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Extraction of high molecular weight insect DNA for long-read sequencing

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Bérénice Lafon[1], Marie-Ka Tilak[1], Fabien L. Condamine[1]

[1]CNRS, Institut des Sciences de l'Evolution de Montpellier, Place Eugène Bataillon, Montpellier, France

Bérénice Lafon
CNRS

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We use this protocol and it's working

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Abstract

The objective of this protocol is to extract insect DNA with minimal fragmentation to optimize long fragment sequencing with *Oxford Nanopore Technology* (ONT). The DNA manipulation and centrifugation steps have been minimized all along the protocol, which have also been simplified for implementing its application in the field. We have applied this protocol to a wide range of insects such as butterflies (Papilionidae [adults and larvae] and Nymphalidae), moths (Uraniidae), ants (Formicidae), beetles (Carabidae), and cicadas (Cicadidae). We have generally obtained on average 7 µg (ranging from 2 to 13 µg) of high-quality DNA for long-read sequencing. As the obtained DNA pellet is not purified during this extraction, it will require purification on beads before constructing the library. Such DNA extractions have further been successfully used for ONT sequencing on non-model insect species.

Image Attribution

Bérénice Lafon & Fabien L. Condamine

Materials

Equipments:

- Centrifuge
- Incubator (Eppendorf ThermoMixer F1.5)
- Dumont No. 5 forceps
- Scissors or scalpel
- Petri dishes
- Sterile compresses
- Mortar and pestle
- Pipettes P1000, P200, P10 + matching cones
- 2 mL LoBind tubes

Reagents:

- Buffer ATL Qiagen
- Proteinase K
- RNase A
- Ehanol absolute and 70% ethanol
- Sodium acetate (3 M, pH 5)

Step 1: Preparing and selecting the tissues

15m

1 Select tissues for extraction (avoid cuticle, bristles, gut, defensive glands, eggs...). Give priority to muscles and respiratory system (tracheae).

Pictured is a beetle specimen of *Carabus hispanus* for which we extracted high molecular weight insect DNA for long-read sequencing.

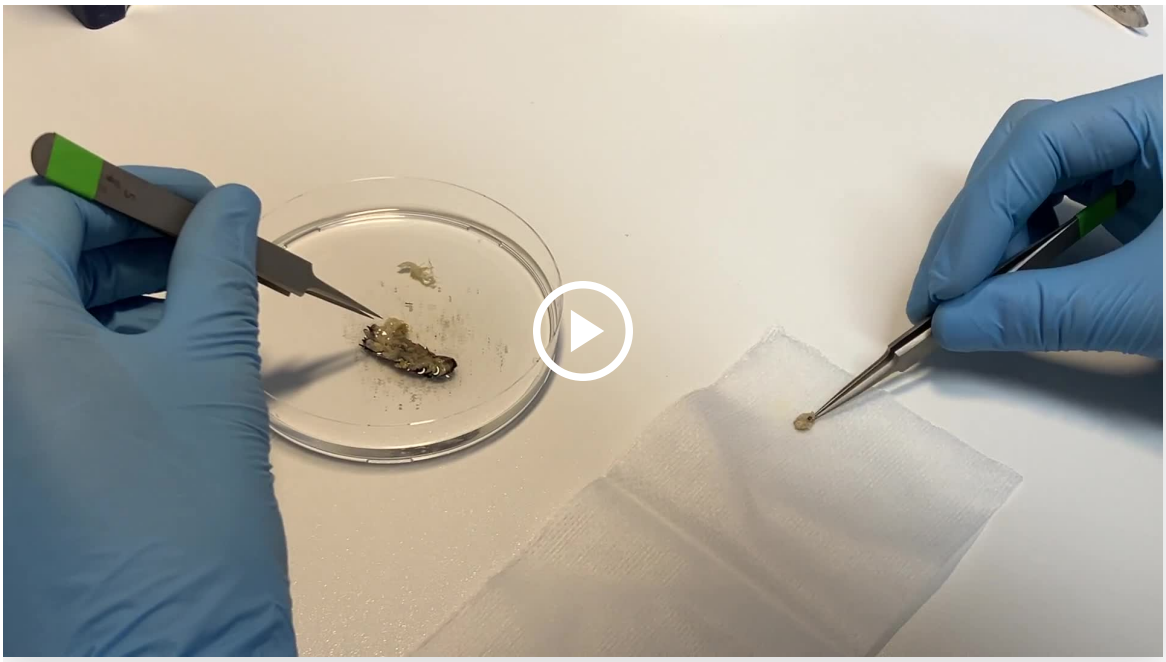
2 Depending on how the specimen has been sampled and preserved, one may adapt the DNA extractions as follows:

2.1 If the specimen is alive or has been killed and stored at -80°C, dissect it in absolute ethanol in a Petri dish. Lightly blot the tissue and cut it into small pieces on a sterile compress before placing it in the mortar.

2.2 If the specimen was preserved in ethanol, pour the contents of the tube into a Petri dish. Before placing the tissue in the mortar, lightly blot it with a sterile pad.

2.3 If the tissue sample was preserved in RNAlater, pour the contents of the tube into a Petri dish. Blot the RNAlater onto a sterile pad, then place the tissue pieces into a 1.5 mL tube with **1 mL of PBS** to wash out the RNAlater. Blot again on a sterile pad before placing the tissue in the mortar.

3 Video showing the selection of tissues as in the **case 2.1**. This is a specimen of *Carabus hispanus* that was freshly collected and dissected in absolute ethanol. Here we see the selection of internal tissues (mostly muscles) after the specimen has been cut into two parts. The tissues are lightly blotted before being placed in the mortar.



Step 2: Grinding the tissues

5m

4 Place **500 µL lysis buffer** (ATL from the *Qiagen DNeasy Blood & Tissue Kit* containing EDTA and SDS) in a mortar.

5 Grind with a pestle for a few minutes until the tissue is well crushed.

6 Recover all the crushed tissue with a P1000 pipette. If the grind is not sufficiently fluid, cut off the tip of the P1000 cone to facilitate collection. Transfer to a 2 mL tube.

7 Depending on the amount of tissue, adjust the volume by adding lysis buffer to completely submerge the tissue.

Step 3: Digesting the tissues

2h

8 Add Proteinase K (included in the *Qiagen DNeasy Blood & Tissue Kit*) at 10% of the buffer volume (e.g. **50 µL Proteinase K to 500 µL buffer**).

9 Gently mix by swirling the tube (do not vortex).

10 Add RNase A to digest the RNA. *Qiagen* recommends **4 µL RNase A for 200 µL** (buffer + Proteinase K), so adjust the volume of RNase A accordingly (e.g. add 11 µL RNase A for 550 µL volume).

11 Mix by gentle swirling of the tube (do not vortex). **Centrifuge for a maximum of 3 seconds.**

12 **Incubate at 56°C** (in a Eppendorf ThermoMixer F1.5 **at 300 rpm;** agitation is not mandatory) for 1 to 2 hours, depending on the progress of the digestion.

Step 4: Precipitating DNA

15m

13 **Centrifuge at 11,000 g for 10 minutes** at room temperature to pellet the remaining tissue.

14 Collect the supernatant, avoiding any floating fat layer, and transfer to a fresh 2 mL tube. **Tip: Pipet in 100 µL** steps to determine the final volume of supernatant (e.g. you hope to pipet between 450 and 500 µL).

15 Important for subsequent steps: If the volume of supernatant obtained exceeds 600 µL, separate this volume into two tubes.

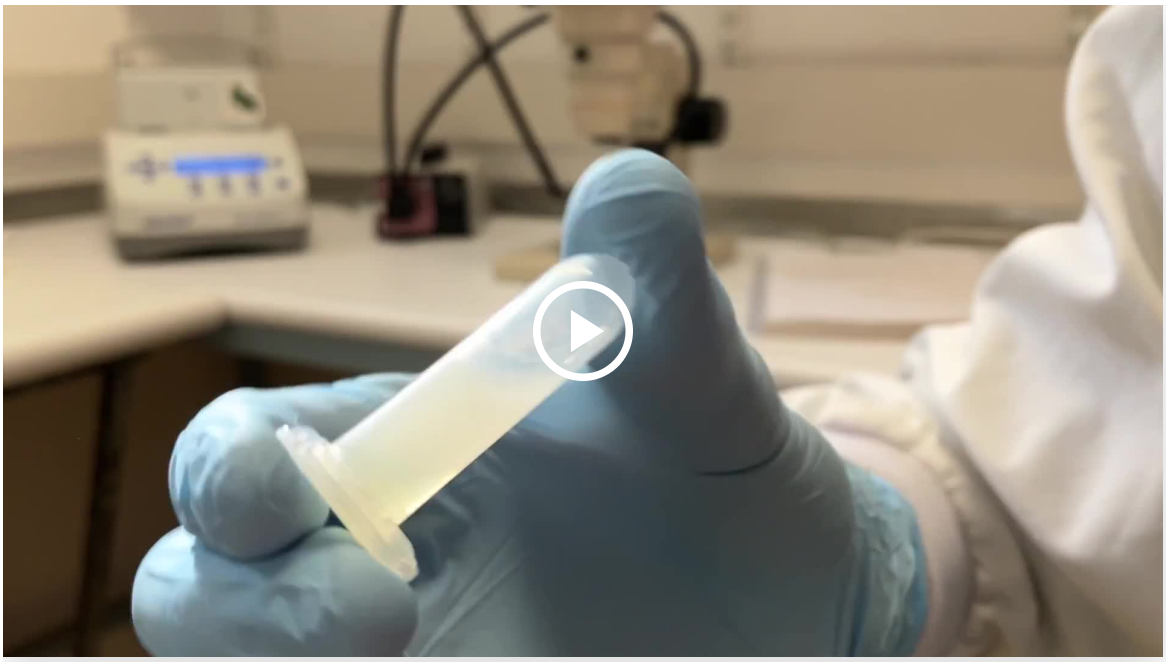
16 **Add sodium acetate (3 M) to 10%** of the supernatant volume (e.g. 50 µL for 500 µL supernatant).

17 Mix gently by swirling the tube (do not vortex).

18 **Add absolute ethanol equal to twice the volume** of the supernatant (e.g. 1,000 µL ethanol for 500 µL supernatant).

19 Mix gently by inversion of the tube until the DNA pellet forms (**approximately 20 inversions**).

20 Video showing the formation of the DNA pellet as expected from a high molecular weight insect DNA.



Step 5: Washing DNA

5m

21 **If the DNA forms a pellet:**

21.1 Collect it and transfer it to a 1.5 mL tube containing **1 mL of 70% ethanol**. *Tip:* Collect the DNA pellet by aspiration with a P1000 cone, taking care not to aspirate the pellet completely so as not to break it. Otherwise, remove the supernatant by gentle pipetting without aspirating the pellet.

21.2 Gently mix by inverting the tube.

21.3 Repeat the previous step a second time to make two washes with 70% ethanol.

21.4 Remove the 70% ethanol by pipetting without aspirating the DNA pellet.

21.5 **Centrifuge for a maximum of 3 seconds.**

21.6 Remove the remaining ethanol with a P10 pipette.

21.7 Allow to **dry for 5 minutes** with the tube open to allow the 70% ethanol to evaporate.

22 **If a DNA pellet is not present:**

22.1 **Centrifuge the tube at 11,000 g for 10 minutes** to pellet.

22.2 Pipette off the supernatant.

22.3 Add **1 mL of 70% ethanol** to wash the DNA pellet. Tap the tube to dislodge and resuspend.

22.4 Remove the 70% ethanol and repeat the previous step to perform two washes.

22.5 Remove the ethanol by pipetting without aspirating the DNA pellet.

22.6 **Centrifuge for a maximum of 3 seconds.**

22.7 Remove the remaining ethanol with a P10 pipette.

Step 6: Eluting DNA

13h

23 Resuspend the DNA pellet in water (between **100 µL and 200 µL**, depending on the size of the pellet) to allow it to settle properly.

24 If the DNA pellet does not dissolve completely (presence of fat or protein), **incubate at 37°C for 30 minutes** with minimal agitation (Thermomixer).

25 Leave it overnight at room temperature.

26 The next day, if impurities remain, **centrifuge for 5 minutes at 11,000 g** and collect the supernatant. If any fat remains, pipette the supernatant, taking care not to collect the fat, and transfer to a new 1.5 mL tube.

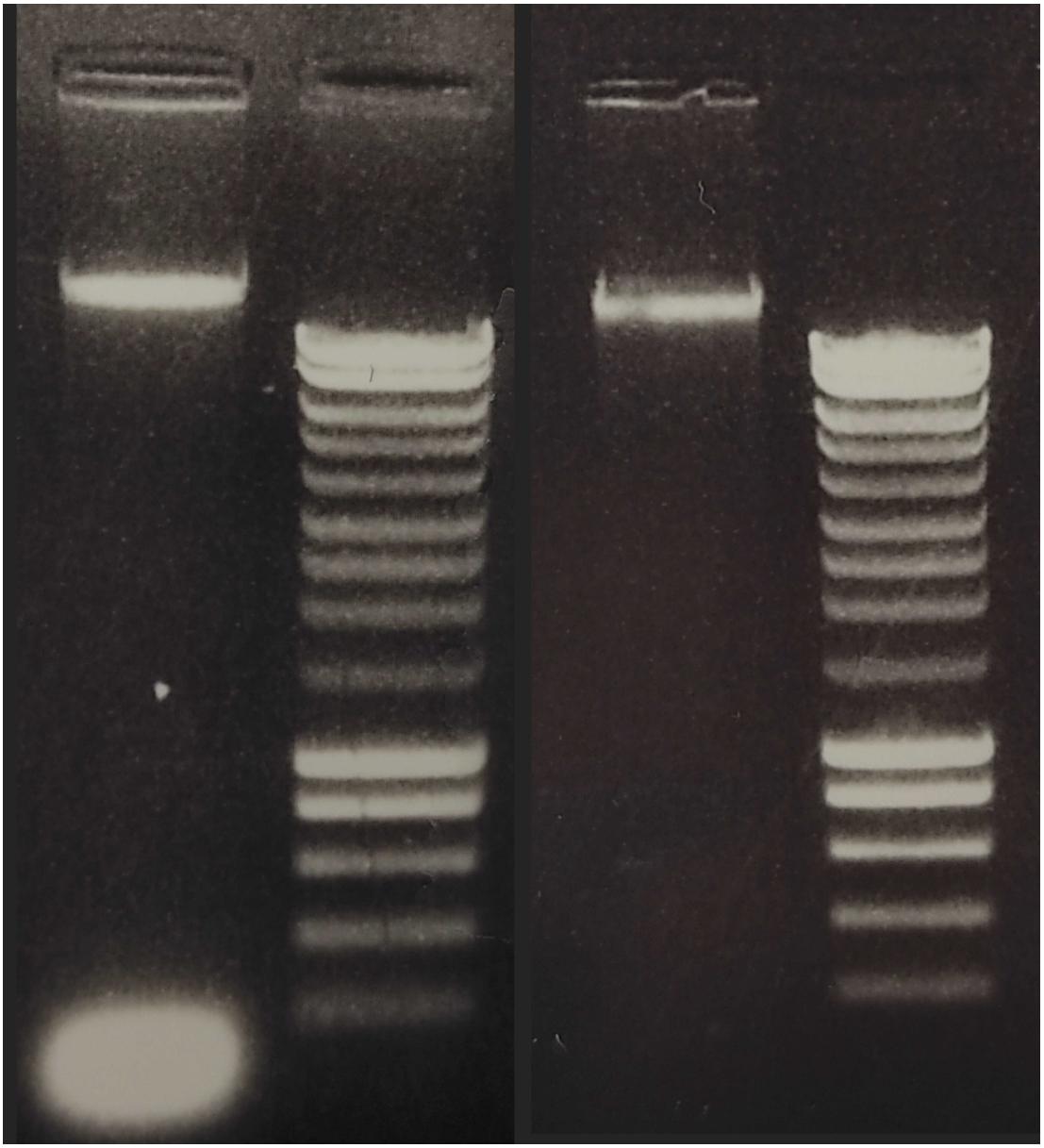
Step 7: DNA quality controls

1h

27 Perform a Qubit BR DNA assay.

28 Load on a 1% agarose gel. A large band above 10 kb should normally be observed. Degraded RNAs (bands below 200 bp) are often left over for purification during library construction.

29 Purification with AMPure 1X beads. Make a Qubit BR and Nanodrop assays.



Pictured is an agarose gel showing DNA after extraction (left) and DNA after purification with beads (right).