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DNA extraction and genomic sequencing library preparation for individual root-knot nematodes V.1

Graham S Sellers[1], Dave H Lunt[1]
[1]University of Hull

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Graham S Sellers

ABSTRACT

A simple, high molecular weight DNA extraction and long read genomic sequencing library preparation from individual root-knot nematodes (RKN). This protocol successfully sequences juvenile stage 2 (j2) RKN individuals, which are less than 500 µm long, and therefore the protocol is applicable for other meiofauna.

DNA extraction follows a highly modified Mu-DNA: Tissue protocol (Sellers et al., 2018) using a solid phase reversible immobilization (SPRI) magnetic bead capture method, adapted from Rohland and Reich, (2012). Library preparation follows Oxford Nanopore Technologies' Rapid PCR Barcoding Kit (SQK-RPB004), modified for extremely low input DNA template and converted for Flongle flow cell sequencing.

The method is capable of generating sufficient reads for successful taxonomic assignment from juvenile stage 2 (j2) to adult individuals using our bioinformatics workflow available at: https://github.com/Graham-Sellers/RKN_genomic_taxonomic_assignment.

Rohland N, Reich D (2012) Cost-effective, high-throughput DNA sequencing libraries for multiplexed target capture. Genome research 22:939–946.
Sellers GS, Di Muri C, Gómez A, Hänfling B (2018) Mu-DNA: a modular universal DNA extraction method adaptable for a wide range of sample types. Metabarcoding and Metagenomics 2:e24556. (dx.doi.org/10.17504/protocols.io.qn9dvh6)

THIS PROTOCOL ACCOMPANIES THE FOLLOWING PUBLICATION

Sellers GS, Jeffares DC, Lawson B, Prior T, Lunt DH (2021) Genomic taxonomic assignment of individual root-knot nematodes. bioRxiv:2021.03.18.435936. doi: 10.1101/2021.03.18.435936

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PROTOCOL CITATION

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KEYWORDS

Nematode, DNA extraction, Oxford Nanopore, Flongle, Library preparation, Sequencing, Meiofauna

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MATERIALS TEXT

Reagents
Sera-Mag SpeedBead Carboxylate-Modified Magnetic Particles (Hydrophobic), 15 mL (GE Healthcare: 65152105050250)
Rapid PCR Barcoding Kit (Oxford Nanopore: SQK-RPB004)
LongAmp Taq 2X Master Mix (NEB: M0287)
Qubit High-sensitivity (HS) dsDNA assay (Thermo Fisher Scientific: Q32851)

Chemicals
5M HCl (Fisher Scientific: 10695872)
Tris HCl (Alfa Aesar: J67233.22)
Disodium EDTA dihydrate (Sigma Aldrich: E5134-250G)
Trisodium phosphate dodecahydrate (Sigma Aldrich: 04277-1KG)
Sodium chloride (Sigma Aldrich: S7653-250G)
Sodium dodecyl sulphate (Alfa Aesar: J75819.22)
Proteinase K (Thermo Fisher Scientific: AM2542)
Ammonium acetate (Sigma Aldrich: A1542-500G)
Aluminium ammonium sulphate dodecahydrate (Alfa Aesar: 13802.22)
Calcium chloride dihydrate (Sigma Aldrich: 1.02382.0250)

Plastics
1.5 ml Eppendorf DNA LoBind Tubes (Fisher Scientific: 10051232)
10, 100, 200 and 1000 µl pipette tips (brand of choice)

Equipment
Hula mixer (Thermo Fisher Scientific: 15920D) or similar
Eppendorf ThermoMixer C (Eppendorf: 5382000031) or similar
Magnetic racks for 1.5 ml tubes and 0.2 ml PCR tubes
Qubit 3.0 fluorometer (Fisher Scientific: 15387293) or similar

Stock solutions

5 M NaOH:
Do this on ice as the reaction is exothermic. Dissolve 20 g sodium hydroxide in 75 ml ddH2O by adding in small amounts of sodium hydroxide pellets until dissolved. Bring to 100 ml final volume with ddH20.

1 M Tris HCl (pH 8):
Add 15.7 g of Tris HCl to bottle. Add 75 ml ddH2O. Add 5 ml ***5 M NaOH*** to adjust to pH 8. Bring to 100 ml final volume with ddH2O. Place on Hula Mixer until all solids dissolve.

0.5 M EDTA (pH 8):
Add 18.6 g of disodium EDTA dihydrate to bottle. Add 75 ml ddH2O and 10.2 ml ***5 M NaOH*** to adjust to pH 8. Bring to 100 ml final volume with ddH2O. Place on Hula Mixer until all solids dissolve.

5 M Ammonium acetate:
Add 38.6 g ammonium acetate to bottle. Bring to 100 ml final volume with ddH2O. Place on Hula Mixer until all solids dissolve.

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180 mM Aluminium etc.:
Add 8.2 g aluminium ammonium sulphate dodecahydrate to bottle. Bring to 100 ml final volume with ddH2O. Place on Hula Mixer until all solids dissolve.

3% Calcium chloride:
Add 3 g calcium chloride dihydrate to bottle. Bring to 100 ml final volume with ddH2O. Place on Hula Mixer until all solids dissolve.

5 M NaCl:
Dissolve 29.2 g sodium chloride in 75 ml ddH2O. Bring to 100 ml final volume with ddH20.

50% PEG 8000:
To 50 g polyethylene glycol 8000 add ddH20 to a final volume of 100 ml. Place on Hula Mixer until all solids dissolve.

10% Tween 20:
To 900 µl ddH2O add 100 µl Tween 20. Invert repeatedly to mix.

Note: With the exception of ***10% Tween 20*** all stock solutions can be UV sterilised. Stock solutions can be stored at room temperature. Store ***10% Tween 20*** in the dark.

Working solutions

Lysis Solution:
Add 8.7 g trisodium phosphate dodecahydrate and 0.2 g sodium chloride to bottle. Add 70 ml ddH2O, 6.7 ml ***1 M Tris HCl (pH 8)*** and 5.3 ml ***0.5 M EDTA (pH 8)***. Place on Hula Mixer until all solids dissolve. Add 2.5 ml 5 M HCl to adjust to pH 9. Bring to 100 ml final volume with ddH2O. Invert to mix.

Tissue Lysis Additive:
To 600 ml (2 volumes) ddH2O add 300 ml (1 volume) ***20% SDS***. Invert to mix.

Flocculant Solution:
To 50 ml ***5 M Ammonium acetate*** add 25 ml ***180 mM Aluminium etc.***. Invert to mix before adding 25 ml ***3% Calcium chloride***. Invert to mix.

Wash Solution:
To 80 ml 100% ethanol add 20 ml ddH2O. Invert to mix.

Elution Buffer:
To 1 ml ***1 M Tris HCl (pH 8)*** and 0.2 ml ***0.5 M EDTA (pH 8)***. Bring to 100 ml final volume with ddH2O. Invert to mix.

Library Buffer:
To 1 ml ***1 M Tris HCl (pH 8)*** and 1 ml ***5 M NaCl***. Bring to 100 ml final volume with ddH2O. Invert to mix.

DNA Extraction Bead Solution **(for final 10 ml vol):**
Mix 100 µl ***1 M Tris HCl (pH 8)***, 20 µl ***0.5 M EDTA (pH 8)*** and 3.2 ml ***5 M NaCl***. Add 4 ml ***50% PEG 8000*** and invert to mix. Add 2.53 ml ddH20. Invert to mix thoroughly. Add 50 µl of ***10% Tween 20*** then add 100ul prepared ***Bead suspension*** (see below). Place on Hula Mixer until mixed thoroughly.

Library Prep Bead Solution **(for final 10 ml vol):**
Mix 100 µl ***1 M Tris HCl (pH 8)***, 20 µl ***0.5 M EDTA (pH 8)*** and 3.2 ml ***5 M NaCl***. Add 4 ml ***50% PEG 8000*** and invert to mix. Add 2.43 ml ddH20. Invert to mix thoroughly. Add 50 µl of ***10% Tween 20*** then add 200ul prepared ***Bead suspension*** (see below). Place on Hula Mixer until mixed thoroughly.

Note: Working solutions can be stored at room temperature. Bead solutions should be refrigerated, avoid prolonged exposure to light. For best results, bead solutions should be replaced after 1 month.

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Bead suspension

It is simplest to take aliquots of beads from the Sera Mag SpeedBead bottle at adequate amounts for use, e.g. 100 µl for 10 ml ***DNA Extraction Bead Solution***. Allow Sera-Mag SpeedBeads bottle to reach room temperature. Vortex the bottle until the beads are completely resuspended - this may take some time but it is essential they are fully suspended. Immediately after resuspension transfer the desired volume of Sera-Mag SpeedBeads to a 1.5 ml tube. Store aliquots in the fridge ready for preparation.

Bead suspension preparation:

1. Allow Sera-Mag SpeedBeads aliquot to reach room temperature.
2. Vortex thoroughly to resuspend beads. Centrifuge briefly to remove droplets from tube lid.
3. Place on magnetic stand until supernatant is completely clear and beads are bound towards magnet. This should take approximately ten minutes but can take longer.
4. While on the stand carefully remove and discard supernatant without disturbing beads.
5. Add 500 µl ddH2O. Vortex tube to resuspend beads. Centrifuge briefly to remove droplets from tube lid.
6. Place on magnetic stand until supernatant is completely clear and beads are bound towards magnet. This should take approximately ten minutes but can take longer.
7. While on the stand carefully remove and discard supernatant without disturbing beads.
8. Repeat steps 5 to 7 three more times.
9. Add ***Elution Buffer*** to match the starting volume of aliquot. Vortex tube to resuspend beads. Centrifuge briefly to remove droplets from tube lid.
10. ***Bead suspension*** can now be added to the bead solution

Note: For steps 5 to 7 the amount of ddH2O added needs to be more than the starting volume of bead aliquot. If preparing 500 µl of beads, adding 750 µl ddH2O is adequate.

DNA extraction

1 SPRI based DNA extraction

Before beginning DNA extraction

Thaw samples on ice. Centrifuge 1.5 ml Eppendorf LoBind tube containing nematode sample at ≥ 10,000 xg for 1 min to ensure sample is at bottom of tube. Pipette off excess water if sample is stored in water, be careful not to remove sample. A small volume of water remaining (~10 µl) will not affect extraction.

Take ***DNA Extraction Bead Solution*** from the fridge and allow it to reach room temperature. Vortex to mix thoroughly prior to use.

Create ***Lysis Master Mix***: 730 µl ***Lysis Solution***, 250 µl ***Tissue Lysis Additive*** and 60 µl ***PK***. Vortex to mix. Incubate at 55°C until required.

Incubate ***Elution Buffer*** at 55°C until required.

Lysis

1. Add 200 µl ***Lysis Master Mix*** to 1.5 ml LoBind tube containing nematode sample. Vortex briefly to mix and centrifuge tube for 1 sec
2. Place in Thermomixer at 55°C for 90 mins at 550 rpm.

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3. Centrifuge tube for 1 sec

Inhibitor removal

1. Add 70 µl (0.3 X volume) of ***Flocculant Solution***, invert several times to mix and incubate on ice for a minimum of 10 min
2. Centrifuge at ≥ 10,000 xg for 2 min at room temperature
3. Without disturbing the pellet, transfer supernatant to a fresh 1.5 ml LoBind tube

SPRI DNA binding

1. Add 400 µl (2 X volume) of ***DNA Extraction Bead Solution***. Place on HulaMixer (continual rotation) for 10 min
2. Place on magnetic stand until supernatant is clear and beads are bound towards magnet
3. While on the stand carefully remove and discard supernatant without disturbing beads

Wash

1. Add 1000 µl ***Wash Solution***. Incubate at room temperature for 30 secs
2. While on the stand carefully remove and discard supernatant without disturbing beads
3. Repeat steps 11 to 12 a further time
4. Centrifuge tube for 1 sec. Place back on magnetic stand ensuring beads are bound towards magnet. Remove all remaining ***Wash Solution*** with a 10 µl pipette. Air dry tube with cap open until beads are completely dry (i.e. no longer shiny)

Elution

1. Add 25 µl ***Elution Buffer*** (55°C) and gently flick to resuspend beads. Ensure all beads are resuspended with no clumps. Centrifuge tube for 1 sec
2. Place in Thermomixer at 55°C for 10 mins at 550 rpm. Centrifuge tube for 1 sec
3. Place on magnetic stand until supernatant is clear and beads are bound towards magnet
4. Carefully transfer eluate to a fresh 1.5 ml LoBind tube without disturbing beads

Store DNA at -20°C until required.

Library preparation and sequencing

2 Library preparation and Sequencing

Library preparation and sequencing is broken down into distinct stages. At the end of the first two stages are suitable stopping points. This allows the process to be carried out over multitiple days if required.

2.1 DNA fragmentation/tagmenting and PCR barcoding

Before beginning fragmentation/tagmenting and PCR barcoding

Thaw samples on ice. Thaw Rapid PCR Barcoding Kit reagents: Barcodes (***RLB*** 01-12a) at room temperature and place Fragmentation Mix (***FRM***) on ice. Keep reagents on ice until required, vortex briefly and spin down prior to use.

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DNA quantification

Quantify 2 µl of each sample on a Qubit 3.0 fluorometer using high-sensitivity (HS) dsDNA assay.

DNA fragmentation/tagmenting

Important: If sample DNA concentration is less than 1 ng/µl (or 'too low') use 5 µl of sample for fragmentation, otherwise use 3 µl. Maximum amount of DNA to be used is 5 ng (as per Oxford Nanopore Rapid PCR Barcoding Kit: SQK-RPB004).

Add appropriate sample volume to 1 µl ***FRM*** in a 0.2 ml PCR tube, close lid and flick gently to mix. Centrifuge briefly to collect droplets from lid. Place on ice until required.

In a thermal cycler, incubate PCR tube for: 1 min @ 30°C, then 1 min @ 80°C, hold at 4°C. DNA template is now tagmented, place on ice until required.

PCR Barcoding

Make a PCR master mix per sample (allow ~5% for pipetting error):

20 µl Nuclease-free water
25 µl LongAmp Taq 2X master mix

Add 45 µl PCR master mix to each tagmented sample and 1 µl of ***RLB*** (01-12a). Flick gently to mix, centrifuge briefly to collect droplets from lid. Place on ice until required.

Thermal cycler conditions

If initial sample DNA template > 0.1 ng/µl, place sample in a thermal cycler with:
3 mins @ 95°C, **15** X (15 secs @ 95°C, 15 secs @ 56°C, 6 mins @ 65°C), 6 mins @ 65°C, hold at 4 °C

Else, place sample in a thermal cycler with:
3 mins @ 95°C, **20** X (15 secs @ 95°C, 15 secs @ 56°C, 6 mins @ 65°C), 6 mins @ 65°C, hold at 4 °C

Stopping point: PCR barcoded samples can be stored in a fridge overnight or up to a week if required.

2.2 Sample concentration

Before beginning sample concentration

A magnetic stand or rack is required for this step. If it cannot accommodate 0.2 ml PCR tubes, transfer each PCR barcoded sample to a 1.5 ml Eppendorf DNA LoBind tube.

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Take ***Library Prep Bead Solution*** from the fridge and allow it to reach room temperature. Vortex to mix thoroughly prior to use.

Create ***Lysis Master Mix***: 730 µl ***Lysis Solution***, 250 µl ***Tissue Lysis Additive*** and 60 µl ***PK***. Vortex to mix. Incubate at 55°C until required.

Incubate ***Library Buffer*** at 55°C until required.

1. Add 50 µl (1 X volume) of ***Library Prep Bead Solution*** to each sample. Mix by pipetting
2. Leave to stand at room temperature for 10 mins
3. Place on magnetic stand until supernatant is clear and beads are bound towards magnet
4. While on the stand carefully remove and discard supernatant without disturbing beads
5. Add 150 µl ***Wash Solution***. Incubate at room temperature for 30 secs
6. While on the stand carefully remove and discard supernatant without disturbing beads
7. Repeat steps 11 to 12 a further time
8. Centrifuge tube for 1 sec. Place back on magnetic stand ensuring beads are bound towards magnet. Remove all remaining Wash Solution with a 10 µl pipette. Air dry tube with cap open until beads are completely dry (i.e. no longer shiny)
9. Add 15 µl ***Library Buffer*** (55°C) and gently flick to resuspend beads. Ensure all beads are resuspended with no clumps. Centrifuge tube for 1 sec
10. Leave to stand at room temperature for 10 mins
11. Place on magnetic stand until supernatant is clear and beads are bound towards magnet
12. Carefully transfer eluate to a fresh 0.2 ml PCR tube without disturbing beads

Stopping point: Concentrated samples can be stored in a fridge overnight or up to a week if required.

2.3 Sample DNA quantification and molarity estimation

DNA quantification

Quantify 2 µl of each concentrated sample on a Qubit 3.0 fluorometer using high-sensitivity (HS) dsDNA assay.

Fragment length estimation

For sample DNA fragment length estimates there are three options we have used:

Option 1 (ideal and accurate but expensive, does not work with low DNA concentration):
1 µl of each sample was measured on a Tapestation 2200 (Agilent Technologies) using genomic tape.

Option 2 (cheap but less accurate, may not work with low DNA concentration):
2 µl of each sample was run on a 1% agarose gel against 0.5 µl Generuler 1 Kb DNA ladder (or similar, diluted 1:20). Estimate fragment length based on gel imaging.

Option 3 (free but less accurate, DNA concentration not an issue):
Assume all samples have a fragment length of 7 Kb.

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Molarity estimation

Based on the sample quantification and fragment length estimates (whichever option chosen), the molarity of each sample can now be calculated in fmol/µl. For this use the following equation:

fmol/µl = DNA concentration / ((DNA fragment length X 1,000 X 617.96) + 36.04) X 1,000,000

DNA concentration is in ng/µl, DNA fragment length is in Kb.

Pool samples

Samples should be pooled as equimolar as possible for best results. However, this is not entirely necessary or sometimes even possible. Aim for a final library concentration of ~ 2 fmol/µl. This will mean that the 5 µl volume of library used for sequencing a full 12 samples will contain 5 - 10 fmol of DNA, ideal for Flongle sequencing.

It is perfectly feasible to concentrate (see sample concentration above) or dilute (using ***Library Buffer***) the pooled library as required. After adjusting the concentration it is advised to quantify 2 µl of the pooled library on a Qubit 3.0 and recalculate molarity (as above) using the average fragment length.

2.4 Sequencing adapter ligation

Allow Rapid Adapter (**RAP**) to reach room temperature. Vortex briefly and spin down.

1. Transfer 5 µl of final library into a 1.5 ml Eppendorf DNA LoBind tube
2. Add 0.5 µl ***RAP*** to the tube. Flick gently to mix and spin down briefly
3. Incubate for 5 - 10 minutes at room temperature
4. Place on ice until required

Loading the Flongle flow cell

Note: It is important to perform a flow cell check prior to loading to ascertain the flow cell's quality.

Follow any Flongle protocol from 'Loading the Flongle flow cell' (e.g. this protocol).

Sequencing

We recommend specifying a directory for sequencing output (i.e. a desktop or external hard drive directory) rather than the hard-to-find default output directory of ONT's MinKNOW software. Disable all base calling options - we basecall the output fast5s with Guppy GPU HAC post sequencing. Run the sequencing for the default 24 hours. A longer sequencing period may allow for more data, however, we have found reads begin to plateau around the 24 hour mark using our protocol.

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