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

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Abstract

The Plant Organic HMW gDNA Extraction (POE) protocol acts as the ToL's mid-throughput, reserve gDNA extraction procedure for recalcitrant, 'routine failure' species within the Plantae kingdom; developed in-house, it 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: direct tissue lysis with an SDS-based buffer containing reducing agents to mitigate oxidative DNA damage, centrifugation and DS-protein complex precipitation using potassium acetate, gDNA isolation by two chloroform phase separations, and gDNA capture/purification using a 1X Sera-Mag™ SpeedBead and 0.45X AMPure® PB double SPRI cleanup/size selection. The protocol generally yields a sufficient quantity of high purity, ultra HMW (uHMW; 100 kb+) gDNA from 65-100 mg of fresh-frozen tissue for high-quality long-read sequencing submissions. However, outcome success is dependent on the plant species, tissue type and sample quality.

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

Health & 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.

Guidelines

- High-grade, young leaf material with no observable damage, disease or other stressors, 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 if required, however outcomes may vary.
    - Recalcitrant sample types (e.g. woody or rigid, fibrous tissues) or tissues with signs of stress should be avoided where possible.

- Plant tissues should be preserved via flash freezing and stored under constant cryogenic conditions thereafter (e.g. 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.
    - 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 the Sanger Tree of Life Sample Homogenisation: Covaris cryoPREP® Automated Dry Pulverizer and Sanger Tree of Life Sample Homogenisation: Cryogenic Bead Beating of Plants with FastPrep-96 protocols.

- An experienced operator 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.

Additional Notes

- It is recommended to split larger numbers of samples (12+) into 2 batches, 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 tubes 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, an alternative HMW gDNA extraction protocol should be performed.

Before Starting

- Ensure adequate volumes of Sera-Mag™ Speedbead solution have been prepared prior to initiating the protocol, 500 µL is required per sample (recipe below).
    - This requires 50% PEG 8000, 10% Tween-20, SpeedBead wash suspension and SpeedBead binding solution to be prepared prior to initiating the protocol.
- Ensure all required equipment and materials are available to the user.

Laboratory Protocol

<u>Sample Lysis</u>

1. Prepare an adequate volume of the 'Direct Plant Lysis Buffer' (recipe below).
    - Preheat the direct plant lysis buffer for 15-30 mins, 65°C at 400 rpm prior to use, ensuring the total dissolution of reagents.
    - Add DTT and Proteinase K to the direct plant lysis buffer <u>immediately prior</u> 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.
3. Transfer 2 mL Lo-Bind tubes containing sample to wet ice for 10 - 15 minutes, allowing sample temperature to equilibrate.
4. Perform the direct sample lysis.

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

4.2. Once all samples are homogenised and have began incubation, inspect each by inverting to mix. Any samples with aggregated tissue that can't be homogenised through inversion should be mixed with wide bore P1000 until homogenous.

4.3. After 15 minutes of incubation, add 4 µL RNase A to each sample and mix by inversion until any aggregated, insoluble or sedimented tissue particles are resuspended. Repeat step 4.2 for severely reaggregated samples.

- 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.

- Do not agitate 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 fresh 2 mL Lo-Bind tubes containing 150 µL of cold potassium acetate (4°C; 5 M; pH 7.4) for each sample, and place on wet ice.

5. Remove the samples from the heat block, allow the lysate to settle for 5 mins at RT, and centrifuge for 10 minutes, 8000 rpm at room temperature.

- Avoid disturbing the settled insoluble tissue prior to centrifugation.

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

- gDNA is highly susceptible from this point; 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 the 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.

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.

- 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 the 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.

- 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 the well containing the sample of 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
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 below in KingFisher™ Apex POE Protocol section/attached file) 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 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 to transfer the 135 µL sample eluate to an appropriate tube for gDNA storage.

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

17. Proceed to appropriate QC checks and downstream processing.

18. Store the DNA at 4ºC.

KingFisher™ Apex POE Protocol:

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

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 (2M stock concentration, pH 8)
- 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
- Proteinase K
- DTT (Dithiothreitol, Stock concentration 1M)
- RNase A
- Potassium Acetate (Stock concentration 5M, pH 7.4, store at 4°C)
- PEG 8000
- Tris-HCl (1M Stock concentration, pH 8.0)
- Tween-20
- 1 x phosphate-buffered saline (PBS)

Equipment:

- Pipettes for 0.5 - 1000 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)
- Fume Hood
- HulaMixer Sample Mixer (Cat. no. 15920D)
- Kingfisher Apex™ instrument (Cat. no. 5400930)
- Cool rack (Corning® CoolRack CF45 Product no. 432051) or equivalent

Below recipes should be prepared as required during the protocol

Add reagents in order as shown in recipes below

Direct Plant Lysis Buffer

Reagent	Target concentration	Molecular weight (g/mol)	Stock concentration	Input from stock per sample (600 µL total)
Nuclease-free H_20	-	-	-	95 µL
Tris, pH 8.0	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 mixing with the sample).				
Proteinase K	-	-	20 mg/mL	20 µL
DTT	5 mM	154.253	1 M	3 µL
(Add RNase A after 30 mins of incubation, 55°C at 600 rpm).				
RNase A	-	-	100 mg/µL	4 µL

- RNAse A and proteinase K are provided with the Qiagen MagAttract HMW DNA Kit.

Potassium acetate (KAc) - Addition to lysate supernatant

Reagent	Target concentration (upon addition)	Molecular weight (g/mol)	Stock concentration	Input from stock per sample
Potassium acetate, pH 7.4, 4°C	1.25 M	98.15	5 M	200 µL
Store stock at 4°C for up to 1 year.				

Below recipes should be prepared prior to starting the protocol

Add reagents in order as shown in recipes below

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 H_20	-	-	-	6 mL
(Incubate for 60 mins, 75°C at 600 rpm, routinely vortexing until fully dissolved).				
Nuclease-free H_20	-	-	-	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 H_20	-	-	-	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 H_20	-	-	-	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 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 H_20 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 H_20 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 H_20	-	-	-	up to 40 mL
Filter sterilise through a 0.45 uM 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:

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