

Aplysia

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)
TissueRuptor II	Qiagen (9002755)
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)
14 mL Round Bottom Tubes	Fisher Scientific (14-956-3B)
Wide Bore 200 μ L 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.

Aplysia

This application note describes the isolation of HMW DNA from the mollusk *Aplysia*. Extraction and sequencing data are provided.

Sample Notes

- A whole *Aplysia* was dissected by tissue type.
- HMW DNA was isolated from muscle, salivary gland, buccal mass, ovotestis, and heart to compare the quality and purity of the DNA for subsequent sequencing.
- Muscle sample was sequenced on PacBio Sequel II.

Protocol Notes

- This application note uses the **Nanobind Tissue Big DNA Kit** (NB-900-701-01).
- This application note describes DNA extraction using TissueRuptor homogenization for disruption.
- Dounce homogenization would also be fine for disruption.
- Due to the very high nuclei density of some tissues such as the ovotestis, the supernatant in step 20 may have very concentrated DNA that forms a gel-like matrix – **this gel-like matrix contains all the DNA!** Transfer the entire gel-like matrix in step 20.

Tissue Dissection

A live *Aplysia* was sacrificed and dissected into individual tissue specimens by type. These included muscle, buccal mass, ovotestis, heart, salivary gland, hepatopancreas, gill, winding gland, and opaline gland.

The tissues were frozen and stored at -80 °C.

Extractions were performed using the TissueRuptor II Protocol.



Protocol

1. Place a 14 mL round bottom tube on ice and chill the centrifuge to 4 °C.
2. Place ~25 mg of tissue on a clean, chilled surface, and finely mince to ≤ 1 mm³ pieces using a scalpel.
 - A plastic weigh boat cleaned with 70% EtOH can be placed on an upside-down, aluminum dry bath incubator heat block sitting in ice.
 - Ovotestis had a liquid consistency and did not require mincing.
3. Transfer minced tissue to the chilled 14 mL round bottom tube. Keep the tube on ice during the entire disruption process.
4. Add 750 μ L of cold Buffer CT.
 - Buffer CT should be kept on ice when removed from refrigerator.
5. Submerge the TissueRuptor probe tip in the buffer and blend at max speed for 10 s.
 - Blending may create foam. Be sure to transfer all the foam in the next step.
6. Transfer homogenate and any foam to a 2 mL Protein LoBind microcentrifuge tube.
7. Pellet homogenate by centrifuging at 1,500 x g and 4 °C for 5 min. Discard supernatant.
8. Add 1 mL of cold Buffer CT and pipette mix 10X with a wide bore P200 pipette to resuspend tissue.
9. Pellet homogenate by centrifuging at 1,500 x g and 4 °C for 5 min. Discard supernatant.
 - For some *Aplysia* samples, the supernatant had a gooey portion. It was discarded.
10. Pulse vortex pellet 1s x 2 times (max setting) to dislodge pellet.
11. Add 20 μ L of Proteinase K to the previous pellet.
12. Add 150 μ L 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 μ L 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 μ L 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.
20. Transfer up to 300 μ L of supernatant to a new 1.5 mL Protein LoBind microcentrifuge tube using a wide bore P200 pipette. (Discard the 2 mL Protein LoBind microcentrifuge tube containing the precipitated pellet.)
 - Typical supernatant volumes will be 225 – 300 μ L.
 - Some tissues may not result in a visible pellet after the spin.

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

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.

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.

For high DNA content tissues, a diffuse, gel-like matrix forms that contains all the DNA – transfer the entire gel-like matrix.

- If there is no visible pellet, transfer supernatant as if there were a pellet present and avoid pipetting from the very bottom of the tube.
- For tissues with high DNA content, such as ovotestis, a gel-like matrix may form. This gel-like matrix contains all the DNA – transfer the entire gel-like matrix.

21. Add 50 μ L of Buffer BL3 to the previous supernatant and inversion mix 10X.

- The solution became cloudy with noticeable precipitates 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 μ L 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 μ L 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 μ L of Buffer CW2, 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 μ L 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.

- 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.

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.**

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 the DNA eluate from 19 mg of mouse spleen, step 36 had to be performed 4 times to get all the DNA off of the disk.
- Tissue types with high DNA content such as spleen may result in a dense, clear gel that adheres strongly to the Nanobind disk. *This clear gel is DNA!* For these tissue types, this spin step is critical for recovering all the DNA. Repeat until all the clear gel has spun off of the Nanobind disk.

Quick Tip

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

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.

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.

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 1.86–2.01 and A260/A230 in the range of 1.86–2.32 for spleen samples.

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

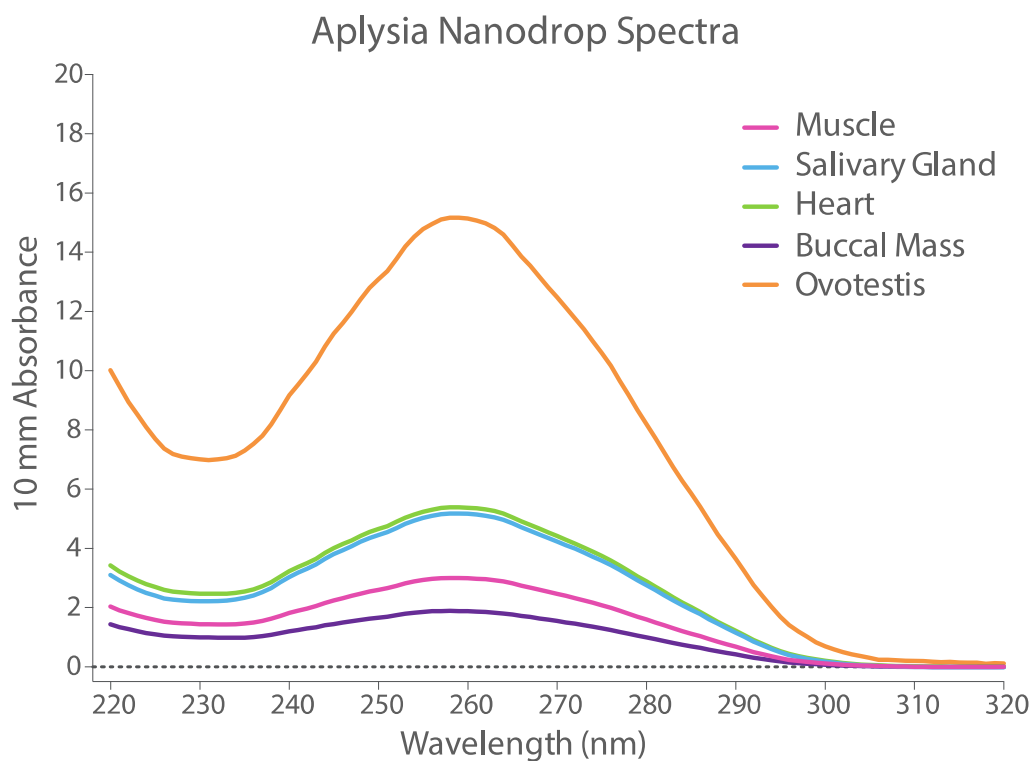
- We recommend making multiple measurements from the top, middle, and bottom of the eluate for an accurate DNA concentration reading.

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

DNA Extraction Yield and Purity

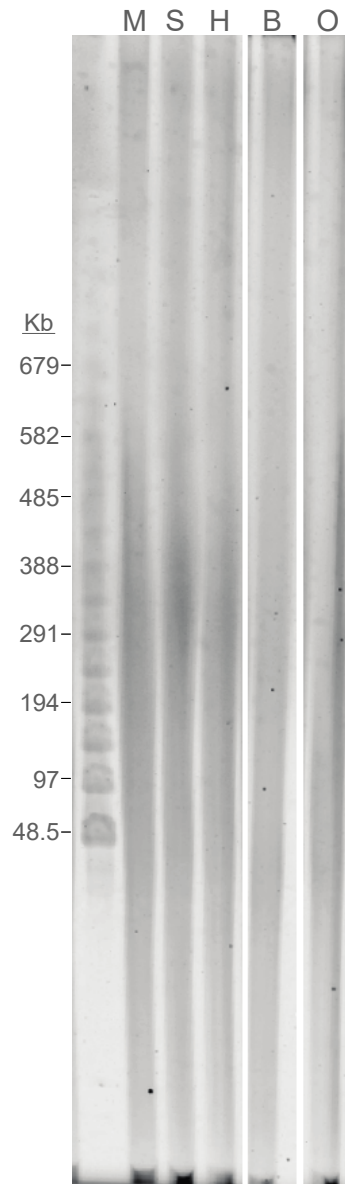
- Extractions were performed on fresh muscle, buccal mass, ovotestis, heart, and salivary gland tissue.
- All tissue types resulted in good extraction yields and ideal UV purity
- Extractions performed on frozen tissues resulted in comparable results to fresh tissues.

Sample	Sample Input	dsDNA Yield	% RNA	A260/A280	A260/A230
<i>Aplysia</i> Muscle	43 mg	9.1 µg	11.7	1.87	2.07
<i>Aplysia</i> Salivary Gland	15 mg	18.3 µg	10.5	1.87	2.32
<i>Aplysia</i> Heart	29 mg	14.5 µg	12.9	1.87	2.17
<i>Aplysia</i> Buccal Mass	20 mg	4.2 µg	15.8	1.89	1.91
<i>Aplysia</i> Ovotestis	38 mg	46.5 µg	10.5	1.85	2.16



DNA Size

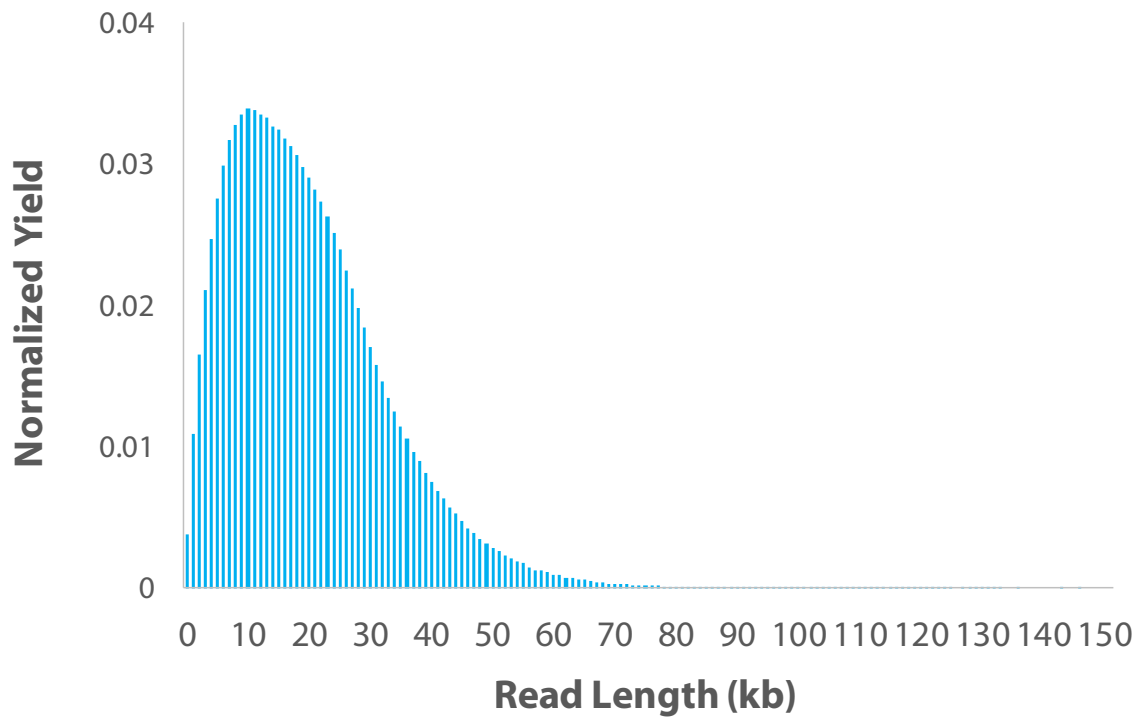
- The DNA size was 50–300+ kb across all tissue types.
- PFGE images displayed smearing making precise sizing challenging.
- Samples displayed some lower molecular weight smearing that can be removed through size selection.



PFGE of DNA extracted from *Aplysia*: (M) muscle, (S) salivary gland, (H) heart, (B) buccal mass, (O) ovotestis.

PacBio Sequencing

- *Aplysia* muscle sample was prepared for sequencing using PacBio Express Template 2.0 kit with no shearing
- 20 kb high pass size selection was performed using BluePippin.
- Sequenced for 15 hours on PacBio Sequel II.
- Sample generated good read length N50 and yield.



Nanobind Tissue Big DNA Kit – PacBio Sequel II Sequencing

Sample	Library Prep	Subread N50 (bp)	Polymerase N50 (bp)	Total Data (Gb)
Aplysia Muscle	No shear, 20 kb BP, Express Template 2.0, Sequel II, 15 hr	18,151	18,868	71.6

Data courtesy of Abrams Lab at University of Maryland School of Medicine