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Effect of DNA freezing on long-read sequencing in butterflies

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We use this protocol and it's working

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Abstract

High molecular weight DNA extractions are a key step in the sequencing of long DNA fragments. As part of a project to sequence reference genomes for genera in the family Papilionidae, we extracted and sequenced several DNAs from non-model species. For one of these, *Graphium doson*, the DNA extraction from a living individual resulted in high molecular weight DNA, but sequencing using *Oxford Nanopore Technology* (ONT) provided an N50 of around 3.3 kb. We expected an N50 of more than 10 kb, as indicated on our agarose gels. A year later, in an attempt to increase the amount of data for this genus, we repeated the sequencing run and obtained an N50 of around 7.7 kb. The difference between the first and second sequencing runs was that the DNA has been stored in a -20°C freezer. We therefore hypothesised that the freezing of the DNA had an effect on the sequencing result. To test this hypothesis, we performed several tests with another species, *Graphium antiphates*, to quantify the difference in N50 obtained with and without this freezing step after DNA extraction. Based on our results, we found a significant difference in N50 suggesting that freezing DNA extractions before DNA library preparations and ONT sequencing increase read lengths. Although we have only thoroughly tested the effect of freezing over non-freezing DNA from a single sample, we strongly recommend that ONT users freeze their DNA extractions for a few days (*i.e.* 2-3 days) after DNA extraction to maximize read lengths.

Image Attribution

AI-generated image with ideas from Bérénice J. Lafon and Fabien L. Condamine.

Materials

Equipments:

- Centrifuge
- Incubator (Eppendorf ThermoMixer F1.5)
- Dumont No. 5 forceps
- Scissors or scalpel
- Petri dishes
- Pipettes P1000, P200, P10 + matching cones
- 2 mL LoBind tubes
- Qubit [®] 2.0 Fluorometer (Thermo Fischer Scientific)
- NanoDrop[™] Spectrophotometer (Thermo Fisher Scientific)
- GridION (Oxford Nanopore Technologies)
- R9 flow cells (Oxford Nanopore Technologies)

Reagents:

- Qiagen Blood & Cell Culture DNA Mini Kit
- RNase I
- Protease
- Isopropanol
- Ethanol absolute and 70% ethanol
- Free nuclease water
- Short Read Eliminator Kit (SRE XS, Pacific Biosciences)
- SQK-LSK109 Kit (Oxford Nanopore Technologies)
- Agencourt AMPure XP beads (Beckman Coulter, France SAS)



Step 1: Preparing the specimen and DNA extraction

4h 59m

1 Preparation of the specimen

1.1 A specimen of the species *Graphium antiphates* (FC1341) was collected alive (see *Acknowledgements*). The butterfly was frozen alive in a papillote placed in a freezer at **-80°C**.

1.2 After separating the wings from the body, the butterfly was dissected in a Petri dish lined with 96% EtOH while taking care not to pick up any bristles or scales from the body. The body was separated into three parts: abdomen, thorax, and head.

5m

1.3 We performed one extraction for the abdomen and another for the thorax using the *Qiagen Blood & Cell Culture DNA Mini Kit*. The abdomen and thorax were ground separately and **1.5 mL of G2 lysis buffer** from the *Qiagen Blood & Cell Culture DNA Mini Kit* was added. Each extraction was performed according to the manufacturer's protocol.

5m

2 Tissue lysis

2.1 Add **3 µL RNase I** per tube and incubate for **30 min** at room temperature.

30m



2.2 Add **110 µL Protease** per tube and incubate for **2 h at 50°C**, inverting the tubes from time to time.
Centrifuge at **11,000 rpm for 15 min at 4°C**.

2h 30m



2.3 Meanwhile, equilibrate the columns by adding **1 mL of QBT buffer** to each column and allow the liquid to pass through by gravity.

1m



2.4 After centrifugation, collect the supernatant and transfer to another 2 mL tube.
Add **400 µL of buffer G2** and vortex at **medium speed for 10 s**.

1m



2.5 Transfer the tube to the column and wait for **5-10 min**.
Wash 4 times with **QC buffer using 4 x 1 mL**.

10m



2.6 Transfer the column to a new 1.5 mL tube and add **750 µL of QF buffer** (previously heated to **50°C**) to elute the DNA.

1m





3 DNA precipitation

3.1 Add **525 µL of isopropanol** and mix by inversion several times. *

5m

***If a DNA 'jellyfish' forms, transfer it to a new tube containing 70% EtOH and centrifuge at 12,000 rpm for 5 min. Remove all EtOH from the tube with a P10 pipette, dry it for 5 min, and elute in 80 µL of nuclease-free water.**



3.2 Centrifuge at **12,000 rpm for 20 min at +4°C**.

20m



3.3 Remove the supernatant and add **1 mL of cold EtOH 70%**.
Remove the pellet and mix by inversion.
Centrifuge for 5 min at 12,000 rpm at 4°C.

5m



3.4 Repeat step 3.3

3.5 Remove the EtOH with a P10 pipette.
Dry the tube for **5 min** at room temperature.

5m



3.6 Elute the DNA into **50 µL of free nuclease water**.

1m



4 Extraction results and DNA quantity and quality control

1h

DNA was quantified using the Qubit® 2.0 fluorometer (Thermo Fisher Scientific) and the NanoDrop™ spectrophotometer (Thermo Fisher Scientific). DNA quality was also checked by running a 1% agarose gel (**Figure 1**).

After these two extractions we obtained 4 tubes of DNA including:

- a DNA pellet for the abdomen (148 ng/µL in 100 µL)
- a DNA 'jellyfish' for the abdomen (78.3 ng/µL in 100 µL)
- a DNA pellet for the thorax (119 ng/µL in 100 µL)
- a DNA 'jellyfish' for the thorax (77.8 ng/µL in 100 µL)

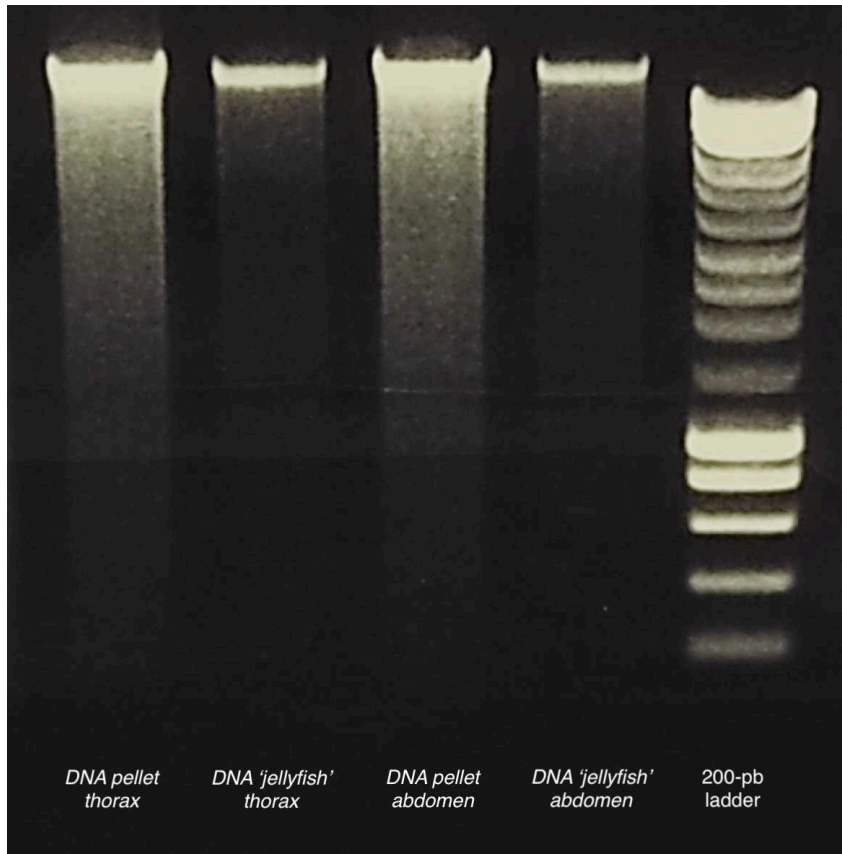


Figure 1. Pictured is an agarose gel showing DNA after extraction of the thorax (two left bands) and after extraction of the abdomen (two right bands).

Step 2: DNA freezing and removal of small fragments

3d

- 5 We tested the effect of freezing on the N50 of ONT sequencing (see **Figure 2** for the details). To do this, we divided each of the 4 tubes into two (50 μ L each), with one tube frozen and the other not. Thus, 4 tubes of DNA were frozen at **-20°C for 72 h**, while the other 4 tubes were kept in the refrigerator at **4°C** for the same period.

3d

After freezing and/or refrigeration, we removed fragments smaller than 10 kb from the DNA extracted from the thorax using the Pacific Biosciences *Short Read Eliminator* (SRE XS), formerly called the "Circulomics" kit, according to the protocol instructions.

DNA was quantified using the Qubit[®] 2.0 fluorometer (Thermo Fisher Scientific) and DNA quality was also checked by running a 1% agarose gel.

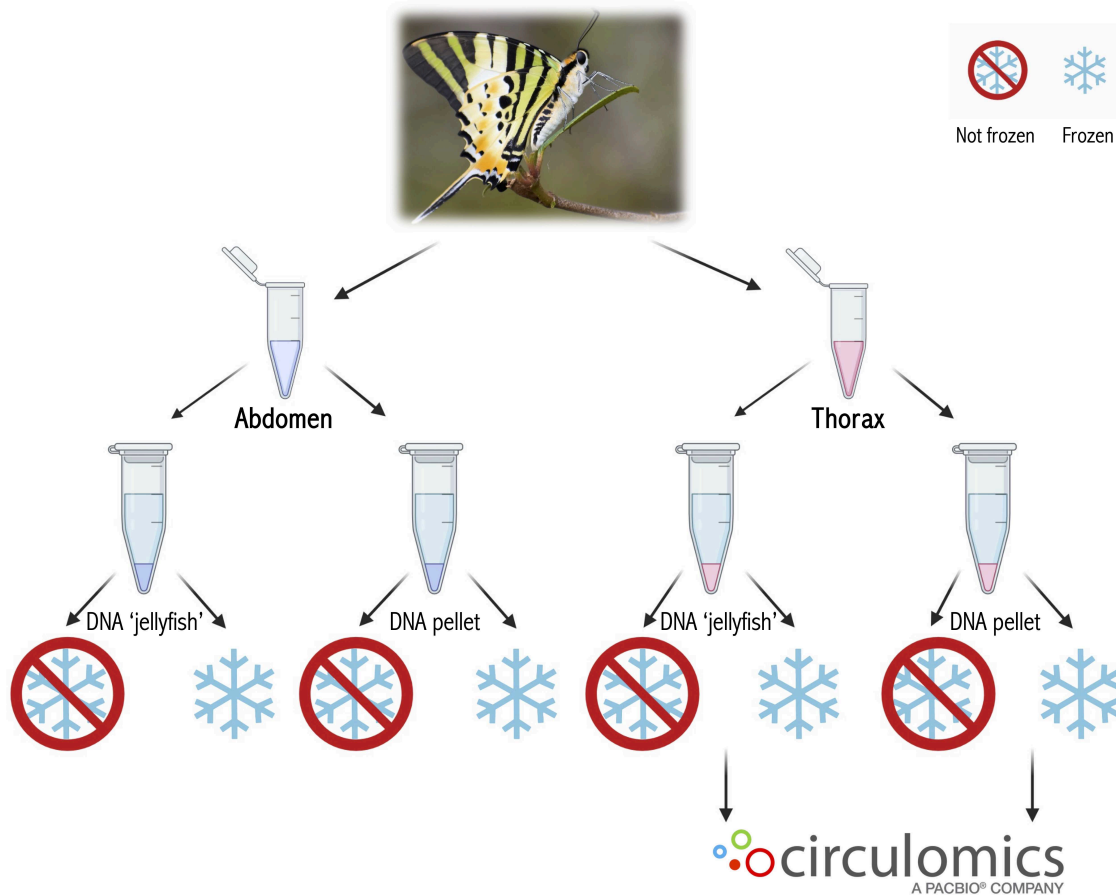




Figure 2. Illustration of the different treatments applied to extracted DNA for testing the effect of DNA freezing. Blue tubes represent DNA extracted from the abdomen, while red tubes represent DNA extracted from the thorax. Pictured is an adult of the five-bar swordtail (*Graphium antiphates*, © Mahesh Baruah) that was used for our study.

Step 3: Library preparation

2h 7m

6 DNA repair (FFPE) and A-tailing

- 6.1 Follow the ONT Genomic DNA by Ligation protocol (SQK-LSK109). We have made a few modifications: **incubate 20 min at 20°C then 5 min at 65°C** and place immediately on ice. 20m 
- 6.2 Purify with a **1.2X ratio** (108 µL of Agencourt AMPure XP beads) and follow the protocol instructions for purification. This ratio allows us to eliminate as few DNA molecules as possible, as we have already selected the largest fragments. 40m 
- 6.3 Elute in a final volume of **65 µL**.

1m



6.4 DNA was quantified using the Qubit® 2.0 fluorometer (Thermo Fisher Scientific) and DNA quality was also checked by running a 1% agarose gel.

7 Preparing the ONT libraries

7.1 Calculate the volume of DNA required to obtain approximately **0.2 pmoles of DNA per library**.

7.2 Follow the ONT Genomic DNA by *Ligation protocol (SQK-LSK109)*.

1h



7.3 Elute into a LowBind tube to give a final volume of **15 µL**.

1m



7.4 Check the concentration of the library with Qubit by taking 2 µL. Add the LB and SQB mix to the remaining 13 µL.

5m



Step 4: Results of ONT sequencing and run monitoring

15h

8 The sequencing of the libraries was performed using a **GridION** from ONT with **R9 flow cells**. We then monitored the sequencing in live thanks to the MinkNOW interface to check the evolution of the N50 length over time (**Figure 3**). We compiled the N50 score every 15 minutes and during 15 hours.

15h

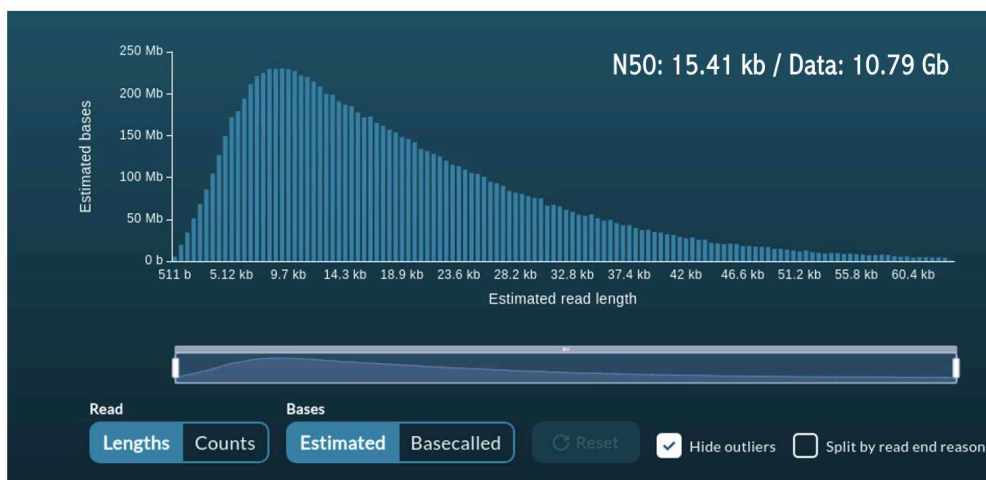
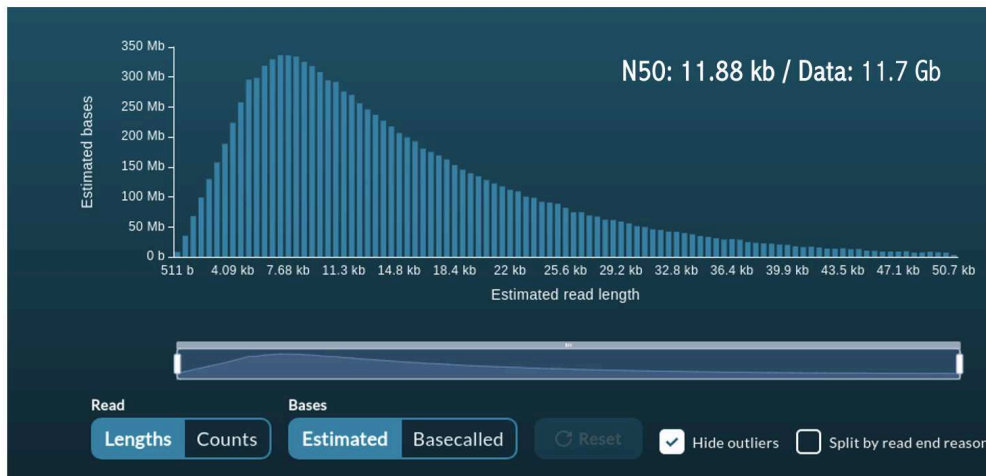


Figure 3. Pictured are two screenshots of the MinKNOW interface during two GridION runs showing the distribution of read lengths and the average N50 length. For illustration purpose, two runs are displayed: one with a non-frozen DNA (top panel) and the other with a frozen DNA (bottom panel) for the library preparation.

- 9 We obtained N50 lengths ranging from 5.63 to 11.39 kb for six R9 MinION flow cells, yielding 53.5 Gb and 51.8 Gb of basecalled data for library preparations made with unfrozen DNA and frozen DNA, respectively (**Figure 4**).

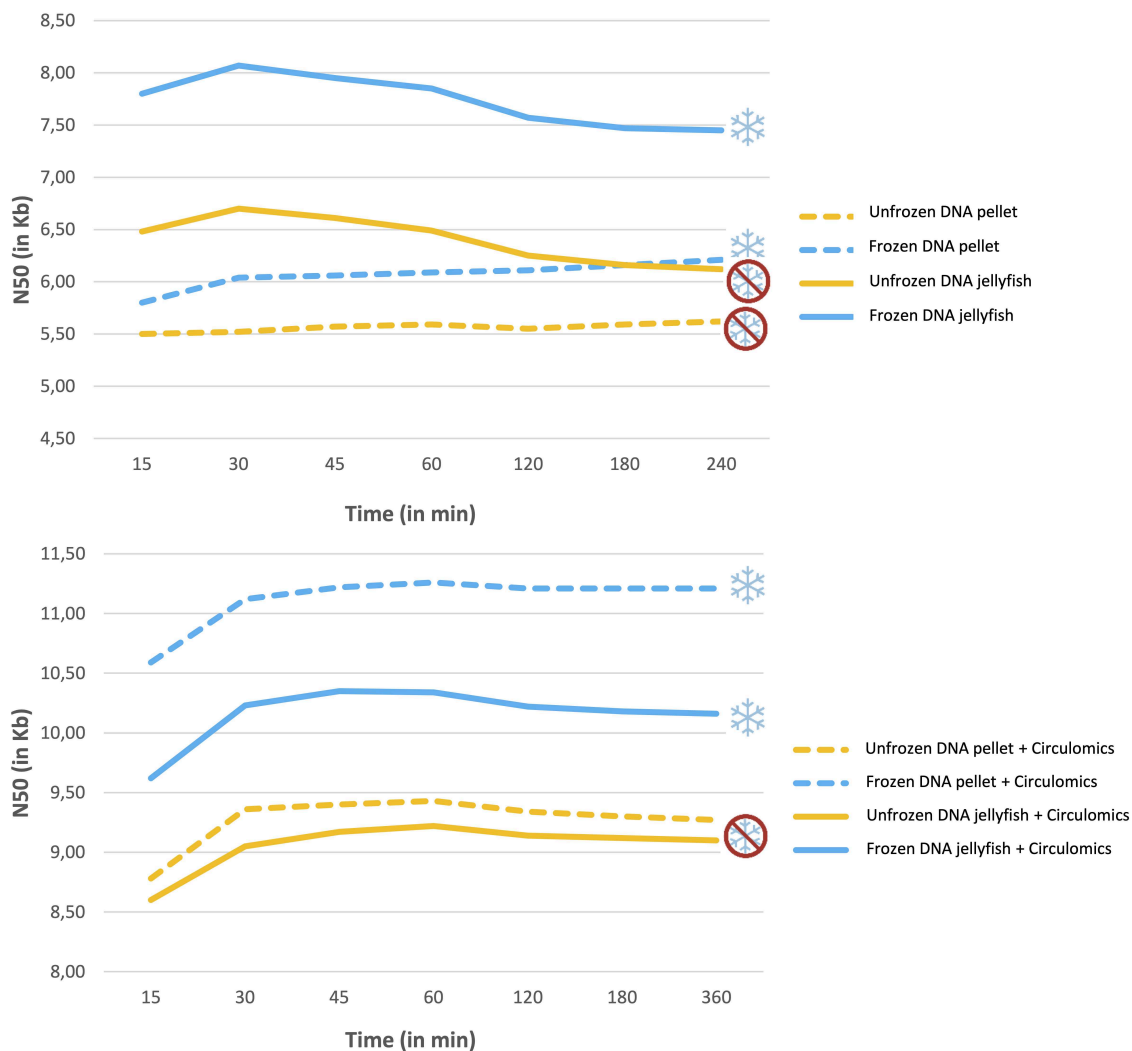


Figure 4. Plots of the N50 lengths through time without (top panel) and with (bottom panel) Circulomics treatment of the DNA. The blue lines stand for the frozen DNA, while the orange lines are for the unfrozen DNA.

The results suggest that freezing has a positive effect on the length of butterfly DNA fragments (higher N50). As expected, these results also indicate that the removal of small fragments using the Circulomics kit also increased N50 (**Figure 4**). The combination of freezing and Circulomics provided the best result for the ONT sequencing (blue lines on the bottom panel).

10 We compared the N50 using the paired samples Wilcoxon test, which is a non-parametric alternative to paired t-test used to compare paired data that are not normally distributed, in R using the function `wilcox.test()`.

- **DNA pellet:** median N50 for unfrozen DNA = 5.57 kb vs. median N50 for frozen DNA = 6.09 kb ($P = 0.0156$, Wilcoxon signed rank exact test)

- **DNA jellyfish:** median N50 for unfrozen DNA = 6.48 kb vs. median N50 for frozen DNA = 7.76 kb ($P = 0.0078$, Wilcoxon signed rank exact test)
- **DNA pellet with Circulomics:** median N50 for unfrozen DNA = 9.34 kb vs. median N50 for frozen DNA = 11.21 kb ($P = 0.0156$, Wilcoxon signed rank exact test)
- **DNA jellyfish with Circulomics:** median N50 for unfrozen DNA = 9.12 kb vs. median N50 for frozen DNA = 10.22 kb ($P = 0.0220$, Wilcoxon signed rank exact test)

Despite the small sample size, all tests showed a significant difference of the treatment with the N50 of frozen DNA being larger than non-frozen DNA.

Step 5: Conclusion

- 11 Based on our results, and although we have only thoroughly tested the effect of freezing DNA on a single butterfly specimen, we recommend ONT users to freeze their DNA extractions for a few days (*i.e.* 2-3 days) after DNA extraction. Interestingly, for another butterfly species, *Papilio machaon*, we sequenced the whole genome of 9 specimens with ONT of which the DNA of one specimen was not frozen. The unfrozen DNA yielded an average N50 ranging between 2.66 and 3.28 kb for three flow cells. In comparison, the eight other frozen DNA produced N50 lengths well above 6 kb (ranging between 6.23 and 16.12 kb).

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