Fish Skeletal Muscle

Application Note v1 (12/2019)

User Supplied Equipment and Reagent List

Equipment	Model
Nanobind Tissue Big DNA Kit	Circulomics (NB-900-701-01)
Magnetic Tube Rack	Thermo Fisher DynaMag-2 (12321D)
Wheaton 1 mL Dounce Tissue Grinder with Tight and Loose Pestles	Fisher Scientific (06-434)
Surgical Scalpel	Fisher Scientific (22-079-712)
ThermoMixer	Eppendorf (5382000023)
Platform Rocker	Thermo Scientific (M48725Q)
Mini-Centrifuge	Ohaus Mini-Centrifuge (FC5306)
1.5 mL Protein LoBind Microcentrifuge Tubes*	Eppendorf (022431081)
2.0 mL Protein LoBind Microcentrifuge Tubes*	Eppendorf (022431102)
Wide Bore 200 µL Pipette Tips	USA Scientific (1011-8410)
Ethanol (96–100%)	
Isopropanol (100%)	
UV/Vis	Thermo Fisher Scientific NanoDrop 2000
Fluorescent DNA Quantitation	Thermo Qubit 3.0, dsDNA BR and RNA BR Assay Kits

*Eppendorf Protein LoBind tubes (Eppendorf #022431081 and #022431102) are highly recommended for all extractions to reduce protein contamination from tube carryover. Protein LoBind tubes are more effective in reducing carryover contamination than DNA LoBind tubes or other tubes and will result in improved UV purity.

Kit Storage

RNase A and Buffer CT should be stored at 4 °C upon arrival.

Nanobind Disks and all other buffers should be stored at room temperature (18–25 °C).

Safety Precautions

Buffer BL3 and Buffer CW1 contain guanidine hydrochloride. Warning! Guanidine hydrochloride is harmful if swallowed or inhaled and causes skin and eye irritation. DO NOT mix with bleach or acidic solutions.

Product Use

Nanobind Tissue Big DNA Kits are intended for research use only.

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This application note describes the isolation of HMW DNA from fish skeletal muscle. Extraction data is provided from a fish skeletal muscle sample processed at Circulomics. Sequencing data from customer extracted and sequenced fish and shrimp tissue are also provided.

Sample Notes

- Fish skeletal muscle is not recommended if other fish tissues are available (*e.g.*, heart, testes, fin clip, kidney).
- Fish skeletal muscle results in low DNA yield compared to other fish tissues due to the low density of nuclei in this tissue.
- Fish skeletal muscle can be a challenging tissue to extract DNA from.
- DNA extraction yields, purity, and quality obtained from fish skeletal muscle can be highly variable across fish species and extractions.
- See **Fish Tissue Sequencing Guide** for additional details.

Protocol Notes

- This protocol uses Dounce homogenizer for disruption.
- Due to the low density of nuclei in skeletal muscle, this protocol uses 48 mg of input.
- Skeletal muscle from some fish species can be difficult to pellet once homogenized.
- Many fish skeletal muscle tissues will swell to fill the full volume of Buffer CT during disruption in step 5. In such cases, this homogenate does not form a pellet when spun using the conditions in the standard tissue protocol. Thus, the tissue disruption in this protocol deviates from the standard tissue process in the following ways:
    1) Higher tissue input (step 2).
    2) Smaller volume of Buffer CT (step 4) + Dounce wash (step 7).
    3) Faster centrifugation speed (step 8).
    4) No secondary wash with Buffer CT.

Protocol

1. **Place the Dounce homogenizer and tight pestle on ice and chill the centrifuge to 4 °C.**
2. **Place 48 mg of fish skeletal muscle on a clean, chilled surface, and finely mince to ≤ 1 mm³ pieces using a scalpel.**
   - 50 mg of tissue is a good starting point for most fish species muscle. Depending on the species, inputs up to 100 mg might be necessary. We recommend using the smallest input that yields enough DNA for your application.
   - A plastic weigh boat cleaned with 70% EtOH can be placed on an upside-down, aluminum dry bath incubator heat block sitting in ice.
3. **Transfer minced tissue to the chilled Dounce homogenizer. Keep the Dounce chamber on ice during the entire disruption process.**
4. **Add 200 µL of cold Buffer CT.**
   - Buffer CT should be placed on ice after removing from the refrigerator.
5. **Gently homogenize the tissue with the pestle 10X.**
   - Push the tissue with the pestle firmly into the bottom of the Dounce chamber with each stroke (Down + Up = 1X).
   - Keep the tissue between tip of pestle and the bottom of the Dounce chamber for thorough homogenization. (
   - It is not necessary to twist the pestle at the bottom of the Dounce chamber to disrupt the tissue.
6. **Transfer homogenate to a 2 mL Protein LoBind microcentrifuge tube using a wide bore P200 pipette.**
   - Tissue homogenate will probably be quite thick and dense.
7. **Add 200 µL of cold Buffer CT to rinse the Dounce homogenizer barrel and recover the residual tissue homogenate. Add residual homogenate to the 2 mL tube in step 6.**
8. **Pellet homogenate by centrifuging at 16,000 x *g* and 4 °C for 5 min. Discard supernatant.**
9. **Pulse vortex pellet for 1s x 2 times (max setting) to dislodge pellet.**
10. **Add 20 µL of Proteinase K to the previous pellet.**
11. **Add 150 µL of Buffer CLE3 and pipette mix 10X with a wide bore P200 pipette.**
12. **Incubate on a ThermoMixer at 55 °C and 900 rpm for 30 min.**
13. **Spin the tube on a mini-centrifuge for 2 s to remove liquid from the cap.**
14. **Add 20 µL of RNaseA.**
15. **Incubate on a ThermoMixer at 55 °C and 900 rpm for 30 min.**
16. **Spin the tube on a mini-centrifuge for 2 s to remove liquid from the cap.**
17. **Add 60 µL of Buffer SB and pulse vortex for 1s x 5 times (max setting) to mix.**
18. **Centrifuge at 10,000 x *g* and RT (18–25 °C) for 5 min.**

Quick Tip

Thorough tissue disruption is key to efficient lysis. It is also important to keep the tissue cold during the entire disruption process.

Quick Tip

See **Tissue Disruption Strategies** section for Dounce Homogenization Tips and Tricks.

Quick Tip

The 2 mL tube is essential for efficient lysis because of its shape; the narrow taper of a 1.5 mL tube prevents proper mixing of the lysate during thermomixing.

Quick Tip

If there are still visible, undigested tissue pieces after step 12, the incubation may be extended up to 2 h. However, if tissue is appropriately disrupted in steps 1-5, then 30 min should be sufficient.

19. **Transfer up to 300 µL supernatant to a new 1.5 mL Protein LoBind microcentrifuge tube using a wide-bore pipette. (Discard the 2 mL Protein LoBind microcentrifuge tube containing the precipitated pellet.)**
    - Typical supernatant volumes will be 225 – 325 µL.
    - Some samples will not result in a visible pellet after this spin. If this happens, remove supernatant as if there were a pellet present and avoid pipetting from the very bottom of the tube.
20. **Add 50 µL of Buffer BL3 to the previous supernatant and inversion mix 10X.**
    - Solution became cloudy but cleared up in step 23.
21. **Spin the tube on a mini-centrifuge for 2 s to remove liquid from the cap.**
22. **Add Nanobind disk to lysate and add 350 µL of isopropanol. Inversion mix 10X.**
    - The Nanobind disk must be added before isopropanol.
    - A large, cloudy mass appeared upon addition of isopropanol and inversion mixing; this adhered to the Nanobind disk and became clear during the next step.
23. **Mix on a platform rocker at 20 rpm for 30 min at RT.**
24. **Place tube rack on the magnetic base using the method described in the Magnetic Rack Handling Procedure section.**
25. **Discard the supernatant with a pipette using the method described in the Pipetting section, taking care to avoid pipetting the DNA or contacting the Nanobind disk.**
26. **Add 500 µL of Buffer CW1, remove tube rack from magnetic base, inversion mix 4X, replace the tube rack on the magnetic base, and discard the supernatant.**
27. **Repeat step 26.**
28. **Add 500 µL of Buffer CW2, remove tube rack from magnetic base, inversion mix 4X, replace the tube rack on the magnetic base and discard the supernatant.**
29. **Repeat step 28.**
30. **Pipette out any residual liquid from the tube cap.**
31. **Spin the tube on a mini-centrifuge for 2 s. With the tube rack already on the magnetic base and right-side-up, place tube on tube rack and remove residual liquid.**
    - If the Nanobind disk is blocking the bottom of the tube, gently push it aside with the tip of the pipette towards the magnet.
32. **Repeat step 31.**
33. **Add 75 µL of Buffer EB directly onto the Nanobind disk and incubate at RT for 10 min.**
    - The Nanobind disk does not need to be fully immersed in Buffer EB – it need only be wetted and sitting atop the liquid.
34. **Collect DNA by transferring eluate to a new 1.5 mL microcentrifuge tube using a wide bore P200 pipette.**
    - Either Protein LoBind or DNA LoBind tubes can be used in this step.

Quick Tip

The narrow taper of the 1.5 mL tube is critical for proper removal of wash buffer in steps 31 & 32 and for thorough recovery of eluate in step 35.

Quick Tip

The Nanobind disk only needs to be wetted in the elution step: **THE DISK DOES NOT NEED TO BE FULLY SUBMERGED IN BUFFER EB**.

- Avoid Axygen tubes as these have been shown to interfere with PacBio sequencing.

35. Spin the tube containing the Nanobind disk on a mini-centrifuge for 5 s. Use a standard P200 pipette to combine any additional liquid that comes off the disk with the previous eluate. Repeat if necessary.

- For this species of fish skeletal muscle, step 35 had to be performed 2 times.

36. Pipette mix 5X with a standard P200 pipette to homogenize the eluate and disrupt any unsolubilized "jellies" that may be present.

- Limited pipette mixing will not noticeably reduce DNA size or sequencing read lengths but is important for accurate quantitation and consistent sequencing performance.
- Take care to disrupt any regions that feel more viscous than other regions.

37. Let eluate rest overnight at RT to allow DNA to solubilize.

- Visible "jellies" should disperse after resting.
- The extracted HMW DNA can be heterogeneous. This is normal and is one of the challenges of working with HMW DNA. The bigger the DNA, the more this will be apparent. See **Heterogeneity and Viscosity** section for detailed tips.

38. Following overnight rest, pipette mix 5X with a standard P200 pipette and perform triplicate NanoDrop measurements by sampling the top, middle, and bottom of the eluate.

- If the concentration %CV exceeds 30%, 5X pipette mix with a standard P200 pipette and allow DNA to rest at RT for 1 hour to overnight. Take care to disrupt any regions that feel more viscous than other regions. Remeasure with NanoDrop.
- Limited pipette mixing will not noticeably reduce DNA size or sequencing read lengths but is important for accurate quantitation and consistent sequencing performance.
- We routinely see A260/A280 in the range of 1.25–1.93 and A260/A230 in the range of 0.68–1.04 for fish skeletal muscle.

39. Use Qubit dsDNA BR Assay to determine DNA concentration.

- We recommend making multiple measurements from the top, middle, and bottom of the eluate for an accurate DNA concentration reading.

40. Run pulsed field gel electrophoresis (PFGE) to size the HMW DNA.

Quick Tip

This **5 s spin is CRITICAL** for recovering the DNA. We do not recommend a 2nd elution

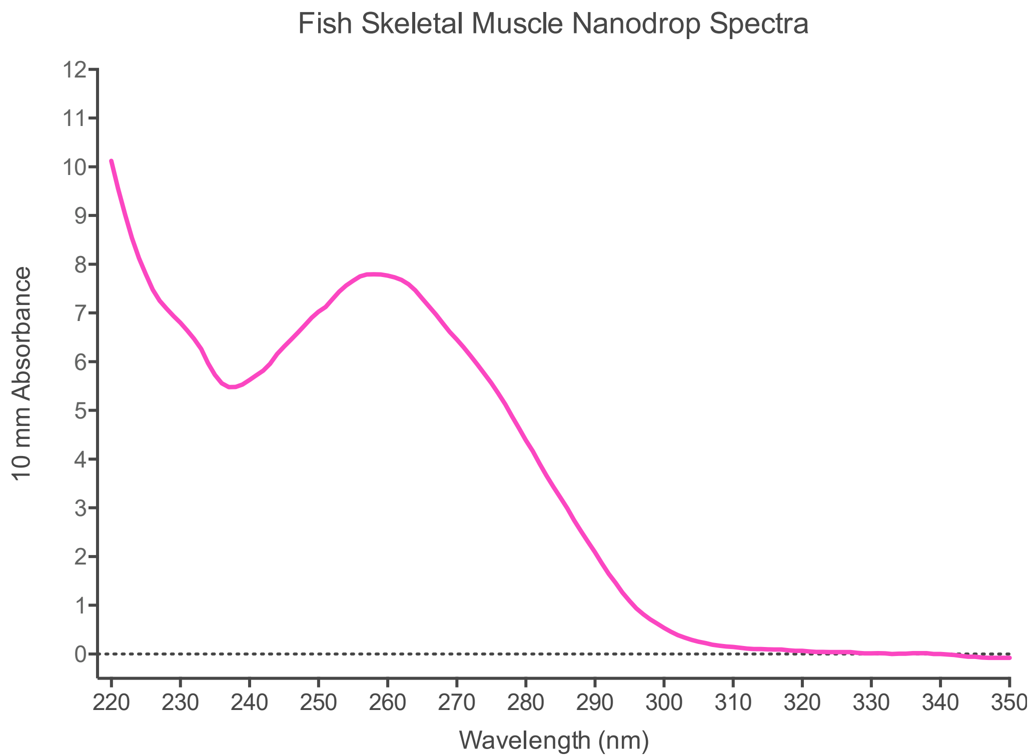
Quick Tip

The DNA will solubilize after resting at RT or by coaxing it into solution using standard P200 pipette mixing. For samples that need to be used immediately, we recommend needle shearing.

DNA Extraction Yield and Purity

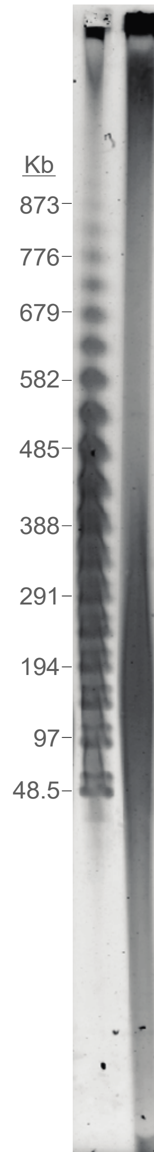
- Fish skeletal muscle results in low extraction yields.
- Purities obtained from fish skeletal muscle are lower than other tissues.

Sample	Sample Input	dsDNA Yield	% RNA	A260/A280	A260/A230
Fish Skeletal Muscle	48 mg	2.7 µg	55.9	1.77	1.04



DNA Size

- Size of DNA extracted from fish skeletal muscle is 50—300+ kb.



PFGE of DNA extractracted from fish skeletal muscle.

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PacBio Sequencing

- Samples were extracted and sequenced by customers on PacBio Sequel I/II.
- Different shearing sizes were used.
- Different BluePippin size selection cutoffs were used.
- Samples generated good subread N50 and good data yields.

Nanobind Tissue Big DNA Kit – PacBio Sequel I/II Sequencing				
Sample	**Library Prep**	**Subread N50 (bp)**	**Polymerase N50 (bp)**	**Total Data (Gb)**
Stickleback Brain/Muscle[1]	60 kb shear, 12.5 kb BP, Express Template 2.0, Sequel I, 10 hr	22,370‡	25,812‡	9.4‡
Antarctic Icefish Muscle[1]	60 kb shear, 27.5 kb BP, Express Template 2.0, Sequel II, 30 hr	29,252*	35,534*	107.7*
Prawn [2]	15 kb shear, 15 kb BP, Express Template 2.0, Sequel I, 20 hr	23,186	53,091	9.8

‡Data is average of multiple flow cells.
[1]Data courtesy of researchers working with the Genomics & Cell Characterization Core Facility (GC3F) at University of Oregon.
[2]Data courtesy of ARC ITRH for Advanced Prawn Breeding Hub, James Cook University and Australian Genome Research Facility.