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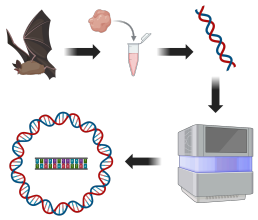
DNA Extraction and mtDNA genome sequencing protocol from museum voucher specimens



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We are still developing and optimizing this protocol

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Abstract

Museum voucher specimens represent an invaluable resource for biodiversity research, serving as permanent, verifiable records of species identities, morphology, and provenance (Suarez & Tsutsui, 2004). In recent decades, these archived specimens have also become critical molecular reservoirs, particularly for DNA-based taxonomic and phylogenetic studies (Wandeler et al., 2007). Among genetic markers, mitochondrial DNA (mtDNA) is especially suited for taxonomic assessment due to its maternal inheritance, relatively rapid mutation rate, and high copy number per cell, which enhances its retrievability even from degraded or historical samples (Avise, 2000; Paabo et al., 2004).
In mammals, sequencing mitochondrial genomes or key regions (e.g., cytochrome b, COI) from voucher specimens enables retrospective species identification, clarifies cryptic diversity, and facilitates phylogeographic and conservation assessments (Tobe et al., 2010; Lorenzen et al., 2011). Integrating molecular data from authenticated voucher specimens strengthens taxonomic resolution and provides reproducibility across studies, underscoring their dual importance in both classical and modern systematics.

Our protocol offers multiple extraction methods for variously stored samples (formallin or ethanol fixed). It makes it widely usable but the method was mainly tested on mammal samples.

References

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Materials

	A	B	C
	Reagents		
	Component	Manufacturer	Catalog number
	DNeasy Blood and Tissue Kit or	Qiagen	69504
	MinElute Reaction Cleanup Kits	Qiagen	28204
	Absolute Ethanol (Molecular Biology Grade)	any	-
	Phenol (Molecular Biology Grade)		-
	Isopropanol (Molecular Biology Grade)	any	-
	Sodium acetate (Molecular Biology Grade)	any	-
	SDS	any	-
	NaOH	any	-
	QIAseq FX DNA Library UDI Kit (24)	Qiagen	180477
	Qubit™ 1X dsDNA High Sensitivity (HS)	Invitrogen	Q33230
	Nuclease free water	any	-

	A	B	C
	Agencourt® AMPure® XP	Beckman Coulter	A63880
	apeStation DNA ScreenTape & Reagents	Agilent	-
	Flow cell	Illumina	-
	Cartridge	Illumina	-

	A	B	C
	Consumables		
	Component	Manufacturer	Catalog number
	Qubit™ Assay Tubes	Invitrogen	Q32856
	1.5 ml tubes	any	-
	Pipette tips 0.1-1000 µl	suitable	-
	PCR tubes 0.2 ml	any	-

	A	B	C
	Equipments		
	Component	Manufacturer	Catalog number
	Pipettes 0.1-1000 µl	any	-
	Centrifuge	any	-
	Magnetic stand or rack	any	-
	Qubit™ 4 Fluorometer	Invitrogen	Q33238

	A	B	C
	End-point PCR machines	any	-
	Tapestation System (4150 or 4200)	Agilent	-
	Illumina sequencer	Illumina	-

Troubleshooting

Safety warnings

Use all reagents based on the manufacturer's information, and check the risks and countermeasures on the safety data sheet!

Before start

If you are using the Phenol-Chloroform extraction, prepare the solutions in advance. I case of kits the handbook contains the necessary preparation before operation.

DNA Extraction from Formalin-fixed Tissue

2d 6h 54m 15s

1 For this version of extraction the **DNeasy Blood and Tissue Kit (Qiagen)** is used.

1.1 Manually chop the tissue and soak in water at room temperature for 06:00:00 hours than remove the water. 6h

1.2 Add 300 µL **ATL buffer** (pre-warmed to 98 °C) to the tissue and incubate at 98 °C for 00:15:00 minutes in a heating block. 15m

1.3 Cool down the samples on ice for 00:02:00 minutes. 2m

1.4 Add 40 µL **Proteinase K** and incubate for 48:00:00 hours at 65 °C . 2d

1.5 If the tissue is not digested completely, **Proteinase K** can be added up to 3 more times in 25 µL increments.

1.6 Vortex the sample for 00:00:15 seconds and briefly spin it down. 15s

1.7 Split the sample into two tubes and proceed with both (load both on the same column).

1.8 Add 200 µL **AL buffer** (for both aliquot), vortex and centrifuge.

1.9 Add 200 µL **Ethanol** (96–100%), vortex thoroughly and centrifuge.

1.10 Load both samples onto the same column, spin at 8000 rpm for 00:01:00 min. 1m

1.11 Discard collection tube, place column in a new collection tube.

1.12 Add 500 µL **AW1** buffer, spin at 8000 rpm for 00:01:00 min. 1m

1.13 Discard collection tube, place column in a new collection tube.

1.14 Add 500 µL **AW2** buffer, spin at 14,000 rpm for 00:03:00 min. 3m

1.15 Place column in a new collection tube, spin empty at 14,000 rpm for 00:01:00 min. 1m

1.16 Place column in a new 1.5 mL tube and add 140 µL AE buffer to the column.

1.17 Incubate for 00:30:00 min at room temperature, spin at 8000 rpm for 00:01:00 min. 31m

Note

For more information you can found the kit handbook here:

HB-2061-004_HB_DNY_Blood_Tiss...

1.18 If you used this method for DNA extraction, go to **Step 4** and proceed with quality check.

DNA Extraction using Phenol-Chloroform 1h 45m

2 For this version of extraction the classical **Phenol-Chloroform method** is used.

2.1 Add 500 µL **Alkaline lysis buffer** (0.1 M NaOH, 1% SDS) to 1.5 mL screw-cap Eppendorf tubes with O-ring.

2.2 Add tissue piece and incubate at 100 °C for 00:40:00 minutes. 40m

2.3 Cool down for 00:05:00 at room temperature. 5m

2.4 Add 500 µL **Phenol/Chloroform/Isoamyl alcohol** (25:24:1), mix gently for 00:05:00 min. 5m

2.5 Centrifuge at 10,000 rpm for 00:05:00 min. 5m

2.6 Transfer the upper aqueous phase to a fresh tube and add 500 µL **Chloroform**.

2.7 Mix gently for 00:05:00 min and centrifuge again at 10,000 rpm for 00:05:00 min. 10m

2.8 Carefully transfer the upper aqueous phase and precipitate the DNA by adding 0.6–1x volume **Isopropanol** and 0.1x volume [M] 3 Molarity (M) **Sodium acetate**.

2.9 Centrifuge at 13,000 g for 00:30:00 min at room temperature. Remove the supernatant carefully. 30m

2.10 Wash the pellet with 500-1000 µL **85% Ethanol**, centrifuge at 13,000 g for 00:05:00 min. 5m

2.11 Remove ethanol completely and carefully. Dry the pellet with open cap for 00:05:00 min. 5m

2.12 Resuspend the pellet in 50 µL **Nuclease-free water**.

2.13 If you used this method for DNA extraction, go to **Step 4** and proceed with quality check.

DNA Extraction from Ethanol-fixed Tissue (MinElute Kit)

2d 0h 33m

3 For this version of extraction the DNeasy Blood and Tissue Kit (Qiagen) is used.

3.1 Wash tissue pieces 3 times with 400 µL nuclease free water for 00:30:00 minutes. 30m

3.2 Remove the water from the last washing step.

3.3 Incubate tissue sample with 180 µL ATL lysis buffer (Qiagen) and 20 µl Proteinase K at 55 °C for 48:00:00 hours in a heat block. 2d

3.4 Add 300 µL ERC buffer to each portion of 100 µL lysate.

3.5 Buffer mix should appear yellow (pH 7.5); if not, add 10 µL [M] 3 Molarity (M) Sodium acetate.

3.6 Transfer the sample to a MinElute spin column.

3.7 Centrifuge at 10,000 g for 00:01:00 min. Discard collection tube and reuse the column with a new sample portion (if applicable). 1m

3.8 Wash bound DNA with 750 µL PE buffer. Centrifuge at 10,000 g for 00:01:00 min. 1m

3.9 Discard flow-through and centrifuge the empty column at maximum speed. Place column into a new 1.5 mL tube.

3.10 Elute DNA with 50 µL EB buffer, incubate 1 min at room temperature and centrifuge at 10,000 g for 00:01:00 min. 1m

Note

For more information you can found the kit handbook here:

EN-MinElute-Handbook.pdf

Quality check

2m

4 For quality check the concentration was measured with Qubit fluorometer using the 1xds High Sensitivity kit.

4.1 Aliquot 199 µL 1x Working solution to Qubit assay tube (equal to the number of your samples). Prepare two extra tubes for the standards with 190 µL 1x Working solution.

4.2 Add 1 µL sample for each test tube and 10 µL from the Standards (Standard 1, Standard 2). The final volume is 200 µL

4.3 Mix each sample vigorously by vortexing and pulse centrifuge to collect the liquid. Incubate at room temperature for 00:02:00 min before measuring.

2m

4.4 Calibrate Qubit fluorometer with the standards based on manufacturers recommendation.

4.5 Read your sample.

Note

If it is possible, fragment length could be checked on Bioanalyzer or Tapestation system.

NGS Library preparation

1d 1h 3m 20s

5 For the library preparation we use the QIAseq FX DNA Library Kit (Qiagen)

Note

The handbook of FX DNA Library Kit.

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5.1 **Sample dilution**

We need 100 ng DNA input for library preparation. Dilute the sample with Nuclease-free water to 100 ng / 35 µL concentration.

5.2 **Fragmentation and End preparation** 32m

Prepare the following reaction:

Component	Volume
FX Buffer 10x	5 µL
FX Enzyme Mix	10 µL
Sample DNA (100 ng)	35 µL
Final volume:	50 µL

Incubate the reaction with the following conditions:

Temperature	Time
4 °C	00:01:00
32 °C	00:01:00
65 °C	00:30:00
4 °C	

Note

DNA fragmentation was minimised due to the high degradation of nucleic acid (DNA ≤ 500 bp) material and low concentration (≤ 3 ng /µl).

6 **Adapter Ligation** 15m

Prepare the following reaction:

Component	Volume
DNA-Ligase Buffer 5x	20 µL
DNA-Ligase	10 µL
Nuclease free H2O	15 µL
Unique Adapter	5 µL
Previous reaction	50 µL

Final volume: 100 µL

Incubate at 20 °C for 00:15:00 minutes.

7 **Clean up**

Clean up the adapter ligated product with Ampure Xp Beads

7.1 Mix 80 µL beads with the previous reaction and incubate for 00:05:00 minutes at room temperature. 5m

7.2 Place on magnetic stand for 00:02:00 and carefully remove the supernatant. 2m

7.3 Wash the beads with 200 µL 80% EtOH for 00:00:30 seconds and and carefully remove the supernatant. 30s

7.4 Repeat the washing step: Wash the beads with 200 µL 80% EtOH for 00:00:30 seconds and and carefully remove the supernatant. 30s

7.5 Allow the beads to dry on the magnetic stand for approx. 5 min (till the point when the pellet lose its shine).

7.6 Add 52.5 µL elution buffer to the dried beads and resuspend well. Incubate for 00:02:00 minutes off the magnetic stand. Place back to the magnetic stand and incubate for additional 00:02:00 . 4m

7.7 Remove 50 µL of the supernatant and transfer to a new PCR tube.

8 **Library amplification** 4m 20s

Prepare the following reaction

Component	Volume
HIFI PCR Master Mix 2x	25 µL
adapter Primer Mix	1.5 µL
Cleaned up DNA	23.5 µL
Final volume	50 µL

Reaction conditions:

Temperature	Time	No. of cycles
98 °C	00:02:00	1
98 °C	00:00:20	8
60 °C	00:00:30	8
72 °C	00:00:30	8
72 °C	00:01:00	1
4 °C	Till further processing	

9 **Library Clean up**
Clean up the amplified library with Ampure Xp Beads

9.1 Mix 50 µL beads with the previous reaction and incubate for 00:05:00 minutes at room temperature.

9.2 Place on magnetic stand for 00:02:00 and carefully remove the supernatant.

9.3 Wash the beads with 200 µL 80% EtOH for 00:00:30 seconds and and carefully remove the supernatant.

9.4 Repeat the washing step: Wash the beads with 200 µL 80% EtOH for 00:00:30 seconds and and carefully remove the supernatant.

9.5 Allow the beads to dry on the magnetic stand for approx. 5 min (till the point when the pellet lose its shine).

9.6 Add 52.5 µL elution buffer to the dried beads and resuspend well. Incubate for 00:02:00 minutes off the magnetic stand. Place back to the magnetic stand and incubate for additional 00:02:00 .

9.7 Remove 50 µL of the supernatant and transfer to a new PCR tube. Storage at 4 °C for a day is possible but for long term storage -20 °C is suitable. 1d

10 **Library Quantification and checking**

Measure the concentration with Qubit Fluorometer according to **Step 4**. If it is possible fragment length could be checked on Bioanalyzer or Tapestation system. Alternative solution is gel electrophoresis (1.5% gel), as the library concentration should be high enough.

11 **Start sequencing**
Pool the sequencing based on the applied flow cell and equipment and start the run. Mitochondrial DNA typically constitutes **<0.5–1%** of total reads in whole-genome Illumina shotgun libraries. The minimum sequencing depth for successful recovery of complete mitochondrial genome is around 10 million reads/sample.