Sanger Tree of Life HMW DNA Extraction: Automated Modified Omega Bio-Tek E.Z.N.A.®

Author

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Abstract

This protocol describes the automated extraction and SPRI of HMW DNA from metazoa samples intended for long-read sequencing using a combination of the Omega Bio-Tek E.Z.N.A.® Mollusc & Insect DNA kit, Sera-Mag™ SpeedBeads and the Thermo Fisher KingFisher™ Apex. It is effective for several metazoa species covered by the Tree of Life Programme, such as isopods, amphipods, decapods, annelids and molluscs. This protocol has resulted in successful extractions for a number of species including *Ligia oceanica*, *Titanethes albus*, *Oritoniscus flavus*, *Hyloniscus adonis*, *Oniscus asellus, Munidopsis polymorpha*, *Nephrops norvegicus, Willemoesia leptodactyla, Lumbricillus lineatus* and the hemichordata *Saccoglossus kowalevskii.*

The output of this protocol is HMW DNA which – depending upon yield and genome size of the species – can be directed towards any of the Sanger Tree of Life HMW DNA Fragmentation protocols for LI or ULI PacBio sequencing.

This protocol was developed through R&D by the Tree of Life Core Laboratory; it was primarily adapted from the Omega Bio-Tek E.Z.N.A.® Mollusc & Insect DNA kit protocol by combining the initial lysis and precipitation (steps 1 to 10), omission of vortexing and the addition of a bead-based extraction using Sera-Mag™ SpeedBeads, as utilised in the 'Sanger Tree of Life HMW DNA Extraction: Automated Plant Organic HMW gDNA Extraction (POE)' protocol.

Health & Safety warnings

- The operator must wear a lab coat, powder-free nitrile gloves and safety specs to perform the laboratory procedures in this protocol. Cotton glove liners are strongly recommended when handling the samples on dry ice.
- Eye protection and silver shield/chemical resistant gloves should be worn when handling chloroform, with all handling performed in a chemical fume hood.
- Waste needs to be collected in a suitable container (e.g. plastic screw-top jar or Biobin) and disposed of in accordance with local regulations.
- Liquid waste needs to be collected in a suitable container (e.g. glass screw-top jar) and disposed of in accordance with local regulations.
- Do not open the door of the KingFisher™ Apex instrument whilst it is in operation.

Guidelines

- Input amounts of 25–35 mg of fresh frozen tissues are required for this protocol. Smaller input amounts can be used, however the yields of DNA obtained may be lower.
- Keep samples on dry ice until the lysis buffer is ready to be added to them to maintain temperature and prevent nucleic acid degradation.
- An experienced operator can expect to comfortably process 24 samples, with approximately 2–3 hours handling time over a start to finish period of 4–5 hours. This estimation excludes subsequent QC checks.

Additional Notes

- FluidX tubes are used throughout the Tree of Life programme in order to track samples, therefore rather than the microcentrifuge tubes which have been mentioned in this protocol for DNA storage, all routine DNA extracts are stored in FluidX tubes.

Before Starting

- Set a heat block to 60 °C.
- Ensure that an adequate amount of Sera-Mag™ SpeedBead solution has been prepared prior to initiating the protocol - 600–800 µL is needed per sample, and requires 50% PEG 8000, 10% Tween-20, SpeedBead wash suspension and SpeedBead binding solution to be prepared prior to initiating the protocol (details in the Materials section).
- Remove the prepared Sera-Mag™ SpeedBead solution from the fridge 30 minutes before starting the extraction step so they can be brought to room temperature.
- Remove the AMPure PB beads from the fridge 30 minutes before starting the 0.45X SPRI step to allow them to be brought to room temperature.

Laboratory Protocol

<u>Sample Lysis & Precipitation</u>

1. Prepare tubes for the samples being processed depending upon the method of disruption:

    1.1. For samples that require powermashing, label 1.5 mL BioMasher tubes for each sample being processed. Save the BioMasher pestles for later use in their sterile packet, also labelling them for each sample.

    1.2. For samples that have been cryogenically disrupted (e.g. via bead beating), label 2 mL microcentrifuge tubes for each sample being processed.

2. Transfer the samples into their corresponding 1.5 mL BioMasher tube or 2 mL microcentrifuge tube.

3. Add 350 µL of ML1 buffer and 25 µL of Proteinase K solution to each sample.

4. Homogenise samples within the lysis buffer according to sample type and disruption method:

    4.1. For samples which require powermashing, use the BioMasher pestle and Diagnocine PowerMasher II tissue disruptor to disrupt the sample within the buffer, following the 'Sanger Tree of Life Sample Homogenisation: PowerMash' protocol.

    4.2. For samples which have been cryogenically disrupted, pipette-mix samples gently with a wide-bore pipette tip 10 times to homogenise.

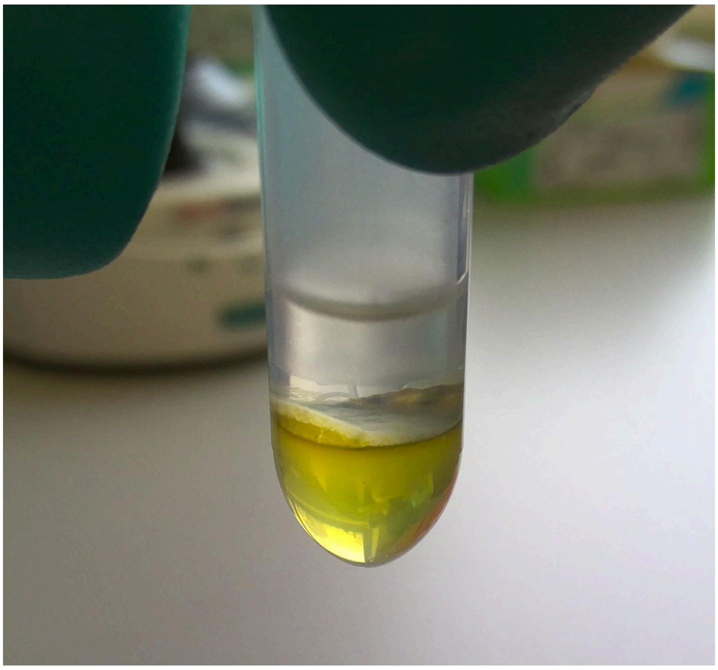
5. Incubate samples for at least 30 minutes at 60 °C. If samples are not completely solubilised after 30 minutes, allow for a longer incubation (45 minutes to 1 hour).

6. After incubation, transfer any samples in 1.5 mL BioMasher tubes to fresh 2 mL microcentrifuge tubes using a wide bore pipette – label these 2 mL microcentrifuge tubes for each sample.
   Samples that are already in 2 mL microcentrifuge tubes do not require transfer into new tubes.

7. Transfer samples to the fume hood for chloroform separation, then add 350 µL of chloroform:isoamyl (24:1) to each sample.

8. Transfer samples to the hula-mixer, rotating at 8 rpm for 5 minutes.

9. Centrifuge samples at 10,000 *g* for 2 minutes at room temperature. The samples will then have three distinct phases: an upper aqueous phase containing the gDNA, a milky interphase containing inhibitors and contaminants, and a lower organic phase containing denatured proteins.



10. Return samples to the fume hood in order to transfer the upper aqueous phase to a clean 2 mL microcentrifuge tube using a wide-bore pipette tip. Avoid transferring the milky interphase containing contaminants and inhibitors.
11. Add 1 X volume of BL buffer to each sample. For example, if 400 µL aqueous upper phase was recovered in Step 10, add 400 µL of BL buffer.
12. Add 10 µL RNase A to each sample. Pipette-mix 15 times with a wide-bore pipette tip.
13. Incubate samples for 10 minutes at 70 ºC.
14. Remove samples from the heat block and allow to cool to room temperature.

Loading and Running the KingFisher™ Apex

15. Label five 1 mL 96-well deep-well KingFisher plates with the following labels, and fill all applicable wells of each plate with their corresponding reagents (see table below).

Plate name	Reagent(s) required
Tip Plate	96-well tip comb (no reagent)
Sample Plate	~500 µL of sample + ~500 µL SpeedBead solution (detailed in Step 17 and 18)
Ethanol wash 1 Plate	1 mL 80% ETOH
Ethanol wash 2 Plate	1 mL 80% ETOH
Elution Plate	400 µL Buffer EB

16. Using a wide bore pipette tip, set the volume to 600 µL, transfer lysate from the now room temperature sample tubes into individual wells in the sample plate, taking care not to transfer large pieces of debris.
    Sample volume varies from 500–600 µL - it is important to note the volume as this will affect the maximum volume of SpeedBead solution that can be added.
17. Add 400–500 µL Sera-Mag™ SpeedBead solution to each sample. The Sera-Mag™ SpeedBead solution should be vortexed before use to ensure that the beads are resuspended.
    Ensure that the total sample + SpeedBead solution volume does not exceed 1 mL e.g. for a 600 µL sample, only 400 µL of SpeedBead solution can be added.
18. Select the required DNA extraction protocol in the protocol list on the KingFisher™ Apex (details below in KingFisher™ Apex DNA Extraction Protocol section/attached file) and select using the play button.
19. Load the filled plates onto the instrument following the instructions provided on screen.

20. Once the final plate is loaded, the protocol will automatically begin; this takes approximately 90 minutes.

21. Once the protocol has completed, follow the on-screen instructions to remove plates from the instrument.

22. Using a wide-bore pipette tip, carefully transfer the 400 µL EB buffer containing purified gDNA from the Elution plate into new 1.5 mL microcentrifuge tubes.
If proceeding to the 0.45X SPRI, samples can remain in the Elution plate – this will then become the 'Sample plate' for the 0.45X SPRI.

<u>Loading and Running the KingFisher™ Apex for the 0.45X SPRI (optional)</u>

23. Set-up the KingFisher™ plates for the 0.45X SPRI as detailed below:

Plate	Plate Type	Reagent(s) required
Tip Plate	1 mL Deep-well	96-well tip comb (no reagent)
Sample Plate	1 mL Deep-well	400 µL DNA + 180 µL AMPure PB beads
Ethanol Wash Plate	1 mL Deep-well	1 mL 80% EtOH (freshly made)
Elution Plate	200 µL standard	135 µL Buffer EB

24. Select the required 0.45X SPRI protocol in the protocol list on the KingFisher™ Apex (details below in KingFisher™ Apex 0.45X SPRI Protocol section/attached file) and select using the play button.

25. Load the filled plates onto the instrument, following the instructions provided on screen.

26. Once the final plate is loaded, the protocol will automatically begin; this will take approximately 45 minutes.

27. Once the protocol has completed, follow the on-screen instructions to remove plates from the instrument.

28. Using a wide-bore pipette tip, transfer the 130 µL of eluate from the elution plate into new microcentrifuge tubes.

29. Incubate the DNA at room temperature overnight and perform the required QC the following morning.

30. Store the DNA at 4 °C.

KingFisher™ Apex MOB Protocol:

1) Pick Up Tip - Tip Plate
2) Bind 1 - Sample Plate

Pre-collect beads:	Off			
Release beads:	On	00:10:00	Medium	
Heating & Cooling:	Off			
Mixing	1#	00:02:00	Slow	Looping: 4
	2#	00:01:55	Paused	Tip position: Tip edge in liquid
	3#	00:00:05	Medium	
Postmix:	Off			
Collect beads:	On	10 Count	30 Seconds	

3) Bind 1 - Sample Plate

Pre-collect beads:	On			
Release beads:	Off			
Heating & Cooling:	Off			
Mixing	1#	00:00:10	Paused	Tip position: Tip edge in liquid
Postmix:	Off			
Collect beads:	On	10 Count	30 Seconds	

4) Ethanol Wash 1 - Ethanol Wash Plate

Pre-collect beads:	Off		
Release beads:	Off		
Heating & Cooling:	Off		
Mixing	1#	00:00:20	Slow
Postmix:	Off		
Collect beads:	On	1 Count	1 Second

5) Ethanol Wash 2 - Ethanol Wash 2 Plate

Pre-collect beads:	Off		
Release beads:	Off		
Heating & Cooling:	Off		
Mixing	1#	00:00:20	Slow
Postmix:	Off		
Collect beads:	On	1 Count	1 Second

6) Air Dry 1 - Ethanol Wash 2 Plate

Duration:	00:01:00	Above well

7) Elute 1 - Elution Plate

Pre-collect beads:	Off			
Release beads:	On	00:00:00		
Heating & Cooling:	On	37℃	Preheat: On	
Mixing:	1#	00:02:25	Slow	Looping: 10
	2#	00:02:00	Paused	Tip position: Tip edge in liquid
	3#	00:00:05	Medium	
Postmix:	On	00:00:30	Slow	
Collect beads:	On	10 Count	30 Seconds	

8) Leave Tip 2 - Tip Plate

KingFisher™ Apex 0.45X SPRI Protocol:

1) Pick Up Tip - Tip Plate
2) Mix - Sample Plate
   Pre-collect beads: Off
   Release beads: On 00:00:00
   Heating & Cooling: Off
   Mixing:
   1# 00:01:00 Slow
   2# 00:01:00 Medium
   3# 00:08:00 Paused
   Looping: 1 Tip position: Tip edge in liquid
   Postmix: Off
   Collect beads: On 10 Count 30 Seconds
3) Wash 1 - Ethanol Wash Plate
   Pre-collect beads: On
   Release beads: Off
   Heating & Cooling: Off
   Mixing 1# 00:00:30 Slow
   Postmix: Off
   Collect beads: Off
4) Wash 2 - Ethanol Wash Plate
   Pre-collect beads: Off
   Release beads: Off
   Heating & Cooling: Off
   Mixing 1# 00:00:30 Slow
   Postmix: Off
   Collect beads: Off
5) Dry - Ethanol Wash Plate
   Duration: 00:01:00 Above well
6) Elute - Elution Plate
   Pre-collect beads: Off
   Release beads: On 00:01:00 Slow
   Heating & Cooling: On 37℃ Preheat: On
   Mixing: 1# 00:00:50 Medium
   2# 00:14:00 Slow
   3# 00:00:10 Fast
   Looping: 2 Tip position: Tip edge in liquid
   Postmix: Off
   Collect beads: On 4 Count 30 Seconds
7) Leave Tip - Ethanol Wash Plate

Materials:

- 1.5 mL DNA Lo-Bind microcentrifuge tubes (Eppendorf Cat. no. 0030108418)
- 2 mL DNA Lo-Bind microcentrifuge tubes (Eppendorf Cat. no. 0030108078)
- 1.5 mL BioMasher II tubes and pestles (sterile) (Cat. no. 9791a)
- 15 mL or 50 mL centrifuge tubes

- Thermo Fisher KingFisher™ 96-well Deep-well plates (Thermo Fisher Cat. no. 95040450)
- Thermo Fisher KingFisher™ 96 Tip Comb (Thermo Fisher Cat. no. 97002570)
- Thermo Fisher KingFisher™ 200 µL standard 96-well Plate (Thermo Fisher Cat. no. 97002084)
- Omega Bio-Tek EZNA® Mollusc & Insect DNA kit (Omega Bio-Tek Cat. no. D3373-00)
- Sera-Mag™ magnetic carboxylate modified particles (Cat. no. GE24152105050250)
- AMPure PB beads (Pacific Biosciences Cat. no. 100-265-900)
- Buffer EB (Qiagen Cat. no. 19086)
- 100% absolute ethanol
- Chloroform:isoamyl alcohol (24:1, v/v) (Cat. no. 25666-100ML)
- Nuclease-free water (Cat. no. AM9932)
- PEG 8000 (Cat. no. P5413-500-G)
- Tris-HCl (1 M stock concentration, pH 8.0)
- EDTA (0.1 M stock concentration, pH 8.0)
- NaCl (5 M stock concentration) (Cat. no. 59222C-500ML)
- Tween-20 (Cat. no. 11332465001)
- Dry ice
- Terumo™ 3-Part 50mL Luer Lock Syringes (Cat. no. 15349067)
- Merck Millex™-HP Sterile Polyethersulfone Syringe Filter Units, 0.45 µm (Cat. no. 16427565)
- Weighing boats (SLS Cat. no. bal1820sp)

Equipment:

- Pipettes for 0.5–1000 µL and filtered tips
- Wide-bore tips (200 µL, filtered if available)
- Diagnocine PowerMasher II tissue disruptor (Product no. 891300)
- Eppendorf ThermoMixer C (Cat. no. 5382000031)
- Eppendorf SmartBlock 2.0 mL (Cat. no. 5362000035)
- Eppendorf SmartBlock 50 mL (Cat. no. 5365000028)
- Mini centrifuge (Cat. no SS-6050)
- Eppendorf Centrifuge 5425/5425 R (Cat. no. 5405000263)
- HulaMixer Sample Mixer (Cat. no. 15920D)
- Mettler Toledo Analytic Balance ME204 (Material No. 30029066)
- Timer
- Chemical Fume Hood
- Kingfisher Apex™ instrument (Cat. no. 5400930)

Reagent Recipes (taken directly from the ‘Sanger Tree of Life HMW DNA Extraction: Automated Plant Organic HMW gDNA Extraction (POE)’ protocol):

50% PEG 8000

Reagent	Target concentration	Molecular weight (g/mol)	Stock concentration	Input from stock (15 mL total)
PEG 8000	50% (w/v)	8000	Powder	7.5 g
Nuclease-free water	-	-	-	6 mL
(Incubate for 60 mins, 75°C at 600 rpm, routinely vortexing until fully dissolved).				
Nuclease-free water	-	-	-	Up to 15 mL
Should be prepared fresh and allowed to cool before use in the Bead Binding solution.				

10% Tween-20

Reagent	Target concentration	Molecular weight (g/mol)	Stock concentration	Input from stock (50 mL total)
Nuclease-free water	-	-	-	44 mL
Tris-HCl, pH 8.0	20 mM	157.60	1 M	1 mL
Tween-20	10% (v/v)	1,227.54	100% (v/v)	5 mL
(Place on a tube rotator for 30 mins, 20 rpm, ensuring Tween is dissolved).				
Store protected from light at RT for up to 1 year (replace if solution is yellowed).				

SpeedBead wash suspension

Reagent	Target concentration	Molecular weight (g/mol)	Stock concentration	Input from stock
Sera-Mag™ speedbead stock solution, 4 °C	0.2% (w/v)	-	0.5% (w/v)	800 µL
Wash beads 4 times before use to remove sodium azide (see below).				
Nuclease-free water	-	-	-	Up to 2.0 mL
Should be prepared fresh before use in the Sera-Mag™ SpeedBead solution.				

1. Allow Sera-Mag™ SpeedBeads aliquot to reach room temperature (~30 minutes).
2. Vortex thoroughly to resuspend the beads.
3. Pipette 800 µL of Sera-Mag™ SpeedBead stock solution into a 2 mL LoBind tube on a magnetic stand and wait for the beads to migrate to the magnet.
4. When the supernatant is completely clear, remove and discard the supernatant from the tube without disturbing the beads.
5. Add 1000 µL nuclease-free water to the tube.
6. Vortex the tube to resuspend beads.
7. Centrifuge briefly to remove droplets from tube lid.

8. Place the tube on a magnetic stand until the supernatant is completely clear and beads are bound towards the magnet.
9. Remove and discard the supernatant without disturbing beads.
10. Repeat steps 5 to 9 three times.
11. Add nuclease-free water up to 2 mL.
12. Vortex tube to resuspend beads.
13. Centrifuge briefly to remove droplets from tube lid.
14. SpeedBead wash suspension can now be added to the SpeedBead solution.

SpeedBead Binding solution

Reagent	Target concentration	Molecular weight (g/mol)	Stock concentration	Input from stock (40 mL total)
Tris-HCl, pH 8.0	10 mM	157.60	1 M	400 µL
EDTA, pH 8.0	1 mM	292.24	0.1 M	400 µL
NaCl	1.6 M	58.44	5 M	12.8 mL
Tween-20	0.05% (v/v)	1,227.54	10% (v/v)	200 µL
PEG 8000	18 % (w/v)	8000	50% (w/v)	14.4 mL
Nuclease-free water	-	-	-	up to 40 mL
Filter sterilise through a 0.45 µM filter into a fresh 50 mL falcon. Should be prepared fresh before use in the SpeedBead solution.				

- Ensure the exact volume of 50% PEG 8000 is added, as this is crucial for gDNA binding (solution is viscous and difficult to pipette).

Sera-Mag™ SpeedBead solution

Reagent	Target concentration	Molecular weight (g/mol)	Stock concentration	Input from stock (40 mL total)
SpeedBead binding solution	-	-	-	38 mL
SpeedBead wash suspension	0.01% (v/v)	-	0.2% (v/v)	2 mL
Store at 4 °C in the dark for up to 3 months.				

- 40 mL of Sera-Mag™ SpeedBead solution is enough for 80 samples.

References:

E.Z.N.A.® Mollusc & Insect DNA Kit Protocol: E.Z.N.A.‱ Mollusc & Insect DNA Kit - (omegabiotek.com)

Jackson, B. and Howard, C. (2024) Sanger Tree of Life HMW DNA Extraction: Automated Plant Organic HMW gDNA Extraction (POE)

Denton, A. and Howard, C. (2025) Sanger Tree of Life HMW DNA Extraction: Manual Modified Omega Bio-Tek E.Z.N.A.