Aug 28, 2024

Wheat leaf high-molecular weight (HMW) DNA extraction

DOI
dx.doi.org/10.17504/protocols.io.bp2l6xpy1lqe/v1

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OPEN ACCESS

DOI: dx.doi.org/10.17504/protocols.io.bp2l6xpy1lqe/v1

Protocol Citation: Maximillian RW Jones, Nikolai Adamski, Cristobal Uauy 2024. Wheat leaf high-molecular weight (HMW) DNA extraction. **protocols.io https://dx.doi.org/10.17504/protocols.io.bp2l6xpy1lqe/v1**

Protocol status: Working

Created: November 23, 2023

Last Modified: August 28, 2024

Protocol Integer ID: 91372

Keywords: HMW, high-molecular weight, DNA extraction, wheat, plant, nuclei extraction, cereal, nuclei, pacbio

Funders Acknowledgement:
UK Biotechnology and Biological Sciences Research Council (BBSRC)
Grant ID: BB/X011003/1
UK Biotechnology and Biological Sciences Research Council (BBSRC)
Grant ID: BB/X01102X/1
European Research Council
Grant ID: 866328

Disclaimer

Abstract

This protocol is for the extraction of high-molecular weight DNA from wheat leaf tissue. The product is suitable for long-read sequencing such as PacBio. The protocol may also be effective for leaves of other cereal species. For example, we have had success using it with tef (*Eragrostis tef*).
The protocol consists of four phases; 1) buffer preparation, 2) harvesting of leaf tissue, 3) crude nuclei extraction, and 4) HMW extraction. The last of these uses the PacBio Nanobind® plant nuclei kit.
Using this protocol we have produced good quality DNA extractions with the following features:

- Fragment size distribution peak >50 kb (Agilent Tapestation, Genomic DNA ScreenTape)
- Concentration between 350 and 600 ng / µl (Invitrogen Qubit 3 fluorometer)
- 260/280 ratio 1.75-1.90 and 260/230 ratio 1.75-1.90 (Thermo Scientific Nanodrop)

This protocol was originally adapted from Dvorak et al., 1988, but has been modified over many years.

Materials

Buffer and filter preparation

Equipment

- Precision balance
- Magnetic stirrer and flea(s)
- pH meter
- Scissors
- Permanent marker pen

Consumables / reagents

- Spermine (powder)
- Spermidine (neat liquid)
- EDTA
- Tris-HCl pH 8.0 solution
- KCl
- 10 M NaOH
- Sucrose crystals/powder
- Triton X-100
- Miracloth
- Cheesecloth

Tissue collection

Equipment

- Scissors
- Forceps
- Dewar containing liquid nitrogen (if freezing tissue)
- Permanent marker pen
- Balance (0.1 g precision)

Consumables/reagents

- 50 mL Falcon tubes (or equivalent)
- 70% ethanol in spray bottle
- Paper towels

Nuclei extraction

Equipment

- Fume hood
- Small food processor or jug blender (*not* a hand/stick blender)
- Three or four 2 L buckets of ice
- P1000 pipette
- P5000 pipette
- 250 mL centrifuge bottles
- Large plastic funnel
- 100 mL measuring cylinder
- Glass rod or blunt forceps
- Scissors

- Small paint brushes (<1/4 width of a microfuge tube)
- Cup of soapy water for used paint brushes
- Timer
- Two 1 L waste beakers
- Chemical sharps bucket
- Solid chemical waste bin
- Permanent marker pen

Consumables / reagents

- β-mercaptoethanol
- 70% ethanol in spray bottle
- 2 mL Eppendorf Protein LoBind tubes (catalog no. 0030108132 or 0030108450)
- 1000 µL tips
- 5000 µL tips
- Paper towels

HMW DNA extraction

Equipment

- Magnetic tube rack
- Vortexer
- Tabletop/mini centrifuge
- Micro centrifuge
- Heat block or water bath
- Microvolume spectrophotometer (e.g. Thermo Fisher Nanodrop)
- P200 pipette
- P1000 pipette
- Chemical sharps bucket
- Permanent marker pen

Consumables / reagents

- PacBio Nanobind® plant nuclei kit (SKU 102-302-000, previously NB-900-801-01)
- 2 mL Eppendorf Protein LoBind tubes (catalog no. 0030108132 or 0030108450)
- 100% isopropanol (2-propanol)
- 96-100% ethanol
- 200 µL nuclease-free tips
- 1000 µL nuclease-free tips

Prepare **10x HB**

	Reagent	Final conc.	e.g. for 1 L
	spermine crystals	10 mM	3.425 g
	spermidine liquid	10 mM	2.545 g
	EDTA	100 mM	200 mL of 0.5 M solution
	Tris-HCl pH 8.0 solution	100 mM	100 mL of 1.0 M solution
	KCl	800 mM	267 mL of 3.0 M solution

- Dissolve / mix above components into 75% desired final volume of deionised H_2O

- Adjust pH to 9.4-9.5 with 10 M NaOH
- Fill to final volume with deionised H_2O
- Store at 4 °C for up to 6 months

Prepare **1x HB**

	Reagent	Final conc.	e.g. for 2 L (~12 samples)
	10x HB	1x	200 mL
	sucrose	500 mM	342.3 g

- Dilute 10x HB 10-fold with deionised water
- Dissolve sucrose via magnetic stirrer
- Store at 4 °C for up to 3 months

Prepare **H+20 buffer**

	Reagent	Final conc.	e.g. for 500 mL (~100 samples)
	10x HB	1x	50 mL
	sucrose	500 mM	85.6 g
	1x Triton X-100	~0.2x	100 mL

- Dilute 10x HB to 3/5 final volume
- Dissolve sucrose via magnetic stirrer
- Top up to 4/5 final volume with de-ionised water
- Reduce stirring speed and add Triton X-100 slowly until final volume is reached. Slow addition and slow stirring is necessary to prevent excessive foaming.
- Store at 4 °C for up to 6 months

Prepare **filtration sandwiches**

Prepare squares of Miracloth sandwiched between two sheets of cheesecloth. These should be large enough to line your plastic funnel.

Safety warnings

β-mercaptoethanol: can be toxic if ingested, and fatal if inhaled or absorbed through the skin. Vapours can irritate the eyes, mucous membranes, and respiratory tract. Symptoms of inhalation exposure may include coughing, sore throat, and/or shortness of breath. Work under a fume hood where indicated in the protocol.

Before start

Please see the Materials section for instructions on buffer preparation, storage conditions, and longevity.
We highly recommend you read through the whole protocol before attempting it as certain steps are complex and time-critical.

Tissue collection

1 Sanitise scissors and/or other tools, plus gloved hands, with 70% ethanol. Collect 2-5 g leaf blade tissue into a 50 mL Falcon tube. Do not use leaf sheath tissue. Avoid using diseased, bleached, or senescing sections. According to PacBio, younger leaves generally produces higher DNA quality and quantity. Avoid bruising tissue as much as possible. Using forceps may help with this. Re-sanitise tools and hands between samples.

1.1 If conducting nuclei extraction immediately, bury Falcon tube up to cap in ice.

1.2 If conducting nuclei extraction at a later date, snap freeze the Falcon tube in liquid nitrogen then store at -70 to -80 °C.

Crude nuclei extraction

2 Set up fume hood with the following:

- Blender (~1 L volume, preferably glass-walled as some plastics may react with β-mercaptoethanol)
- Tissue samples on ice
- **1xHB** on ice (100 mL per sample)
- **H+20 buffer** on ice (5 mL per sample)
- β-mercaptoethanol (400 µL per sample)
- 250 mL conical centrifuge bombs on ice (1 per sample) (e.g. Corning CLS430776) (do *not* use
  flat-bottomed centrifuge bottles)
- Mira/cheese-cloth **filtration sandwiches** (1 per sample)
- Large plastic funnel
- 100 mL graduated cylinder
- Glass rod
- Scissors
- Timer
- 70% ethanol for cleaning
- Blue roll
- 1 L waste beaker

If using frozen tissue, finish preparing the fume hood before removing samples from freezer.

3 Set up centrifuge with correct rotor and swing bucket attachments and begin cooling to 4 °C. Place 5 mL of **H+20 buffer** into a 250 mL centrifuge bottle per sample. Label accordingly. Place
on ice in an ordered manner.

4 Working in the fume hood, add 640 µL β-mercaptoethanol to 160 mL of **1xHBS** for each sample and mix well. This mixture is referred to as **1xHB/β-ME** for the remainder of this protocol.

5 Place funnel into one of the 250 mL centrifuge bombs, still on ice. Insert the Mira/cheese-cloth sandwich and push down with the glass rod or gloved finger.

6 Measure out 100 mL **1xHB/β-ME** into the blender (for 2-5 g tissue - if using more tissue increase to 150 mL)

7 Cut plant tissue into small pieces directly into the blender. Start blending on a low setting until the plant tissue is incorporated into suspension. Then blend on high for one minute.

8 Filter the ground sample through the **filtration sandwich** into the centrifuge bomb. While this is dripping through, place blender under a fast-flowing tap, being sure to also rinse the lid. After filtration is complete, squeeze out remaining sample liquid from cloth into the waste beaker and discard cloth into a chemical waste bin. Thoroughly rinse the funnel and your gloved hands. Wipe scissor blades with a paper towel soaked in 70% ethanol. Cap the centrifuge bomb, gently shake, and place back on ice.

9 Repeat until all samples done. Samples should be incubated on ice for at least 10 minutes before moving onto **Step 14**.

10 Spin samples for 20 minutes at 1500 x g at 4 °C. If necessary for balance, adjust volumes with iced **1xHB/β-ME** and/or use H_2O-filled balance bombs.

10.1 While samples are spinning, tidy the fume hood and re-prepare it with the following:
- **1xHB/β-ME** on ice
- Additional ice buckets with enough space for all samples
- Five-layer stacks of paper towels for blotting sample bottles
- Small paint brushes soaking in **1xHB**
- P1000 pipette
- 1000 µL tips with cut ends
- 1 L waste beaker

11 For each sample bottle, carefully pour off the supernatant into the waste bucket, then invert onto the stacked paper towels to remove excess liquid.

12 Add 1 mL of ice-cold **1xHB/β-ME** to each centrifuge bottle. Use a clean paint brush (soaked in 1xHB) to resuspend the pellet. Next, mix using a P1000 pipette *using a cut-tip* until solution is homogeneous. Transfer sample to a 50 mL pre-labelled Oakridge tube.

13 Bring the volume of each sample to approximately 15 mL using ice-cold **1xHB/β-ME.** Spin for 10 min at 1100 x g at 4°C.

14 Repeat **Steps 15 to 17** twice more. The supernatant should be transparent after the last centrifugation. The same paint brush can be re-used for the same sample.

15 Decant the supernatant into the waste beaker and add 1 mL of ice-cold **1XHB** to the pellet *per gram of tissue* being processed (e.g. 3 mL for 3 g tissue).

16 Resuspend the pellet using a fresh paint brush pre-soaked in ice-cold **1XHB**. Mix with a P1000 pipette *using a cut-tip* until the solution is homogeneous.

17 Transfer each mL of the nuclei suspension into a fresh 2 mL Protein LoBind microcentrifuge tube (equivalent to 1 g tissue/tube).

18 Spin the 2 mL tubes for 5 min at 7,000 x g at room temperature.

19 Either proceed directly to **Step 24**, or snap freeze the nuclei pellets in liquid nitrogen and store at -70 to -80 °C to continue the protocol at a later date. We have only tested using frozen nuclei pellets up to 1 week old, but they may be viable for longer.

HMW DNA extraction

20 For the extraction of HMW DNA from the nuclei prep, we utilised PacBio's Nanobind® plant nuclei kit and protocol with one minor modification.
Overview:
https://www.pacb.com/wp-content/uploads/Guide-overview-Nanobind-plant-nuclei-kit.pdf
Protocol:
https://www.pacb.com/wp-content/uploads/Procedure-checklist-Extracting-HMW-DNA-from-plant-nuclei-using-Nanobind-kits.pdf
Modifications:

- Use 2 mL rather than 1.5 mL Protein LoBind tubes. The more rounded bottom prevents the Nanobind disks becoming lodged.

Protocol references

Jan Dvorak, Patrick E. McGuire, and Brandt Cassidy. 1988. Apparent sources of the A genomes of wheats inferred from polymorphism in abundance and restriction fragment length of repeated nucleotide sequences. *Genome*. **30**(5): 680-689. https://doi.org/10.1139/g88-115