Black Mystery Snail

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 Quantification	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

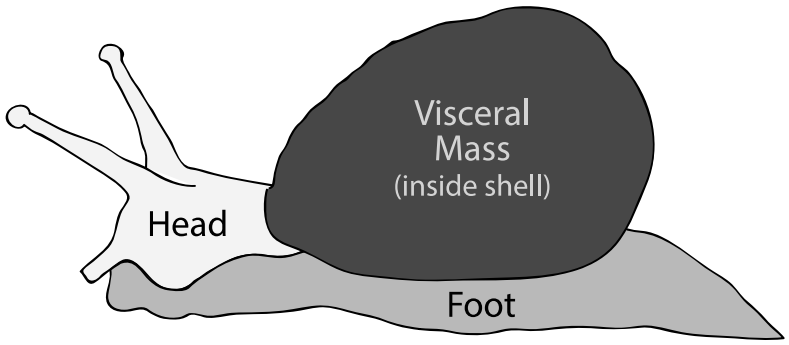
Nanobind Tissue Big DNA Kits are intended for research use only.

Black Mystery Snail

This application note describes the isolation of HMW DNA from frozen foot tissue of Black Mystery Snail which is a freshwater snail.

Sample Notes

- We generally divide the snail into 3 sections: head, foot, and visceral mass:



- We recommend using the foot.
- This sample was validated on Oxford Nanopore MinION.

Protocol Notes

- This application note uses the **Nanobind Tissue Big DNA Kit** (NB-900-701-01).
- This application note describes DNA extraction from 107 mg of Black Mystery Snail foot using a Dounce homogenizer for disruption.
- We **do not** recommend using TissueRuptor for disruption.
- The input for Black Mystery Snail must be increased to at least 59 mg in order to get good recovery. This protocol has been successfully tested using 59–112 mg input.
- Due to the loose, watery consistency of freshwater snail tissue, it was difficult to mince it to pieces ~1 mm^3. In this case, mincing to ~2 mm^3 was sufficient.
- Black Mystery Snail tissue benefits from centrifugation speeds in steps 6 and 8 that are faster (6,000 x *g*) than in the standard tissue protocol.

Protocol

1. **Place the Dounce homogenizer and tight pestle on ice and chill the centrifuge to 4 °C.**
2. **Place 107 mg snail foot tissue on a clean, chilled surface, and finely mince to ~2 mm^3 pieces using a scalpel.**
    - The black mystery snail foot was very watery and loose and did not require mincing as thorough as described in our standard tissue handbook.
    - 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 homogenizer on ice during the entire disruption process.**
4. **Add 750 µL of cold Buffer CT.**
    - Buffer CT should be kept on ice when removed from refrigerator.
5. **Gently homogenize the tissue with the pestle 15X.**
    - Keep the tissue between the tip of the pestle and 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.
    - The snail homogenate became foamy. In the next step, all the foam was transferred.
6. **Transfer homogenate and any foam to a 2 mL Protein LoBind microcentrifuge tube.**
7. **Pellet homogenate by centrifuging at 6,000 x *g* and 4 °C for 5 min. Discard supernatant.**
8. **Add 1 mL of cold Buffer CT. Pulse vortex 1s x 10 times (max setting) to resuspend tissue.**
9. **Pellet homogenate by centrifuging at 6,000 x *g* and 4 °C for 5 min. Discard supernatant.**
10. **Pulse vortex pellet for 1s x 2 times (max setting) to dislodge pellet.**
11. **Add 20 µL of Proteinase K to the previous pellet.**
12. **Add 150 µL of Buffer CLE3 and pipette mix 10X with a wide bore P200 pipette.**
13. **Incubate on a ThermoMixer at 55 °C and 900 rpm for 30 min.**
14. **Spin the tube on a mini-centrifuge for 2 s to remove liquid from the cap.**
15. **Add 20 µL of RNaseA.**
16. **Incubate on a ThermoMixer at 55 °C and 900 rpm for 30 min.**
17. **Spin the tube on a mini-centrifuge for 2 s to remove liquid from the cap.**
18. **Add 60 µL of Buffer SB and pulse vortex for 1s x 5 times (max setting) to mix.**
19. **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 13, 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.

20. **Transfer up to 300 µL of the 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 290 µL – 320 µL.
    - This sample type usually results in a small, thin pellet after this spin.
    - Occasionally, a pellet will not be visible at this step. If this happens, remove supernatant as if there were a pellet present and avoid pipetting from the very bottom of the tube.
21. **Add 50 µL of Buffer BL3 to the previous supernatant and inversion mix 10X.**
    - Solution became cloudy but cleared up in step 24.
22. **Spin the tube on a mini-centrifuge for 2 s to remove liquid from the cap.**
23. **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.
24. **Mix on a platform rocker at 20 rpm for 15 min at RT.**
25. **Place tube rack on the magnetic base using the method described in the Magnetic Rack Handling Procedure section.**
26. **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.**
27. **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.**
28. **Repeat step 27.**
29. **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.**
30. **Repeat step 29.**
31. **Pipette out any residual liquid from the tube cap.**
32. **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.
33. **Repeat step 32.**
34. **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.
35. **Collect DNA by transferring eluate to a new 1.5 mL microcentrifuge tube using a wide bore P200 pipette.**

Quick Tip

The narrow taper of the 1.5 mL tube is critical for proper removal of wash buffer in steps 32 & 33 and for thorough recovery of eluate in step 36.

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**.

- Either Protein LoBind or DNA LoBind tubes can be used in this step.
- Avoid Axygen tubes as these have been shown to interfere with PacBio sequencing.

36. 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 freshwater snail, step 36 usually had to be performed 2 times.
- The extracted DNA had a slight gray color, likely due to pigment within the tissue of this snail. This seems to have had a slight impact on purity but did not affect sequencing (see Sequencing Results below).

37. 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.

38. 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.

39. 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 1.79–1.98 and A260/A230 in the range of 1.40–2.00 for freshwater snail tissue.

40. 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.

41. 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.

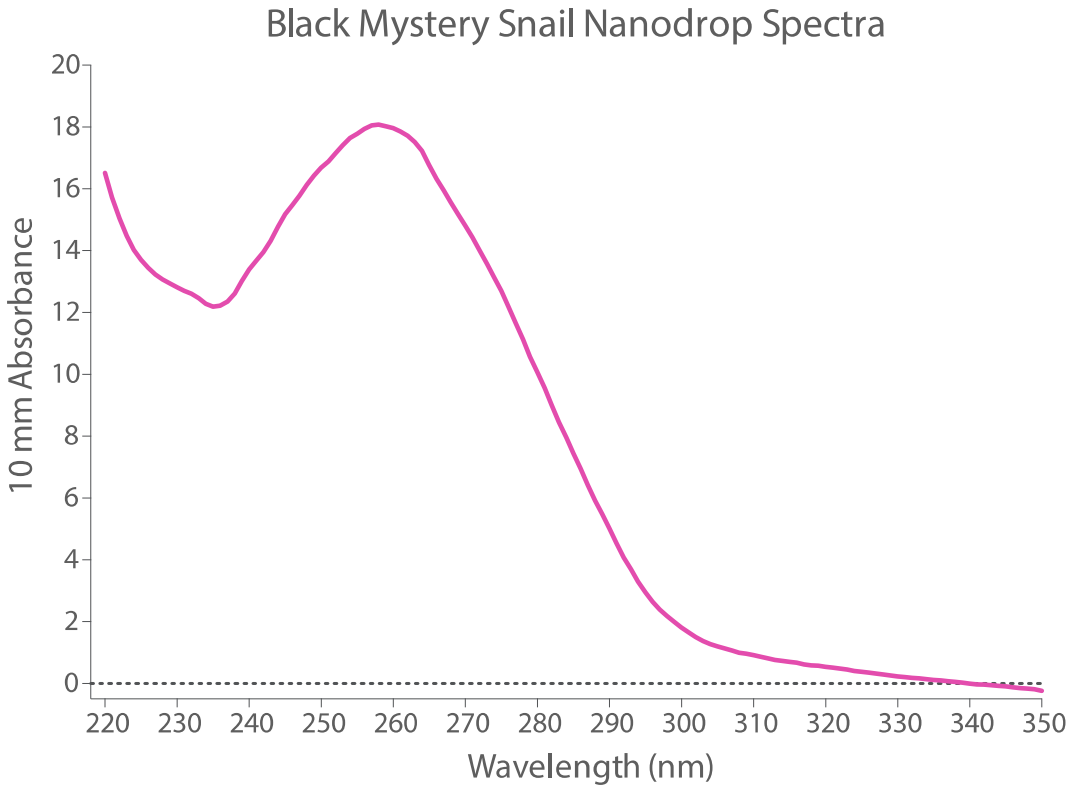
Quick Tip

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

DNA Extraction Yield and Purity

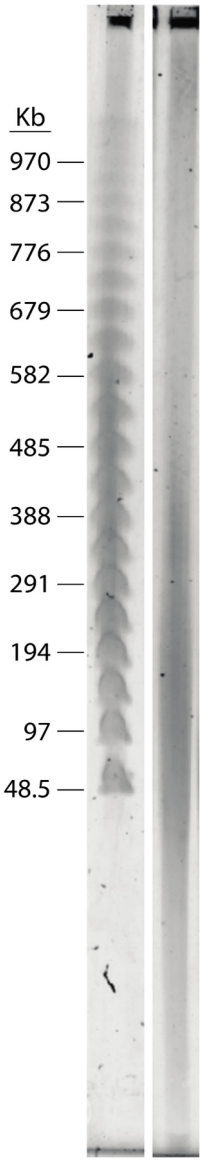
- DNA yield from the Black Mystery Snail was good.
- Purities from the snail were acceptable. The lower than ideal purity is likely due to a small amount of dark pigment carryover from the input tissue. Although the purity is lower than ideal, sequencing was not affected (see Sequencing Results below).

Sample Name	Sample Input	dsDNA Yield	%RNA	A260/A280	A260/A230
Black Mystery Snail Foot	107 mg	16.1 µg	26.2	1.79	1.41



DNA Size

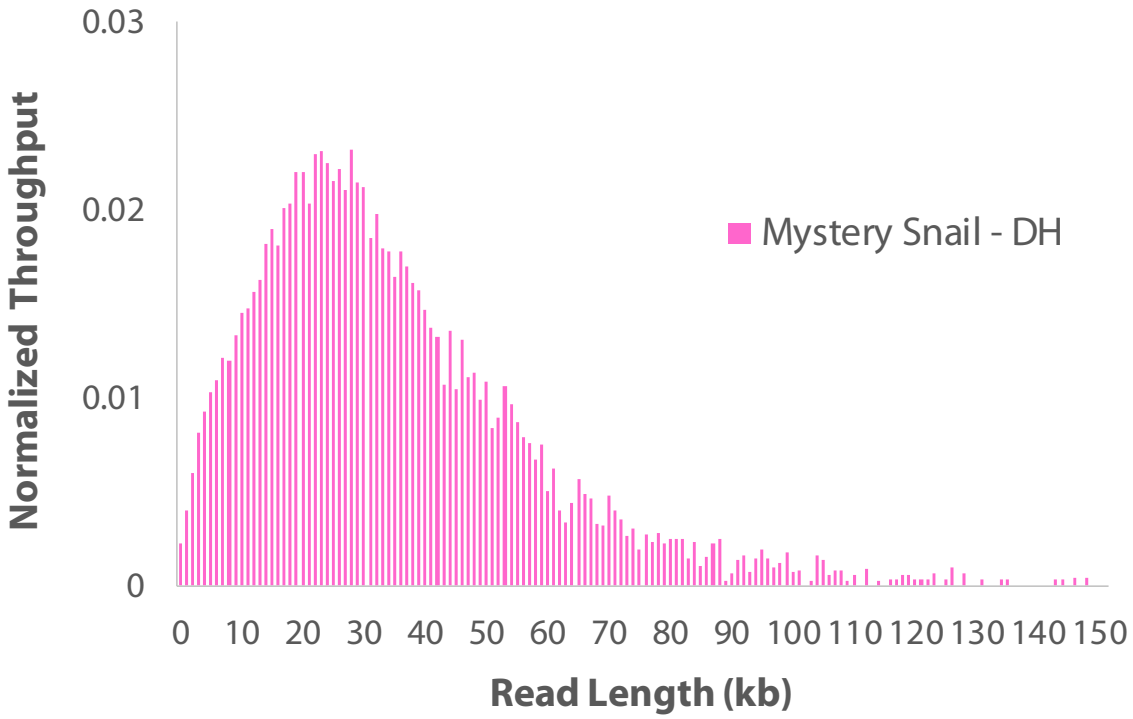
- Size of DNA extracted from a Black Mystery snail was 50–300+ kb.



PFGE of DNA extracted from Black Mystery Snail.

Sequencing Results

- DNA was needle sheared 5X using a 26g needle and then size selected using Short Read Eliminator XL prior to library preparation.
- Library preparation was performed using Oxford Nanopore Ligation Sequencing Kit.
- DNA sample was sequenced on Oxford Nanopore MinION for 1 hour.
- Generated a very good read length N50 and good yield for 1 hour of sequencing.



Nanobind Tissue Big DNA Kit – ONT MinION Sequencing							
Sample	**Sequencing**	**Shearing**	**Size Selection**	**Sequencing Time**	**Read Length N50 (bp)**	**% Data >100 kb**	**Total Data (Gb)**
Snail Foot	SQK-LSK109 FLO-MIN106D	5X Needle Shear	SRE	1 hr	30,600	0.31%	0.40