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Workflow for generating HMW plant DNA for third generation sequencing with high N50 and high accuracy

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1 Works for me dx.doi.org/10.17504/protocols.io.bafmibk6

High molecular weight DNA extraction from all kingdoms
Tech. support email: See@each.protocol

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ABSTRACT

We have developed a method based on the Qiagen Genomics-Tip kit for the extraction of HMW DNA from a wide range of plants suitable for third generation sequencing platforms such as PacBio and Oxford Nanopore. This HMW plant DNA was used to make sequencing libraries with high insert N50 (30-40 Kb) for GridION and PromethION Oxford Nanopore machines and Sequel I and Sequel II PacBio machines. This HMW DNA is also suitable for PacBio HiFi libraries. The resulting sequencing data is ideally suited for de novo assembly of plant genomes.

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KEYWORDS

plant, high molecular weight DNA, third generation sequencing, PacBio, Oxford Nanopore, HiFi, CLR, Genome Assembly

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GUIDELINES

Liquid nitrogen is used for grinding samples for DNA. Take the necessary precautions and training when using liquid nitrogen. Appropriate personal protective equipment is essential when working with liquid nitrogen including lab coat, safety glasses and suitable gloves.

MATERIALS TEXT

MATERIALS

RNase A **Qiagen Catalog #19101**

liquid nitrogen **Contributed by users**

Fresh 80% Ethanol **Contributed by users** In 2 steps

Qubit™ dsDNA BR Assay Kit **Thermo Fisher Scientific Catalog #Q32853** Step 26

Proteinase K **Qiagen Catalog #19131** Step 7

2-Propanol **Sigma Aldrich Catalog #190764** Step 18

Coors™ porcelain mortar Coorstek 60316 capacity 145 mL O.D. × H 90 mm × 70 mm **Sigma Aldrich Catalog #Z247472**

QIAGEN Genomic-tip 100/G **Qiagen Catalog #10243**

QIAGEN Genomic-tip 500/G **Qiagen Catalog #10262**

Genomic DNA Buffer Set **Qiagen Catalog #19060**

TE buffer pH 8.0 **Contributed by users** In 2 steps

Lysis Buffer:

- per 100/G column: 9.5 ml Buffer G2 + 19 µl RNase A (100 mg/ml) in a 50 ml tube
- per 500/G column: 19 ml Buffer G2 + 38 µl RNase A (100 mg/ml) in a 50 ml tube

Alternative suppliers can be used. For example, other sources of proteinase K can be used, such as from Macherey-Nagel (catalog # 740506, Duren, Germany) as long as the stock solution is made to the same concentration (20mg/ml; reconstituted with 3.75ml PB)

Sample Preparation 20m

1 ⚠

For best results use leaves that have been dark-treated for > 48 hours, freshly picked, weighed, flash frozen in liquid nitrogen and stored at -80°C

- **Our empirical experience with non-dark treated leaves have resulted in lower DNA yields and shorter sequencing N50 lengths. As a result of this we recommend dark-treated leaves for optimal results.**

2 In a tray add excess liquid nitrogen to mortar and pestle. Allow most of the liquid nitrogen to evaporate and bring mortar and pestle down to cryogenic temperatures. 10m

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3 Grind > 1 g of frozen leaves to a very fine powder in mortar and pestle. 10m

- Add small amounts of liquid nitrogen, as necessary, to maintain sample, and mortar and pestle at cryogenic temperatures.
- Resuspending powder in a small amount of liquid nitrogen is also very effective for pouring and transferring samples to storage tubes.

4 Process sample straight away using below protocol or return to -80°C for storage.

DNA Extraction: Preparation 10m

5 The following protocol is based on the "QIAGEN Genomic DNA Handbook" available at www.qiagen.com. We follow the recommendations for tissue with modifications.

For 1 g of ground leaf tissue prepare enough **Lysis Buffer** for 4 x 100/G, or 1-2 x 500/G tip columns.

- The suitable number of columns per gram of tissue is plant species-dependent. The optimal loading for the best yield should be determined for each species taking care to not to over-load the columns.

6 Add the **Lysis buffer** to the ground powder depending on quantity and type of columns to be used for purification.

- Resuspend by inverting gently end-over-end
- Dispense to multiple tubes if necessary.

7 Add **Proteinase K**

Proteinase K **Qiagen Catalog #19131**

- **500 µl** per **100/G** column **or** **1000 µl** per **500/G** column
- Tighten cap, cover with Parafilm, and mix by inversion.

DNA extraction: Lysis 3h 30m

8 Incubate > 3.5 h at 50°C with slow rocking. Place the tube with a slight tilt so that the liquid moves very slightly during rotations. 3h 30m

50 °C **03:30:00**

- *Note: The tube can easily leak, check the tube condition periodically*
- We use a platform rocker in a preheated incubator/shaker (with NO SHAKING)

DNA Extraction: Pre-Purification

9 Preheat buffer **QF** in falcon tube in a **50 °C** waterbath:

- **5 mL** per **100/G** column or
- **15 mL** per **500/G** column

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DNA Extraction: Purification

10 Take the lysed samples from the incubator

11 Centrifuge the 50ml tubes containing the samples for 15 minutes at room temp at maximum speed (3220g / 4000 rpm) with acceleration and deceleration at maximum (=9)

3220 x g, Room temperature , 00:15:00

- *After centrifugation minimal debris should be in the supernatant*
- *If large amounts of debris still present repeat centrifugation up to 15 min*

12 **Calibrate** the **100/G** or **500/G** columns

- Put the column inside the placolumneeve on top of a 50ml Falcon tube (see figure in Supplementary section)
- Calibrate the column with the calibration buffer **QBT**:

- Add **4 mL** of buffer **QBT** per **100/G** column
- Add **10 mL** of buffer **QBT** per **500/G** column
- All QBT needs to go through the column. *This step is quite quick*

13 Pour the supernatant in the pre-calibrated Genomic tip column and allow the supernatant to pass through the column.

- This is a slow step, it takes about on average 10-20 mins for the supernatant to elute through the column

14 Add 1 ml of wash buffer **QC** by running along the walls of the tubes in order to precipitate any debris stuck on the walls. Remove the debris from the bottom by aspirating

- If debris still present wash with another **1 mL** of **QC** buffer

15 Wash the column twice with buffer **QC**:

- 2 x **7.5 mL** of buffer QC **100/G** column.
- 2 x **15 mL** of buffer QC **500/G** column.

- *This can take at least 15 mins for each wash*

16 Transfer the column to a clean 50 ml Falcon tube.

17 Elute with elution buffer **QF** previously pre-heated at 50°C

- **5 mL** of buffer **QF** per **100/G** column
- **15 mL** of buffer **QF** per **500/G** column
- *This step usually takes 15 min but can take up to 1 hour per sample*

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18 Remove column and precipitate the DNA by adding **2-propanol** (isopropanol) along the wall of the tube:

2-Propanol **Sigma Aldrich Catalog #190764**

- **3.5 mL** per **100/G** column
- **10.5 mL** per 5**00/G** column

19 Mix gently by slowly inverting the tube to form a visible mass of **HMW DNA** floating in solution **(**a **"jellyfish"**)

- You should observe the formation of the jellyfish during the process of inverting the tubes.
- Once the pellet HMW DNA is compacted slightly, stop mixing.

- *Note*: If the **jellyfish is not formed**, proceed to **Precipitation of DNA with cold centrifugation** (see below).
-

20 Capture the pellet HMW DNA with a disposable inoculation loop.

- This can be difficult and the pellet can easily be lost during transfers between tubes
- Try to trap the DNA pellet in the centre of the loop.

21 Transfer the DNA on the loop to a 1.5ml tube containing **1 mL** of fresh **80% ethanol.**

Fresh 80% Ethanol **Contributed by users**

- This step should remove starch and improve the quality of the DNA.
- **Use separate tubes of ethanol for different samples**

21.1 Soak for 1 min

21.2 Air dry for 30 seconds approximately

21.3 Repeat twice more in two separate tubes of **80% ethanol**

22 Air dry the DNA pellet for 30-60 seconds allowing most of the ethanol to evaporate

23 Dissolve DNA pellet in **50 µl** to **100 µl** of **TE buffer** in a new 1.5ml tube.

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TE buffer pH 8.0 **Contributed by users**

- Cut off the end of the loop with scissors
- Volume of TE can be modified according to the amount of estimated DNA (size of the pellet)
- Several pellets can be combined

24 Leave overnight at room temperature to allow resuspension of the DNA.

Overnight

Day 2: Quantification

25 Remove loop and carefully homogenize the DNA solution with **wide bore tips**. Use slow aspiration and discharge: 3 seconds up and 3 seconds down.

- DNA should be into solution; look for a clear solution
- If DNA is still not resuspended continue with resuspension
- Resuspension on a slow shaker or incubation at 4°C
- **Don't apply heat** (even 37°C can degrade HMW DNA)

26 Quantify the DNA and check the quality

- Use ds BR Qubit assay for DNA quantification

Qubit™ dsDNA BR Assay Kit **Thermo Fisher Scientific Catalog #Q32853**

- Use Nanodrop to check the quality:
- A 260/280 ratio of 1.8-2.0 and 260/230 ratio of 2.0-2.2 is ideal
- This measures contamination with carbohydrates and other contaminants. The 260/230 ratio must be > 2 as sugars disrupt the polymerase during sequencing.

- If 260/230 is low proceed with a gentle AMPure PB Bead clean and re-do the Nanodrop. Only very high molecular weight DNA is lost.

27 Run Pulse Field Gel Electrophoresis or FEMTO Pulse analysis of your DNA to check the size distribution of DNA.

27.1 Pulse Field Gel Electrophoresis

- 1% agarose TBE gel over 24 hours (Initial switch 1 sec, finial switch 25 sec, 6 volt/cm, 120° included angle, Chef III, Bio-Rad, Hercules, CA, USA)
- Midrange PFG and Lambda PFG markers (N0342S, N0341S, NEB, Ipswich, MA, USA).

27.2 FEMTO Pulse analysis

- gDNA 165 kb kit (FP-1002-0275) on the Femto Pulse system (Agilent, Santa Clara, CA, USA).

Precipitation of DNA with cold centrifugation **(Optional)** 25m

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28 Note: If a **jellyfish of DNA was not present** after addition of 2-propanol **go to step #19** **this may indicate a poor quality extraction**, dependent on species of plant, and a re-extraction may be appropriate.

29 Prechill a high speed centrifuge to **4 °C**

30 Centrifuge the samples 15m
5000 x g, 4°C, 00:15:00

31 Remove the supernatant and wash with **1 mL** 80% ethanol.

Fresh 80% Ethanol **Contributed by users**

31.1 Carefully pour off supernatant, **do not re-suspend.** Drain at 45° on a paper towel to dry.

31.2 Wash two more times.

32 Centrifuge tha samples 10m
5000 x g, 4°C, 00:10:00

32.1 Remove the supernatant and dry

32.2 Drain on paper towel at 45°

33 Resuspend in **TE buffer**

TE buffer pH 8.0 **Contributed by users**

Continue with resuspension and quality control of extracted DNA **go to step #24**

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