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An optimized protocol for sequencing mammalian roadkill tissues with Oxford Nanopore Technology (ONT) V.1

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High molecular weight DNA extraction from all kingdoms
Tech. support email: **See@each.protocol**

Frédéric Delsuc

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Abstract

This protocol was developed to optimize DNA sequencing from mammalian roadkill tissue samples using Oxford Nanopore Technology (ONT). Roadkill tissues contain necrotized cells and impurities that result in DNA degradation. First, we observed that DNA preservation was generally better in RNAlater than in 95% EtOH preserved tissues. Second, we found that physically removing necrotized and epidermal cells before DNA extraction resulted in better DNA quality and purity. Finally, adjusting the ratio of AMPure beads to 0.4x at the DNA purification step permitted optimizing size selection for subsequent ONT library construction. These optimization steps allowed to significantly increase both read length and yield per flow cell for roadkill samples sequencing on the ONT MinION device.

Materials

MATERIALS

- NanoDrop spectrophotometer **Thermo Fisher Scientific Catalog #**ND-1000
- Agencourt Ampure XP **Beckman Coulter Catalog #**A63880
- RNAlater **Thermo Fisher Scientific Catalog #**AM7020
- Phenol-chloroform-isoamyl alcohol 25:24:1 (PCI) **Invitrogen - Thermo Fisher Catalog #**15593049
- Proteinase K (2 ml) **Qiagen Catalog #**19131
- RNase A, DNase and protease-free **Thermo Fisher Scientific Catalog #**EN0531
- Qubit **Invitrogen - Thermo Fisher**
- DNA LoBind Tubes **Eppendorf Catalog #**#022431021
- g-TUBE **Covaris Catalog #**520079

Lysis buffer:
Stock solution (all solutions were sterilized even SDS):
Tris Hcl 1M 1ml
Nacl 5M 0,2ml
EDTA 0,5M 0,5ml
SDS 20% 10 ml
qsp sterilized water 100ml
Final concentration:
Tris-Hcl 10mM
Nacl 10mM
EDTA 2,5mM
SDS 2%
Lysis buffer will be preserved at 4°C. SDS will be precipitated. Keep the lysis buffer at least 1 hour at room temperature before use it.

Troubleshooting

1 Tissue sampling and preservation

Tissue were preserved in RNAlater Thermo Fisher® or 95% EtOH. We have observed that RNAlater preservation was often better to conserve DNA from internal organ tissues than 95% EtOH (Figure 1).

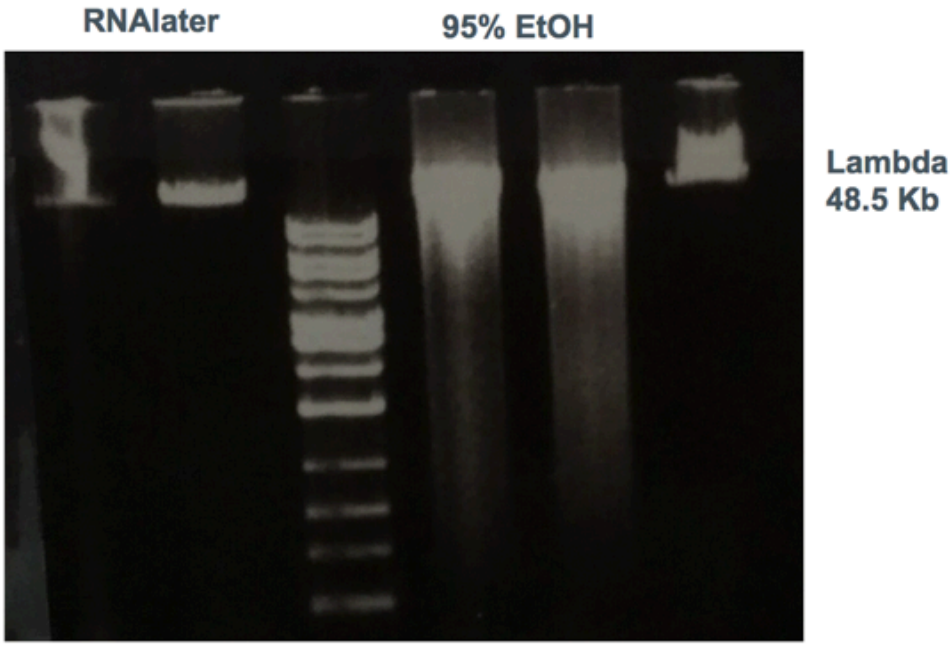

Figure 1: DNA extractions from spleen tissue of a fresh roadkill southern anteater (*Tamandua tetradactyla*) collected and preserved at the same time in either RNAlater or 95% EtOH.
Wells 1-2: extraction from spleen tissue preserved in RNAlater.
Wells 4-5: extraction from spleen tissue preserved in 95% EtOH.
Well 3: 200 bp-10 Kb ladder.
Well 6: lambda ladder (48.5 Kb).

2 Tissue preparation

Before the lysis step put the tissue in a new fresh preservation liquid (RNAlater or 95% EtOH). Under a binocular magnifier, remove all necrotized or epidermal cells, hairs, or dust particles using a scalpel blade. Try to keep the tissue with the best possible integrity in order to limit DNA degradation.

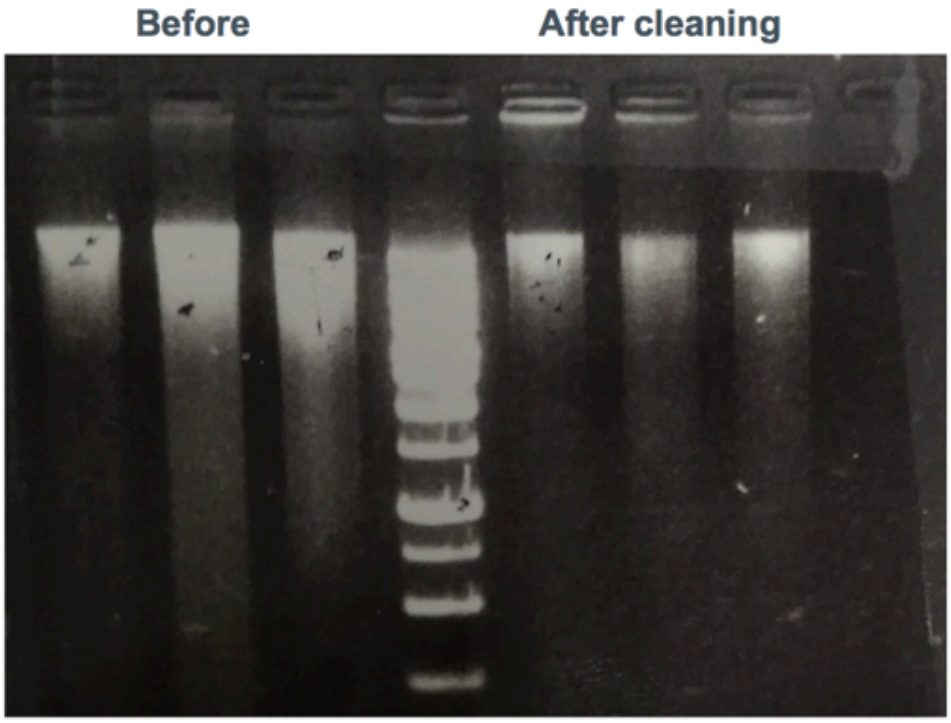

Figure 2: DNA extractions from an ear biopsy of a roadkill bat-eared fox (*Otocyon megalotis*) preserved in 95% EtOH.
Wells 1-2-3: DNA extractions from raw ear biopsies with hairs and epidermal cells.
Well 4: 200 bp-10 Kb ladder.
Wells 5-6-7: same tissue after removing hairs and epidermal cells using a scalpel under a binocular magnifier.

3 DNA extraction

3.1-Tissue lysis

1/ Use 25mg of tissue for 225 mL of lysis buffer with 25 mL of proteinase K.
2/ Incubate at 56°C for at least 2 hours. Tissue must be fully digested.
3/ Add 4 mL of RNAse and incubate at Room Temperature (RT) for 30 min.
Optional: Check tissue lysis under the binocular magnifier. If there are still particles (hairs, dust...) in the solution, centrifuge 5 min at 14000 rpm and transfer the supernatant in new tube.
4/ Add an equal volume of UltraPure Phenol: chloroform:Isoamyl Alcohol (Invitrogen).
5/ Mix by inversion 30 times until an emulsion is formed.
6/ Separate the two phases by centrifugation at 12000 rpm for 5 min at RT.
7/ Gently transfer the upper phase in a new tube without dislocating the interphase.
8/ Add an equal volume of chloroform-isoamyl-alcohol (24:1).
9/ Mix by inversion 30 times until an emulsion is formed.
10/ Separate the two phases by centrifugation at 12000 rpm for 5 min at RT.

11/ Gently transfer upper phase in a new tube without dislocating the interphase.

3.2-DNA precipitation

1/ Add 1/3 vol. of Amonium Acetate 7.5M (to improve protein precipitation: Crouse J. & Amorese D. 1987).
2/ Incubate at RT for 30 min.
3/ Centrifuge at 14500 rpm for 15 min. Sometimes a DNA pellet can appear.
4/ Under a binocular magnifier, gently transfer the supernatant in a new tube without dislocating the pellet.
5/ Check under the binocular magnifier if the supernatant is clear without suspension of slight particles.

At this stage the lysate must be clear to prevent the particles from condensing with DNA during the precipitation step. The presence of particles could disrupt the nanopores on the flow cell during DNA sequencing.

6/ Add 2.5 vol. of RT isopropanol and mix by inversion a couple of times. You must see a mass of DNA (condensate DNA fragments).
7/ Fish the DNA pellet with a 200 ml tip cut at the end to avoid damaging DNA.
8/ Transfer the DNA in 1 ml of fresh 70% EtOH heated at 50°C.
9/ Repeat steps 8 and 9 three more times.
10/ Place the DNA pellet in a low binding 2 ml tube.
11/ Remove residual EtOH with a 10 mL tip.
12/ Air-dry for 5 min (to evaporate EtOH).
13/ Add between 50 mL to 150 mL depending on the size of the condensed DNA.
14/ Place the DNA solution in the fridge at 4°C for 3-4 days so that it resuspends entirely before using it.

Crouse J. & Amorese D. (1987). Ethanol precipitation: ammonium acetate as an alternative to sodium acetate. *Focus* 9(2), 3-5.

4 DNA quality control

Quantify the DNA with Qubit® 2.0 Fluorometer (Thermo Fischer Scientific) and NanoDrop™ Spectrophotometer (Thermo Fisher Scientific).

1/ NanoDrop ratios must be: 1.8 < 260/280 < 2 and 2 < 260/230 < 2.2.
2/ Qubit/Nanodrop ratio must be between 0.6 and 1. Under 0.6, the DNA is not clean enough.
3/ Visualize DNA quality by migration on a 1% agarose gel.

5 Homogenization of DNA fragments size with Covaris G-tubes

1/ Load 4-5 mg (27 to 35 ng/mL) diluted in 150 mL of ultrapure water in a Covaris G-tube.
2/ Centrifuge the G-tube at 5000 rpm for 1 min.
3/ Reverse the G-tube and centrifuge at 5000 rpm for 1 min.
4/ Transfer the fragmented DNA in a new tube.

6 A-tailing and FFPE repair

1/ Transfer 3×48 µL of DNA in three 0.2ml DNA LoBind tubes.
2/ Prepare mix for 3 tubes following 1D Genomic DNA by Ligation (SQK-LSK-109) protocol by ONT with slight modifications: Incubation time is increased up to 1 hour at 20°C followed by 5 min at 65°C before putting on ice.
3/ Pool 3 tubes in one and purify with 0.4x ratio (72 µL) of Agencourt AMPure XP beads following the manufacturer instructions. Elude in maximum 80 µL of ultrapure water. This ratio allows removing the small DNA fragments.
4/ Check Qubit concentration
5/ Visualize DNA quality by migration on a 1% agarose gel (Figure 3).

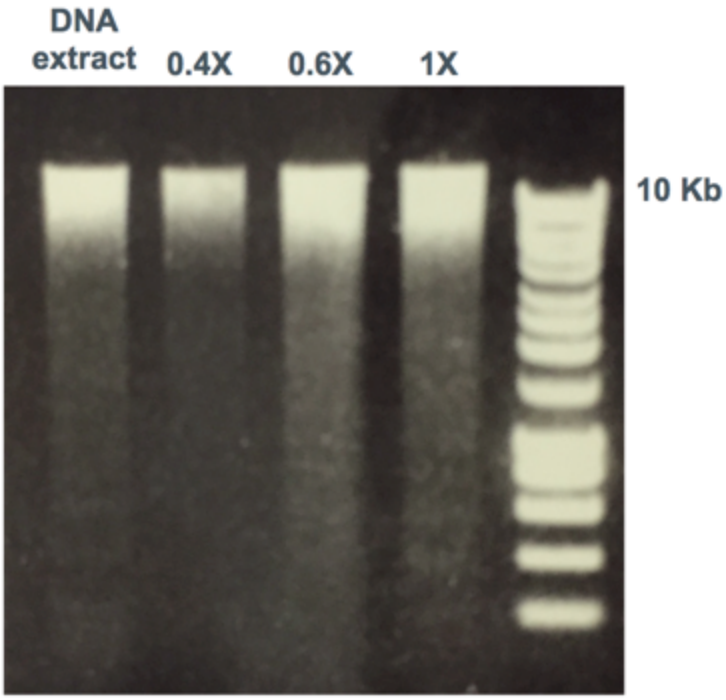

Figure 3. DNA size selection with different ratios of AMPure beadson extractions of a kidney tissue from a roadkill pygmy anteater (*Cyclopes didactylus*) preserved in 95% EtOH.
Well 1: Original DNA extract.
Wells 2-3-4: Size selection with 0.4x, 0.6x, and 1x of AMPure beads.
Well 5: 200 bp-10 Kb ladder.

7 ONT Library preparation

Using this protocol for Covaris G-tubes (step 5), the average size of DNA fragments was between 3000pb and 5000pb.

1/ Use 0.2 pmole of DNA
2/ Follow 1D Genomic DNA by Ligation (SQK-LSK-109) protocol with increasing incubation time up to 1h at RT.
3/ Increase elution volume to 16 µL instead of 13 µL.
4/ Transfer 15.5 µL of the supernatant (library) in a new tube placed on the magnetic rack.
5/ Wait 5 min, very often a little pellet of residual magnetic beads appears.
6/ Gently transfer 13 µL of supernatant without beads in SQB+LB mix.
7/ Check Qubit concentration of library using 2 µL of residual supernatant.

8 ONT MinION sequencing

MinIONqc

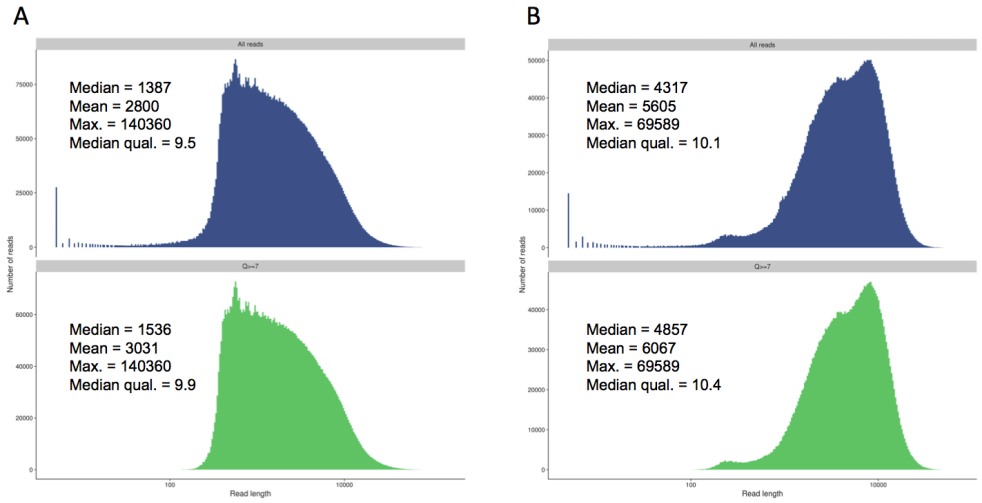

Figure 4: Read length distributions (log-scale) from MinION sequencing of a roadkill pygmy anteater (*Cyclopes didactylus*) obtained with MinIONQC (Lanfear et al. 2019). A. Results obtained from 10 flow cells with SQK-LSK108 ligation kit (total: 17.5 Gb) with basic Phenol/Chloroform extraction and following ONT library preparation protocol (FFPE+1x AMPure beads). B. Results obtained from 7 flow cells with SQK-LSK109 ligation kit (total: 22.2 Gb) following this optimized protocol.

Lanfear R., Schalamun M., Kainer D., Wang W. & Schwessinger B. (2019). MinIONQC: fast and simple quality control for MinION sequencing data. *Bioinformatics* 35(3):523-525.