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Sanger Tree of Life HMW DNA Extraction: Hypertonic Washing of Plant Tissue Homogenates

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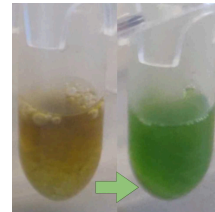
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We use this protocol and it's working

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Abstract

The problematic characteristics of exceptionally recalcitrant plants in extraction are often attributed to the overwhelming abundance of sugars, oils or other endogenous secondary metabolites that may accumulate in a specimen's cells. Whilst alternative specimens and/or tissue types can sometimes rectify these issues by avoiding the use of samples with excessive accumulations of such substances, many species have an innate, systemic predisposition to contaminant concentrations high enough to overload standard methods of nucleic acid extraction.

The most prevalent and interfering irreversible interactions associated with recalcitrant plant extractions are DNA oxidation and lysate viscosity, both of which are correlated with lower DNA quantities and quality that perform substantially worse during ToL's downstream applications. Oxidative DNA damage is induced by the overabundance of oxidising agents such as polyphenols, and is observable through the characteristic browning of sample lysates. However, oxidative gDNA damage is otherwise generally undetectable prior long-read sequencing, where a sizable reduction in performance is observed. The overabundance of viscosity-inducing agents, such as polysaccharides, is easily observable following cell decompartmentalization due to the formation of heavily aggregated, glue-like lysates that co-precipitate with DNA and heavily impede ToL's standard protocols.

To overcome these issues, this protocol utilises a hypertonic sorbitol wash buffer to remove interfering contaminants present within the cytosol prior to cell decompartmentalization, meaning the aforementioned interfering, irreversible interactions are unable to occur due to a lack of contact with gDNA. Sorbitol is an osmotically active sugar alcohol capable of 'drawing out' the cytosol of homogenised (cryogenically disrupted) plant tissues without interrupting cellular or nuclear membranes. If successful, a previously recalcitrant sample's lysate should be absent of either viscosity or browning.

However, it should be noted that the presence of interfering contaminants outside of the cytosol (e.g. extracellular proteoglycans) will not be affected by this protocol. In these instances, an alternative method of pre-lysis purification (e.g. nuclei isolation) is recommended if standard procedures are inadequate.

This protocol is performed before the Plant Organic HMW gDNA Extraction (POE) protocol, which acts as the Tree of Life programme's mid-throughput, reserve gDNA extraction procedure for all recalcitrant species within the Plantae kingdom.



Guidelines

- Input amounts of 40 to 130 mg of cryogenically disrupted plant tissue are required for this protocol.
- Keep samples on dry ice prior to initiating protocol to maintain temperature and prevent nucleic acid degradation.
- An experienced operator can expect to comfortably process 24 samples, with approximately 1 hour handling time over a start to finish period of 1 hour. This estimation excludes the subsequent DNA extraction protocol and QC checks.

Additional Notes

- In the Tree of Life Programme, bead beating using the FastPrep-96 is used routinely for the cryogenic disruption of plant tissue. This process is detailed in the protocol Sanger Tree of Life Sample Homogenisation: Cryogenic Bead Beating of Plants with FastPrep-96 dx.doi.org/10.17504/protocols.io.rm7vzxk38gx1/v1.
- In the Tree of Life Programme, the DNA extraction protocol routinely used following the hypertonic washing of plant tissue homogenates is Automated Plant Organic HMW gDNA Extraction (POE). This process is detailed in the protocol Sanger Tree of Life HMW DNA Extraction: Automated Plant Organic HMW gDNA Extraction (POE) dx.doi.org/10.17504/protocols.io.e6nvwd227lmk/v1.

Materials

- Wet ice
- Dry ice
- 2 mL DNA Lo-Bind microcentrifuge tubes (Eppendorf Cat. no. 0030108078)
- 50 mL centrifuge tubes (Cat. no. 227261)
- 200 mL flask, for example 200 mL DURAN Erlenmeyer Flask (Cat. no. 212263209)
- D-sorbitol (Cat. no S9378-1kg)
- PVP-40 (Cat. no PVP40-50G)
- Ultra-pure H₂O (AM9932)
- Tris (2M stock concentration, pH 8) (Cat. no 10376933)
- KCl (1M stock concentration, pH 7.4) (Cat. no. 40120948-1)
- EDTA (0.1M stock concentration, pH 8) (Cat. no AM9912)
- EGTA (Merck Cat. no. 324626-25GM)
- Dithiothreitol (DTT) crystalline powder (Merck, Cat. no. D0632-5G)
- 1x phosphate-buffered saline (PBS) (Cat. no. 10010023)

Equipment:

- Pipettes for 0.5 - 1000 µL and filtered tips
- Wide bore pipette tips (200 and 1000 µL)
- Sterile Polyethersulfone Syringe Filter Units, 0.45 µm (Cat. no 16427565)
- Terumo 3-Part 50mL Luer Lock Syringes (Cat.no. 15349067)
- Vortex (Vortex Genie 2 SI-0266) (Cat. no. 444-0486)
- Eppendorf Refrigerated Centrifuge 5425 (Cat. No. 5405000760)
- Mettler Toledo Analytic Balance ME204 (Material No. 30029066)
- HulaMixer Sample Mixer (Cat. no. 15920D)
- Cool rack (Corning CoolRack CF45 Product no. 432051) or equivalent

Recipes:

Below recipes should be prepared as a stock prior to starting the protocol

Sorbitol stock buffer

	Reagent	Target concentration	Molecular weight (g/mol)	Stock concentration	Input from stock (500 mL total)	Input from stock (1 L total)
	D-Sorbitol	0.35 M	182.17	Powder	31.88 g	53.76 g
	PVP-40	1% (w/v)	40,000	Powder	5.0 g	10.0 g
	EGTA	6 mM	380.35	Powder	1.14 g	2.28 g
	Ultra-pure H ₂ O	-	-	-	100 mL	200 mL
	Ensure powders are dissolved by thorough homogenisation					
	Tris pH 8.0	50 mM	157.60	2 M	12.5 mL	25 mL
	KCl, pH 7.4	50 mM	74.5513	1 M	25 mL	50 mL
	EDTA pH 8.0	10 mM	292.24	0.1 M	50 mL	100 mL
	Filter sterilise through a 0.45 µm filter into an autoclaved duran bottle					
	Ultra-pure	-	-	-	(Up to 500 mL)	(Up to 1 L)

Reagent	Target concentration	Molecular weight (g/mol)	Stock concentration	Input from stock (500 mL total)	Input from stock (1 L total)
H2O					
Store stock at 4°C for up to 3 months					

Below recipes should be prepared as required during the protocol

Sorbitol Homogenisation Wash Buffer (SHWB)

Reagent	Target concentration	Molecular weight (g/mol)	Stock concentration	From stock to 50 mL total	From stock to 100 mL total
Sorbitol buffer base solution (4°C)	1X	-	1X	50 mL	100 mL
DTT	3 mM	154.253	Powder	23.16 mg	46.32 mg
Ensure powders have fully dissolved before use by thorough homogenisation					
(Should be prepared fresh; store at 4°C whilst in use)					

PBS-DTT wash buffer

Reagent	Target concentration	Molecular weight (g/mol)	Stock concentration	Input from stock (25 mL total)
PBS 1X	1X	-	1X	25 mL
DTT	3 mM	154.253	Powder	11.58 mg
Ensure powder has fully dissolved before use				
(Should be prepared fresh; keep the buffer at 4°C whilst in use)				

1 mL of 'PBS wash buffer' is required per sample.

Protocols PDF:  Sanger Tree of Life HMW DNA Extr... 116.7KB

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.
- 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 an adequate volume of 'sorbitol buffer stock' has been prepared prior to starting the protocol - 3.5 mL of ready-to-use 'sorbitol wash buffer' is required per sample (2 washes of 1.75 mL).
- Ensure cryogenically disrupted plant tissue is completely disrupted into a fine powder; avoid matted/clumped powder. Complete disruption is crucial to ensure sorbitol washing performance and optimal subsequent gDNA yield and integrity; poorly disrupted tissue drastically decreases extraction efficiency and all quantifiable outcomes.
- The day before the protocol, aliquot the recommended 70 - 90 mg of cryogenically disrupted frozen tissue samples into individual, labelled 2 mL Lobind tubes on a cold block on dry ice, then store at -70°C. **(Optional)**



Protocol

- 1 Aliquot 40 - 130 mg of cryogenically disrupted frozen tissue samples into individual, labelled 2 mL Lobind tubes on a cold block on dry ice. 70 - 90 mg is generally recommended.
OPTIONAL: Doing this the day before (then storing at -70°C) saves time.
- 2 Prepare 100 mL (for 24 samples) 'SHWB', by adding 46.32 mg DTT powder to 100 mL 'Sorbitol stock buffer' (prepare in advance, recipe below), then store on wet ice.
 - Ensure the DTT has fully dissolved before use.
 - It is crucial to ensure the sorbitol wash buffer is kept at 4°C whilst in use by placing it back onto wet ice between washes. Use at room temperature may result in sample degradation.
- 3 Prepare 25 mL (24 samples) of cold 'PBS-DTT wash buffer' (recipe below), by adding 11.58 mg DTT powder (4°C), then storing on wet ice.
 - Ensure the DTT has fully dissolved before use, by 'squishing DTT blobs'.
- 4 Transfer the samples to a cold block on wet ice and incubate for 5-10 minutes.
 - Ensure a 2 mL centrifuge is pre-chilled to 4°C, in preparation for step 5.5.
- 5 Perform the sorbitol wash:
 - 5.1 Add 1.75 mL of cold sorbitol wash buffer (4°C) to all samples using a multipipette.
 - 5.2 Vortex the first sample continuously for 3 seconds at full speed until homogenous, then return it to the cold block on wet ice. Repeat for each sample.
 - 5.3 Once all samples are homogenised, inspect each by inverting to mix. Any samples with aggregated tissue that can't be suspended through vortexing should be thoroughly mixed with wide bore P1000 until fully homogeneous.
 - 5.4 Incubate the samples on the cold block on wet ice for 5 minutes.
 - 5.5 Centrifuge the samples for 5 minutes, 10,000 x rpm at 4°C.
 - 5.6 Remove the supernatant by decanting and/or pipetting with a wide bore P1000, then return the sample to a cold block on wet ice. Repeat for each sample.
 - It is essential to remove the gelatinous layer that may have formed above the pelleted tissue for select specimens. Do this by carefully aspirating with an appropriately sized wide bore tip (likely P200).
 - Avoid disturbing the pelleted tissue when aspirating.
- 6 Repeat step 5 once.
- 7 Perform the PBS wash:



- 7.1 Add 1.0 mL of cold 'PBS-DTT wash buffer' (4°C) to all samples using a multipipette.
- 7.2 Vortex the first sample continuously for 3 seconds at full speed, then return it to the cold block on wet ice. Repeat for each sample.
- 7.3 Immediately centrifuge the samples for 5 minutes, 10,000 x rpm at 4°C.
- 7.4 Remove the supernatant by decanting and/or pipetting with a wide bore P1000, then return the sample to a cold block on wet ice. Repeat for each sample.
 - Avoid disturbing the pelleted tissue when aspirating.
- 8 Proceed directly to the extraction method of choice.
 - Keep samples on a cold block on wet ice until the extraction lysis buffer is added to the pellet.

Protocol references

Peter W Inglis, Marilia de Castro R Pappas, Lucileide V Resende & Dario Grattapaglia (2018). [Fast and inexpensive protocols for consistent extraction of high quality DNA and RNA from challenging plant and fungal samples for high-throughput SNP genotyping and sequencing applications - PMC](https://doi.org/10.1186/s13052-018-0361-3)