1 hour)

Step 6.

Pour the supernatant into a clean 50-ml Falcon tube and repeat the centrifugation.

TEMPERATURE

4 °C Additional info: 800g

NOTES

Marita A. Isokallio 01 Feb 2018

In case a fat layer is formed on top of the liquid, dip a pipet tip gently into the solution and remove as much fat as possible.

Mitochondria isolation (~1 hour)

Step 7.

Pour the supernatant carefully into a clean 50-ml Falcon tube.

NOTES

Marita A. Isokallio 01 Feb 2018

Do not disturb the pellet.

Mitochondria isolation (~1 hour)

Step 8.

Collect mitochondria by centrifugation at 8,500 g 4 ℃ for 10 min.

TEMPERATURE

4 °C Additional info: 8500g

Mitochondria isolation (~1 hour)

Step 9.

iscard supernatant by pouring and carefully pipet out all leftover liquid. Continue to the next step immediately.

Nuclear DNA removal (~3 hours)

Step 10.

Add 0.03 mg/ml DNase I and 0.02 mg/ml RNase A into ice cold Mito-DNase buffer.

Nuclear DNA removal (~3 hours)

Step 11.

Dissolve mitochondria pellet carefully into 600 µl Mito-DNase buffer by pipetting up and down. Keep the tubes on ice.

NOTES

Marita A. Isokallio 01 Feb 2018

Use wide-orifice pipet tips when pipetting mitochondria-containing solutions.
Marita A. Isokallio 01 Feb 2018

Harsh pipetting will break mitochondria and decrease the overall yield, but thorough dissolving is required for DNase I to access and digest any nDNA leftovers increasing the purity of the final mtDNA sample.

Nuclear DNA removal (~3 hours)

Step 12.

Divide dissolved mitochondria solution into subfractions: the number of fractions depend on the input tissue amount such that each fraction represents ca. 100-150 mg tissue in 600 µl Mito-DNase buffer. For example, for a full brain tissue (ca. 430 mg) add 2x 600 µl Mito-DNase buffer (final volume 1.8 ml), mix well by pipetting and divide the solution into three 1.5-ml microcentrifuge tubes.

NOTES

Marita A. Isokallio 01 Feb 2018

Use wide-orifice pipet tips when pipetting mitochondria-containing solutions.

Nuclear DNA removal (~3 hours)

Step 13.

Incubate the tubes at 37 ℃ for 1-1.5 hours.

TEMPERATURE

37 °C Additional info:

Nuclear DNA removal (~3 hours)

Step 14.

Collect mitochondria by centrifugation at 13,000 g 4 ℃ for 30 min.

Nuclear DNA removal (~3 hours)

Step 15.

Remove supernatant thoroughly.

Nuclear DNA removal (~3 hours)

Step 16.

Wash out the leftovers of Mito-DNase buffer by carefully re-suspending the mitochondria pellet into 500 µl MIB02, and re-pelleting by centrifugation at 13,000 g 4 ℃ for 15 min.

TEMPERATURE

4 °C Additional info: 13000g

Nuclear DNA removal (~3 hours)

Step 17.

Repeat the previous wash step.

TEMPERATURE

4 °C Additional info: 13000g

Nuclear DNA removal (~3 hours)

Step 18.

Remove the supernatant, spin down the leftover liquid and pipet out any traces of liquid.

Nuclear DNA removal (~3 hours)

Step 19.

Freeze the pellet in liquid nitrogen and store at -80 ℃ until DNA extraction.

NOTES

Marita A. Isokallio 01 Feb 2018

Mitochondria pellets can be stored at -80 ℃ at least a few hours. Longer storage times (weeks/months) is not expected to affect the mtDNA extraction, but has not been tested during this protocol optimization.

DNA extraction (~3-20 hours)

Step 20.

Add 0.02 mg/ml Proteinase K and 0.02 mg/ml RNase A into lysis buffer preheated to 56 ℃.

TEMPERATURE

56 °C Additional info:

ANNOTATIONS

Marita A. Isokallio 19 Apr 2018

Correction:
The concentrations should be **0.2 mg/ml**.

DNA extraction (~3-20 hours)
Step 21.

Dissolve frozen mitochondria pellets into 400 µl lysis buffer by pipetting up and down vigorously.

DNA extraction (~3-20 hours)
Step 22.

Incubate the tubes at 56 ℃ overnight (ca. 14-16 hours).

TEMPERATURE
56 °C Additional info:

NOTES
Marita A. Isokallio 01 Feb 2018

Shorter lysis time can be used, even 1-hour lysis should be sufficient, but has not been tested during this protocol optimization.

DNA extraction (~3-20 hours)
Step 23.

Let the solution cool down to room temperature.

DNA extraction (~3-20 hours)
Step 24.

Add 100 µl potassium acetate.

NOTES
Marita A. Isokallio 01 Feb 2018

White precipitate forms when potassium reacts with SDS.

DNA extraction (~3-20 hours)
Step 25.

Add 500 µl of chloroform:isoamylalcohol (24:1) and shake the tubes for 20 s.

DNA extraction (~3-20 hours)
Step 26.

Separate the phases by centrifugation at 16,000 g at room temperature for 10 min.

TEMPERATURE
22 °C Additional info: 16000g

DNA extraction (~3-20 hours)
Step 27.

Transfer the upper aqueous phase carefully to a clean 1.5-ml microcentrifuge tube.

DNA extraction (~3-20 hours)
Step 28.

Add 100 or 200 µg of RNase A (i.e. 11 or 22 µl for brain or liver sample, respectively).

NOTES

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Complete removal of RNase A might require additional chloroform:isoamylalchol purification step instead of EtOH precipitation alone. However, additional purification step may decrease the final yield and is not necessarily required for a successful Illumina sequencing.

DNA extraction (~3-20 hours)

Step 29.

Incubate the tubes at 37 ℃ for 45-60 min.

TEMPERATURE

37 °C Additional info:

DNA extraction (~3-20 hours)

Step 30.

Add 1 ml (2 volumes) ice cold absolute EtOH and invert the tubes gently for 5 times.

NOTES

Marita A. Isokallio 01 Feb 2018

If, for example, smaller amount of tissue than recommended in this protocol is used and low concentration of DNA expected, glycogen can be used as a carrier to increase the DNA yield in EtOH precipitation. Even glycogen with a dye (e.g. Glycoblue) is compatible with Illumina sequencing.

DNA extraction (~3-20 hours)

Step 31.

Incubate the tubes at -80 ℃ at least for 30 min.

TEMPERATURE

-80 °C Additional info:

NOTES

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Incubation can be even 2-3 hours. Alternatively, prolonged (e.g. overnight) incubation at -20 ℃ is possible.

DNA extraction (~3-20 hours)

Step 32.

Pellet DNA at 16,000 g at room temperature for 15 min.

TEMPERATURE

22 °C Additional info: 16000g

DNA extraction (~3-20 hours)

Step 33.

Remove supernatant by pipetting and wash DNA with 500 µl 70 % EtOH. Pellet DNA by centrifugation at 16,000 g at room temperature for 5 min.

TEMPERATURE

22 °C Additional info: 16000g

DNA extraction (~3-20 hours)

Step 34.

Repeat the wash step in order to increase the purity of the DNA.

TEMPERATURE

22 °C Additional info: 16000g

NOTES

Marita A. Isokallio 01 Feb 2018

Do not disturb the pellet.

DNA extraction (~3-20 hours)

Step 35.

After the last wash, spin down the leftover EtOH and remove all traces by pipetting.

DNA extraction (~3-20 hours)

Step 36.

Air-dry the pellet briefly (1-2 min) until all trace ethanol has evaporated.

NOTES

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Over-drying the DNA might lead to a non-dissolving pellet or damage the DNA.

Drying the pellet with heat is not recommended as higher temperatures may also damage the DNA.

DNA extraction (~3-20 hours)

Step 37.

Dissolve the DNA pellet by adding 18-30 µl elution buffer AE (5 mM Tris/HCl, pH 8.5) depending on the DNA pellet size and required final concentration. Incubate the tubes at room temperature overnight.

NOTES

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Dissolving the pellet with heat is not recommended as higher temperatures may damage the DNA.
Marita A. Isokallio 01 Feb 2018

DNA is less stable in H~2~O and pH of H~2~O might not be optimal for DNA storage, whereas EDTA of TE buffer might not be suitable for downstream applications such as Illumina sequencing.
Marita A. Isokallio 01 Feb 2018

High yield of mtDNA might appear as a brown pellet and brown color might be visible also in the dissolved DNA solution. This affects spectrophotometric DNA concentration measurement, thus, fluorometric method should be used in order to have more reliable concentration measurement.
Marita A. Isokallio 01 Feb 2018

If higher concentration of DNA is required, it is advisable to use 5 mM Tris buffer. Doubling the concentration of DNA can then be obtained by evaporating half of the sample volume, giving a final concentration of 10 mM Tris which is suitable for most downstream applications.

DNA extraction (~3-20 hours)

Step 38.

Short-term storage is recommended at 4 ℃, long-term storage at -20 ℃.

NOTES

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Multiple freeze-thaw cycles should be avoided.
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Continue to the next step earliest on the following day of dissolving the DNA, or stop the protocol here and store the DNA at 4 or -20 ℃ according to the length of the pause. However, it is recommended to continue with the quality control measurements and combination of the final samples before long-term storage of the DNA sample at -20 ℃ in order to avoid unnecessary freeze-thaw cycles.

DNA quality control (~3-4 hours)

Step 39.

Measure the concentration of 1 µl sample with Qubit HS kit twice and take the average as the sample concentration. If the sample is too concentrated, remeasure with suitable dilution.

DNA quality control (~3-4 hours)

Step 40.

Test for nDNA contamination by performing 20-µl PCR reaction with primers designed for an nDNA-encoded target (here, we use primers specific for PolgA (expected product size 520 bp).

Master mix for a single 20-µl PCR reaction:

µl	Reagent	Final concentration
8.9	H_2O	adjust according to the sample volume
4.0	5x Green GoTaq reaction buffer	1x
3.2	dNTPs (1.25 mM each)	200 µM
1.2	$MgCl_2$ (25 mM)	1.5 mM
0.8	PolgA_F (10 µM)	0.4 µM
0.8	PolgA_R (10 µM)	0.4 µM
0.1	GoTaq DNA polymerase (5u/µl)	0.5 units
1.0	mtDNA sample	see note

PCR programme:

94 C - 60 s

---

30 cycles of:

94 C - 30 s

58 C - 30 s

72 C - 45 s

---

72 C - 3 min

8 C - Inf

NOTES

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Generally, 1 µl of the sample is suitable for the PCR, however, if sample concentrations are highly variable between samples, it is advisable to normalize the input DNA amounts.
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Use genomic DNA, such as a quick tail extract, as a positive control.
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For more accurate quality control, quantitative PCR is recommended as described by Kennedy et al. (2013) [11]
Marita A. Isokallio 01 Feb 2018

Heavy RNA contamination inhibits the PCR reaction and might give false-negative result.

DNA quality control (~3-4 hours)

Step 41.

Load the 20-µl PCR reaction fully on 1-% agarose gel in 0.5x TBE buffer containing 0.5 µg/ml EtBr. Only load 5 µl or less of the positive control sample.

NOTES

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No or only very slightly visible DNA band should be observed on the gel (see Figure 1A). nDNA contaminated fractions should not be used for further analysis. The isolation protocol should be optimized, often starting from the homogenization step, in order to reduce the nDNA contamination.
Marita A. Isokallio 01 Feb 2018

Mitochondrial DNA enrichment can be verified by PCR reaction with primers specific for a mtDNA-encoded gene.

DNA quality control (~3-4 hours)

Step 42.

Combine fractions from the same tissue which show no or extremely minor nDNA contamination and re-measure the final sample concentration with Qubit HS. Dispense the sample into aliquots and store at -20 ℃.

NOTES

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If higher (maximum double) concentration is needed for the downstream application, concentrate the sample by vacuum at 45 ℃.

DNA quality control (~3-4 hours)

Step 43.

Test the quality of the mtDNA sample by running 100 ng (according to the Qubit HS concentration measurement) on 1-% agarose gel.

EXPECTED RESULTS

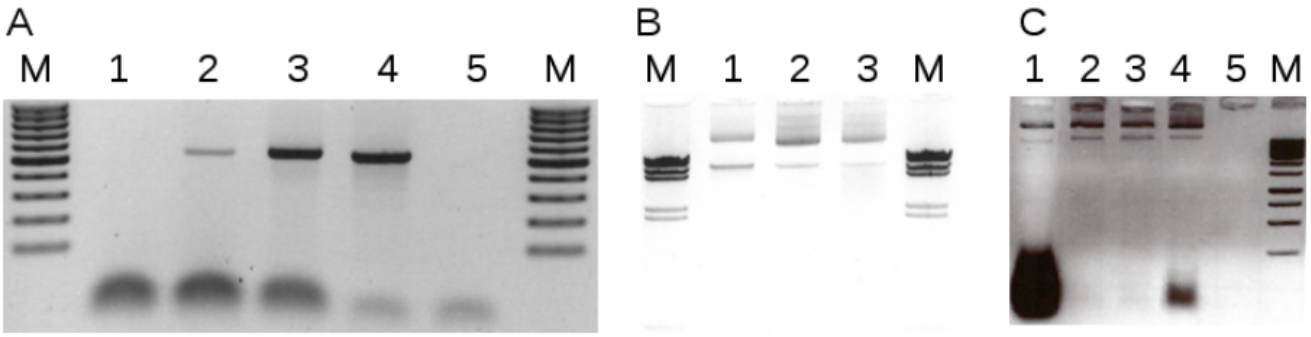

Figure 1. DNA quality control results and troubleshooting.

A) PCR-test of nuclearDNA (nDNA) contamination. An example of 20-µl PolgA-PCR samples from pure liver and brain mtDNA samples (lanes 1 and 2, respectively), contaminated brain mtDNA sample (lane 3) and 5 µl of controls (lane 4 wild-type mouse genomic DNA and lane 5 H_2O). M = 100-bp DNA Ladder. B) An example of 100 ng of liver (lane 1) and brain (lanes 2 and 3) mtDNA samples (same samples as used for PCR in A). M = Lambda DNA/HindIII Marker. C) Examples of RNA contaminated liver mtDNA samples: mtDNA extraction without RNase A treatment (steps 28 and 29) and insufficient RNase A treatment in lane 1 and 4, respectively. Lane 2 and 3 = good mtDNA samples, lane 5 = empty, M = 1-kb DNA Ladder. All gels contained 1 % agarose and 5 µg/ml EtBr.

From liver tissue, total yield of 2-3 µg of good quality mtDNA should be obtained: nuclear DNA specific PCR should not give a visible product on the gel and mtDNA should show distinct bands on the gel.

From brain tissue, total yield of 200-500 ng of highly enriched mtDNA should be obtained. Often slight nDNA contamination can be detected by PCR, but mtDNA should show distinct bands on the gel.

See Figure 1 for the examples of PCR results and gel runs.

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Circular mtDNA shows multiple bands on the gel according to different forms: supercoiled plasmid, relaxed plasmid and/or linear DNA. There should not be strong smears.

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Especially in a short gel run (ca. 30 min), RNA contamination can be seen as two distinct low-molecular weight bands, or more significant RNA contamination might also appear as a huge blob (see Figure 1C, lanes 1 and 4).