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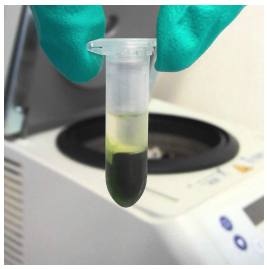
Sanger Tree of Life HMW DNA Extraction: Automated Plant Organic HMW gDNA Extraction (POE)



In 1 collection

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Tree of Life at the Wellco…

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We use this protocol and it's working!

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Abstract

The Plant Organic HMW gDNA Extraction (POE) protocol acts as the Sanger Tree of Life's programmes mid-throughput, reserve gDNA extraction procedure for all plant species too recalcitrant to yield HMW gDNA of adequate quality or quantity with the Plant MagAttract v.4 protocol. Developed in-house, the POE protocol is highly efficient at isolating pure, high-quality and high molecular weight (HMW) gDNA from the majority of plant species to an extent adequate for long-read sequencing.

The POE protocol is divided into four stages: (1) direct lysis of tissue homogenates with an SDS-based buffer containing reducing agents to mitigate oxidative DNA damage, (2) centrifugation and KDS-protein complex precipitation using potassium acetate, (3) gDNA isolation by two chloroform phase separations, (4) and gDNA capture/purification using a 1X Sera-Mag™ SpeedBead and 0.45X AMPure® PB double SPRI cleanup/size selection. Outcomes from 65–100 mg of fresh-frozen tissue homogenates are generally sufficient quantities (1-20 µg) of high purity, ultra HMW (uHMW; 100 kb+) gDNA adequate for multiple high-quality long-read sequencing events. However, outcome success is dependent on the plant species, tissue type and sample quality used.

The output of this protocol is uHMW gDNA, which depending upon yield and genome size of the species can be directed downstream towards HMW DNA Pooling, HMW DNA Fragmentation: Diagenode Megaruptor®3 for LI PacBio or HMW DNA Fragmentation: g-Tube for ULI PacBio.

Acronyms
HMW: high molecular weight
uHMW: ultra high molecular weight
gDNA: genomic DNA
SPRI: solid-phase reversible immobilisation
LI: low input
ULI: ultra-low input

Guidelines

High quality, young leaf material with no observable damage, disease or other stressors, of which generally have less contaminant accumulation and are most pliable, should be preferentially selected for optimal outcomes downstream.

- Alternative pliable tissue types (e.g. herbaceous stem, petiole or flower) may be selected when required.
- Recalcitrant sample types (e.g. woody or rigid, fibrous tissues) or tissues with signs of stress can be selected, but results may vary.

Plant tissues should be preserved via flash freezing and stored under constant cryogenic conditions thereafter (e.g. on dry ice or within a –80 °C freezer). Alternative preservation techniques can be performed but outcomes may vary.

- Whole-frozen or disrupted plant tissues can be stored long term at –80 °C with minimal detrimental effects observed.
- Premature thawing of material or repeat freeze-thaw cycles can drastically decrease extraction efficiency due to gDNA degradation.

Ensure plant tissue is completely disrupted into a fine powder; avoid matted/clumped powder or fibrous material. Increased quality tissue homogenates result in increased quality and quantities of HMW gDNA.

- Complete disruption is crucial to ensure optimal DNA yield and integrity; poorly disrupted input material can drastically decrease extraction efficiency and all quantifiable outcomes.
- Plant tissues can be disrupted via cryogenic grinding (mortar and pestle), with the CP02 cryoPREP Automated Dry Pulverizer, or by cryogenic bead beating – it is recommended to follow the standardised steps outlined in either the 'Sanger Tree of Life Sample Homogenisation: Cryogenic Bead Beating of Plants with FastPrep-96 protocols' or 'Sanger Tree of Life Sample Homogenisation: Covaris cryoPREP® Automated Dry Pulverizer' protocols.

An experienced operator of the POE protocol can expect to comfortably process up to 24 samples per session, with 2–3 hours of handling time required over a start to finish period of 4–6 hours. This estimation excludes the overnight incubation of eluates to solubilise gDNA, subsequent QC checks, and includes the utilisation of the KingFisher™ Apex Instrument to perform the double SPRI cleanup/size selection – alternatively, this can be performed manually, which can be viewed in the manual version of this protocol.

Additional Notes

- It is recommended to split larger numbers of samples (12+) into 2 batches during phase separation, starting step 8 on the second batch once the first is almost ready to come off the tube rotator (step 8.2).

- Tri-coded FluidX tube are used throughout the Tree of Life programme in order to track samples, therefore all routine DNA extracts are stored in FluidX tubes.

- Both the KingFisher™ Apex protocol script and the KFX.file have been made available for this protocol – the KFX.file requires 'BindIx software for KingFisher Apex' to allow the KingFisher™ Apex protocol to be viewed on a PC or laptop. Alternatively, the file can be transferred directly onto a KingFisher™ Apex instrument using a USB.

- Whilst highly effective at isolating gDNA from endogenous contaminants, this protocol is generally unsuitable for species with exceptionally high polysaccharide concentrations that form viscous lysates. For these, a pre-lysis hypertonic sorbitol wash of tissue homogenates should be performed, this will be included as an optional step in subsequent versions of the POE protocol.

Materials

Materials

- Wet ice
- Dry ice
- Weighing boats (SLS Cat. no. bal1820sp)
- 2 mL DNA Lo-Bind microcentrifuge tubes (Eppendorf Cat. no. 0030108078)
- 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)
- Chloroform:isoamyl alcohol (24:1, v/v)
- 100% absolute ethanol
- Buffer EB (Qiagen Cat. no. 19086)
- AMPure PB beads (Pacific Biosciences Cat. no. 100-265-900)
- Sera-Mag™ magnetic carboxylate modified particles (Cat. no. GE24152105050250)
- Nuclease-free water
- Tris Base
- EDTA (0.1M stock concentration, pH 8)
- NaCl (5 M stock concentration)
- SDS (Sodium Dodecyl Sulfate Solution 10%)
- PVP-40 (polyvinylpyrrolidone, M.W. 40,000)
- Sodium metabisulphite
- QIAGEN Proteinase K
- DTT (Dithiothreitol, Stock concentration 1M)
- QIAGEN RNase A
- Potassium acetate
- PEG 8000
- Tris-HCl (1M Stock concentration, pH 8.0)
- Tween-20
- 1 x phosphate-buffered saline (PBS)

Equipment

- Pipettes from 0.5 to 5000 µL and filtered tips
- Wide-bore pipette tips (200 and 1000 µL)
- Eppendorf ThermoMixer C (Cat. no. 5382000031)
- Eppendorf SmartBlock 2.0 mL (Cat. no. 5362000035)
- Eppendorf SmartBlock 50 mL (Cat. no. 5365000028)
- Vortex (Vortex Genie™ 2 SI-0266)
- Eppendorf Refrigerated Centrifuge 5425 (Cat. No. 5405000760)
- Mettler Toledo Analytic Balance ME204 (Material No. 30029066)
- Chemical fume Hood
- HulaMixer Sample Mixer (Cat. no. 15920D)

- Kingfisher Apex™ instrument (Cat. no. 5400930)
- Cool rack (Corning® CoolRack CF45 Product no. 432051) or equivalent

Recipes

Add reagents in order as seen below

Direct Plant Lysis Buffer should be prepared when starting the protocol

Direct Plant Lysis Buffer

	Reagent	Target Concentration	Molecular weight (g/mol)	Stock concentration	Input from stock per sample (600 µL total)
	Nuclease-free water	-	-	-	95 µL
	Tris base solution, pH 8.0 (recipe below)	100 mM	157.60	2 M	30 µL
	EDTA, pH 8.0	50 mM	292.24	0.1 M	300 µL
	NaCl	500 mM	58.44	5 M	60 µL
	SDS	1.5% (v:v)	-	10%	90 µL
	PVP-40	1% (w:v)	40,000	Powder	6 mg
	Sodium metabisulphite	1% (w:v)	190.107	Powder	6 mg
	(Add Proteinase K and DTT to the lysis buffer directly prior to use).				
	Proteinase K	-	-	20 mg/mL	20 µL
	DTT	5 mM	154.253	powder	0.46 mg
	(Add RNase A after 15 mins of incubation, 55 °C at 600 rpm).				
	RNase A (17,500 U)	-	-	100 mg/µL	4 µL

- RNAse A and Proteinase K are both supplied by Qiagen.
- DTT is unstable in solution; only appropriately stored crystalline powder or freshly prepared DTT solutions should be used.

Below recipes should be prepared prior to starting the protocol

2M Tris base solution (pH 8.0)

	Reagent	Target concentration	Molecular weight (g/mol)	Stock concentration	Input from stock (500 mL)
	Tris Base	2 M	121.14	Powder	121.14 g
	Nuclease-free water	-	-	-	up to 500 mL
	(Adjust pH to 8.0).				
	Store stock at RT for up to 3 years.				

Potassium acetate solution (KAc; pH 7.4)

	Reagent	Target concentration	Molecular weight (g/mol)	Stock concentration	input from stock (500 mL)
	Potassium acetate	5 M	98.14	powder	245.35 g
	Nuclease-free water	-	-	-	up to 500 mL
	(Adjust pH to 7.40).				
	Store stock at 4 °C for up to 3 years.				

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 with nuclease free water before use to remove sodium azide).				
	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 mins).
2. Vortex thoroughly to resuspend the beads.
3. Pipette 800 µL of Sera-Mag™ SpeedBead stock solution into a 2 mL Lo-Bind 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 Speed Bead 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.

KingFisher™ Apex POE Protocol Script:

KFX file: Plant Organic Extraction.kfx 2KB

1. Pick Up Tip - Tip Plate 1
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 8 Count 30 Seconds
3. Ethanol Wash 1.1 - Ethanol Wash 1.1 Plate
   Pre-collect beads: Off
   Release beads: On 00:00:10 Bottom mix
   Heating & Cooling: Off
   Mixing 1# 00:00:20 Medium
   Postmix: Off
   Collect beads: On 1 Count 1 Second
4. Ethanol Wash 1.2 - Ethanol Wash 1.2 Plate
   Pre-collect beads: Off
   Release beads: On 00:00:10 Bottom mix

Heating & Cooling: Off
Mixing 1# 00:00:20 Medium
Postmix: Off
Collect beads: On 1 Count 1 Second

5. Air Dry 1 - Ethanol Wash 1.2 Plate
   Duration: 00:01:00 Above well
6. Elute 1 - Elution Plate 1
   Pre-collect beads: Off
   Release beads: On 00:00:00
   Heating & Cooling: On 37°C Preheat: On
   Mixing: 1# 00:01:00 Slow Looping: 6
   2# 00:01:25 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
7. Leave Tip 1 - Ethanol Wash 1.2 Plate
8. Pick Up Tip - Tip Plate 2
9. Dispense - Elution Plate 1
   Automatic numbering: If required, aliquot 10µL voucher before continuing.
   Dispense to plate: AMPure PB 180µl
10. Bind 2 - Elution Plate 1
    Pre-collect beads: Off
    Release beads: On 00:05:00 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 8 Count 30 Seconds
11. Ethanol Wash 2 - Ethanol Wash 2 Plate
    Pre-collect beads: Off
    Release beads: Off
    Heating & Cooling: Off
    Mixing 1# 00:00:30 Slow Looping: 2
    2# 00:00:10 Paused Tip position: Above well
    Postmix: Off
    Collect beads: Off
12. Air Dry 2 - Ethanol Wash 2 Plate
    Duration: 00:01:00 Above well
13. Elute 2 - Elution Plate 2
    Pre-collect beads: Off
    Release beads: On 00:00:00
    Heating & Cooling: On 37°C Preheat: On

Mixing:	1#	00:01:00	Slow	Looping: 6
	2#	00:01:29	Paused	Tip position: Tip edge in liquid
	3#	00:00:01	Medium	
Postmix:	On	00:00:30	Slow	
Collect beads:	On	10 Count	30 Seconds	

14. Leave Tip 2 - Ethanol Wash 2 Plate

Protocol PDF: Sanger Tree of Life HMW DNA Extra… 125KB

Safety warnings

- Powder-free nitrile gloves, eye protection and a lab coat should be worn by the operator when performing this procedure.
- Glove liners are strongly recommended when handling cryogenic substances.
- 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.

Before start

Ensure all appropriate reagents in the 'materials' section have been prepared before starting the protocol:

- 500 µL of Sera-Mag™ SpeedBead solution is needed per sample; this requires the preparation of 50% PEG 8000, 10% Tween-20, SpeedBead wash suspension and SpeedBead binding solution before starting the protocol.
- Prepare the 1M Tris base solution (pH 8.0) and potassium acetate solution (KAc; pH 7.4) before starting the protocol.

Sample lysis

1 Prepare an adequate volume of the 'Direct Plant Lysis Buffer' (recipe in Materials).

- Preheat the direct plant lysis buffer for 15–30 mins, 65 °C at 400 rpm prior to use, ensuring that all reagents are completely dissolved.
- Add DTT and Proteinase K to the direct plant lysis buffer immediately prior to use, ensuring both reagents are thoroughly mixed.

2 Aliquot 65–100 mg of cryogenically disrupted tissue samples into individual 2 mL Lo-Bind tubes on dry ice.

- Lower than recommended quantities of tissue homogenate (down to >15 mg) can be used, but gDNA yield may vary.

3 Transfer the 2 mL Lo-Bind tubes containing sample to wet ice for 10 minutes, allowing sample temperature to equilibrate.

4 Perform the direct sample lysis.

4.1 Add 550 µL of preheated direct plant lysis buffer (65 ºC) to the first sample - immediately pulse vortex 5 times at full speed until homogenous, and place on a heat block at 55 ºC, 600 rpm. Repeat for each sample.

- Some samples will not homogenise through pulse-vortexing. It is recommended that these samples are continuously vortexed for 5 seconds. If this does not homogenise the sample either, proceed to step 4.2.

4.2 Once all samples are homogenised and have begun incubation, inspect each by inverting to mix. Any samples with aggregated tissue that can't be homogenised through inversion should be thoroughly mixed with a wide bore P1000 tip until clumps are separated and the sample is homogeneous.

4.3 After 15 minutes of incubation, add 4 µL RNase A to each sample and mix by pulse-vortexing 5 times until any aggregated, insoluble or sedimented tissue particles are resuspended. Repeat step 4.2 for samples that have reaggregated and remain clumped after vortexing.

- The 4 µL RNase A can be diluted in 6 µL PBS per sample and added to the sample with a Multipette to improve ergonomics.

4.4 Incubate for another 45 minutes, 55 °C at 600 rpm.

- Samples can be routinely resuspended by inversion, or pipette-mixed with a wide bore P1000 tip to remove aggregates, to improve lysis performance. However, this is not essential.

- Do not agitate the samples by mixing for the last 15 minutes of lysis; allow any unlysed sediment to settle at the bottom of the tube.

4.5 Whilst the samples are incubating, prepare a fresh 2 mL Lo-Bind tubes containing 150 µL of cold potassium acetate solution (4 °C; 5 M; pH 7.4) for each sample, and incubate on wet ice until temperature equilibrates.

5 Remove the samples from the heat block, allow the lysate to briefly settle (1 to 5 minutes), and then centrifuge for 10 minutes, 8,000 rpm at room temperature.

- Avoid disturbing the insoluble sediment prior to centrifugation, as this may reduce the loss of uHMW gDNA captured in the pellet.

6 Use a wide bore P1000 tip to transfer the supernatant to its corresponding 2 mL Lo-Bind tubes containing 150 µL cold potassium acetate solution (4 °C; 5 M; pH 7.4), and carefully mix until homogeneous by inversion or pipetting with the same wide bore tip.

- gDNA is highly susceptible to mechanical degradation from this point onwards; handle samples with care.

- The precipitate should appear whitish, opaque and slightly viscous.

7 Incubate the samples on wet ice for 5 minutes (precipitated samples can be left on wet ice for up to an hour if a break is required).

- 500 µL of Sera-Mag™ Speedbead solution and 175 µL of AMPure® PB beads per sample should now be removed from the fridge to equilibrate to room temperature.

- A tabletop centrifuge should now be pre-chilled to 4 °C.

Chloroform phase separation

8 Perform the first chloroform separation (C:IA) in a chemical fume hood:

8.1 Add 700 µL cold chloroform:isoamyl alcohol (–20 °C; 24:1, v/v) to the samples.

8.2 Mix on a tube rotator at 25 rpm for 10 minutes at room temperature.

8.3 Centrifuge at 13,000 rpm for 5 minutes at 4 °C.

8.4 Transfer up to 700 µL of the aqueous phase (top layer) to a fresh 2 mL Lo-Bind tube using a wide bore P1000 tip.

- Carefully aspirate from the top of the aqueous phase to avoid 'dragging' contaminants from the interphase into the pipette.

9 Perform the second chloroform separation (C:IA) in a chemical fume hood:

9.1 Add 700 µL cold chloroform:isoamyl alcohol (–20 °C; 24:1, v/v) to the sample.

9.2 Mix on a tube rotator at 25 rpm for 10 minutes.

9.3 Centrifuge at 13,000 rpm for 5 minutes at 4 °C.

9.4 Transfer up to 600 µL of the aqueous phase (top layer) to the applicable empty well of the 'Sample Plate' (see step 12) using a wide bore P1000 tip.

- Carefully aspirate from the top of the aqueous phase to avoid 'dragging' contaminants from the interphase into the pipette.

10 Add 500 µL Sera-Mag™ SpeedBead solution to each sample in the 'POE Sample Plate'.

Loading and Running the KingFisher™ Apex

11 Label seven KingFisher™ 1 mL 96-well deep-well plates and one KingFisher™ 200 µL standard 96-well plate with the following labels, and fill all applicable wells of each plate with their corresponding reagents (see table below).

	Plate name	Plate type	Reagent(s) required
	POE Tip Plate 1	1 mL	96-well tip comb (no reagent)
	POE Sample Plate	1 mL	Up to 600 µL aqueous phase of sample + 500 µL Sera-Mag™ Speedbead solution (Steps 9.4 & 10)

	Plate name	Plate type	Reagent(s) required
	POE Ethanol Wash 1.1	1 mL	1 mL 80% ETOH
	POE Ethanol Wash 1.2	1 mL	1 mL 80% ETOH
	POE Elution Plate 1	1 mL	400 µL Buffer EB
	POE Tip Plate 2	1 mL	96-well tip comb (no reagent)
	POE Ethanol Wash 2	1 mL	1 mL 80% ETOH
	POE Elution Plate 2	200 µL	135 µL Buffer EB

12 Select the required DNA extraction protocol in the protocol list on the KingFisher™ Apex (details in KingFisher™ Apex POE Protocol Script/attached KFX file in the Materials section) and select using the play button.

13 Load the filled plates onto the instrument following the instructions provided on screen and initiate once ready.

14 The instrument will prompt once the inital 1X SpeedBead SPRI is finished: add 175 µL (0.45X) AMPure PB beads to each well containing sample of the 'POE Elution Plate 1', place the plate back into the instrument, and continue the run.

15 The instrument will prompt when the 0.45X AMPure PB SPRI is finished: remove the 'POE Elution Plate 2' and use a wide bore p200 tip to transfer the 135 µL sample eluate to an appropriate tube for gDNA storage.

16 Incubate the sample at RT overnight to allow the uHMW gDNA to solubilise.

17 Proceed to appropriate QC checks and downstream processing.

17.1 The gDNA extract can be stored long-term at 4 °C.

Protocol references

Mayjonade, B. et al. (2016) 'Extraction of high-molecular-weight genomic DNA for long-read sequencing of single molecules', BioTechniques, 61(4), pp. 203–205. doi:10.2144/000114460.