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High Molecular Weight DNA extraction for long-read sequencing v.1

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We use this protocol and it's working

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Abstract

Third generation long-read sequencing requires high quality DNA. Here, we developed a protocol for the extraction of high quality, high molecular weight(HMW) DNA from plants. This protocol was developed and optimized forStreptocarpus, a plant possessing high amounts of secondary metabolic compounds, such as polysaccharides. The extracted DNA was used for PacBio Sequel II continuous long reads (CLR) and Oxford Nanopore Technologies PromethION sequencing with the resulting reads averaging > 25,000 base-pairs.

References

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Souza HA, Muller LA, Brandao RL, Lovato MB (2012) Isolation of high quality and polysaccharide-free DNA from leaves of *Dimorphandra mollis* (Leguminosae), a tree from the Brazilian Cerrado. Genet Mol Res 11: 756-764

Guidelines

This is the protocol used for *Streptocarpus* long-read whole genome sequencing with PromethION (Oxford Nanopore Technologies) and Sequel II CLR (Pacific Biosciences).

The protocol described below is optimized for 30 g of starting material (*i.e.* fresh leaves) of *Streptocarpus*.

Materials

MATERIALS

- Proteinase K **Qiagen Catalog #**19131
- RNaseA **Thermo Fisher Catalog #**12091021
- Genomic-tip 100/G **Qiagen Catalog #**10243
- RNaseA/T1 Mix **Thermo Fisher Catalog #**EN0551
- Buffer G2 **Qiagen Catalog #**1014636
- Buffer QBT **Qiagen Catalog #**19054
- Buffer QC **Qiagen Catalog #**19055
- Buffer QF **Qiagen Catalog #**19056
- Cell strainer 40 µm **Corning Catalog #**431750
- Cell strainer 100 µm **Corning Catalog #**431752

*See above for specific materials` details

Equipments

Fume hood
Water bath
Heat block
Centrifuges for 50 ml Falcon tubes
Centrifuges for 1.5 ml and 2.0 ml Eppendorf tubes
Spectrophotometer: Nanodrop or DeNovix
Fluorometer: Qubit or DeNovix
Pipetman (1 ml, 200 µl, 20 µl)
500 ml glass beakers
Pestle and mortar (1 set per 1-2 g of fresh leaf material)

Reagents & Consumables

50 ml Falcon tube
15 ml Falcon tube
2 ml Eppendorf tube
1.5 ml Eppendorf tube
2 ml LoBind Eppendorf tube

1ml Pipetman tips
1ml Wide orifice tips (or cut off tip ends)
200 µl Pipetman tips
20 µl Pipetman tips

Nylon mesh 100 µl mesh size (e.g. Corning cell strainer*)
Nylon mesh 40 µl mesh size (e.g. Corning cell strainer*)

QIAGEN Genomic-tip 100/G columns*
QIAGEN Buffer G2*
QIAGEN Buffer QBT*
QIAGEN Buffer QC*
QIAGEN Buffer QF*
Nuclei isolation buffer (NIB) base solution[1]
Nuclei isolation buffer (NIB)[2]
NIB base-Triton mix (10% Triton X-100 in NIB base solution)[3]
Sorbitol buffer base solution (2 ×)[4]
Sorbitol buffer[5]
Proteinase K*
RNaseA[6] or RNase A/T1 Mix*
Liquid Nitrogen
Isopropanol
3 M NaOAc (pH 5.2)
70% ethanol
Polyvinyl polypyrrolidone (PVPP)
PVP-40
β-Mercaptoethanol
4 M spermidine (store at −20 °C)[7]
1 M Tris-HCl pH 8.0
500 mM EDTA pH 8.0
Sucrose
KCl

1. Nuclei isolation buffer (NIB) base solution
Final concentrationsof NIB base solution

Tris-HCl pH 8.0	10 mM
EDTA pH 8.0	10 mM
KCl	100 mM
Sucrose	500 mM

To prepare 1 L stock of NIB base solution:
Add the chemicals below in >1 L autoclavable bottle.

10 ml	1 M Tris-HCl pH 8.0
20 ml	500 mM EDTA pH 8.0
7.4 g	KCl
172 g	Sucrose

Add distilled water to makeup 1 L

Mix until sucrose and KCl are dissolved
Autoclave and store at 4 ºC

2. Nuclei isolation buffer (NIB)
Note: Prepare 200 ml NIB fresh on the day of DNA extraction

Final concentrations of NIB

Tris-HCl pH 8.0	10 mM
EDTA pH 8.0	10 mM
KCl	100 mM
Sucrose	500 mM
Spermidine	4 mM
β-mercaptoethanol	0.1%

To prepare 200 ml NIB
Pre-cool a 500ml beaker on ice and add:

200 ml	NIB base solution
200 µl	β-mercaptoethanol
200 µl	4 M spermidine

Keep NIB cooled on ice.

3. NIB base-Triton mix (10% Triton X-100 in NIB base solution)
In a 50 ml Falcon tube, add:

5 ml	Triton X-100
45 ml	NIB base solution

Gently mix
It can be stored at 4 ºC
Note: Pour Triton X-100, as it is a sticky liquid and pipetting does not work.

4. Sorbitol buffer base solution (2 × stock)
Final concentrations of 2 × Sorbitol buffer base solution

Tris-HCl pH 8.0	200 mM
EDTA pH 8.0	10 mM
Sorbitol	700 mM

To prepare 500 ml of Sorbitol buffer base solution (2 × stock)
Ina 500 ml autoclavable glass bottle, add:

100 ml	1 M Tris-HCl pH 8.0
20 ml	500 mM EDTA pH 8.0
128 g	Sorbitol

Add distilled water to makeup 1 L
Mix to dissolve sorbitol
Autoclave and store at 4 °C

5. Sorbitol buffer
Final concentrations of Sorbitol buffer

Tris-HCl pH 8.0	100 mM
EDTA pH 8.0	5 mM
Sorbitol	350 mM
β-mercaptoethanol	1%
PVP-40	1%

Prepare 60 ml Sorbitol buffer on the day of DNA extraction

To prepare 60 ml Sorbitol buffer
Prepare **two** 50 ml Falcon tubes in a rack and add the following chemicals in each tube:

15 ml	Sorbitol buffer base solution
300 µl	β-mercaptoethanol
0.3 g	PVP-40

Add distilled water to makeup 30 ml

Note: Increasing the concentration of Sorbitol (100 mM Tris-HCl pH8.0; 5 mM EDTA pH8.0; 700 mM sorbitol) worked well for *Streptocarpus*.

Reference: **Souza HA, Muller LA, Brandao RL, Lovato MB** (2012) Isolation of high quality and polysaccharide-free DNA from leaves of *Dimorphandra mollis* (Leguminosae), a tree from the Brazilian Cerrado. Genet Mol Res 11: 756-764

6. RNase A
We recommend to incubate RNase A at 95 °C for 5 minutes in a thermocycler prior to use.

7. 4 M spermidine
Elute spermidine powder in distilled water to makea4 M solution. The molecular weight of spermidine is 145.25 g/mol and thus *e.g.* dissolve 5 g spermidine powder in approx. 8.6 ml distilled water. Divide dissolved spermidine solution into several 1.5 ml Eppendorf tubes. Store the tubes at −20 °C.

Safety warnings

Perform experiment in the fume hood for the steps using liquid nitrogen and β-mercaptoethanol.

Before start

Prepare Nuclei isolation buffer base solution and Sorbitol buffer base solution.
Cultivating fresh plant material > 100 g is recommended.

Before starting

1 Prepare 200 ml Nuclei isolation buffer (NIB) and leave on ice.
On ice

2 Prepare 20 ml NIB base-Triton mix and leave on ice.
On ice

3 Prepare 60 ml Sorbitol buffer.

4 Prepare liquid nitrogen in a suitable dewar. Place mortar and pestle in fume hood.

5 Prepare six 50 ml Falcon tubes on ice and place 100 µm gauge cell strainers (Nylon Meshes) on top.
On ice

6 Prepare the other set of six 50 ml Falcon tube layered with 40 µm gauge cell strainers, leave on ice.
On ice

Tissue grinding

7 Pre-cool the pestle and mortar with liquid nitrogen.

8 Cut 1-2g of fresh leaf tissue from living plants and place in liquid nitrogen to shock-freeze.

9 Add frozen tissue to pre-cooled mortar held in a Styrofoam mortar holder.

10 Add some liquid nitrogen and grind carefully with pestle as the liquid nitrogen evaporates.
Once the liquid nitrogen has evaporated, grind more vigorously for 30 seconds, until the material is a fine powder.

Do not allow the tissue powder to thaw.

11 Add liquid nitrogen (enough to cover the tissue) and repeat step10 two more times.



Fig. 1. One gram of leaf sample in mortar with pestle (top-left) and ground with liquid nitrogen (top-right). Home-made mortar holder for liquid nitrogen grinding, made from a Styrofoam block (bottom).

12 Transfer the ground tissue to a beaker of cold NIB placed on ice with a pre-cooled spatula and mix by gently swirling (Fig. 2). Keep on ice.

On ice



Fig. 2. Ground leaf material in NIB

13 Repeat the above steps 7 to 12 until all the leaf tissue is ground. The suspension should be smooth with no clumps of leaf material.

Pre-treatment

14 Filtering to remove particulate material. Proceed steps on ice.

On ice

14.1 Filter the NIB/sample suspension through 100 µm nylon mesh filter by gravity (see step 5). Do not force the sample through the filter. Replace filters when it is clogged.

On ice

14.2 Filter the 100 µm mesh filtrate through the 40 µm nylon mesh (see step 6). Replace filters when it is clogged.

On ice

14.3 Divide the filtrate of step 14.2 equally into six Falcon tubes, with 40– 50 ml in each.

On ice

15 Add 1/20th volume of NIB base-Triton mix, close the tubes with lids and mix the solution by gently swirling and inverting the tubes.

16 Centrifuge at 2,000 × *g* for 10 minutes, if possible at 4 °C.

2000 x g, 00:10:00

17 Carefully discard supernatant without dislodging the pellet.

18 Add 10 ml Sorbitol buffer to the pellet in each tube and swirl the tubes to mix well.

19 Centrifuge at 3,000 × *g* for 10 minutes, if possible at 4 °C.

3000 x g, 00:10:00



Fig. 3. Centrifuged sample after Sorbitol buffer step.
Sample contains a small amount of intact cells (green) and a large amount of nuclei (white).

20 Discard supernatant leaving the pellet undisturbed. Invert the tubes on tissue to remove traces of buffer without losing the pellet.

Note

The nuclei pellet can now be stored at −80 °C, but this is not recommended.

Note

The pellet might include a small amount of intact cells smaller than 40 µm. Intact cells appear as the greenish in the pellet.

Cell Lysis

21 Set water bath to 50 °C.
50 °C

22 Add 5 ml of QIAGEN Buffer G2 to each 50 ml Falcon tube containing the nuclei/ cells and gently resuspend the pellet.

Note

The amount of QIAGEN Buffer G2 can be reduced or increased depending on the size of the pellet. Different plant species or tissues may yield different amounts of nuclei/ cells.

23 Add 0.1% β-mercaptoethanol (5 µl) and a pinch of PVPP to the tube.
Swirl the tube to mix well.

24 Incubate the tubes at 50 °C for 5 minutes.
50 °C

25 Add 10 µl RNase A or 50 µl RNase A/T1 Mix to the tube.
Gently swirl the tubes to mix.

26 Incubate the tubes at 50 °C for 5 minutes for RNase A, or 30 minutes for RNase A/T1 Mix.
50 °C

27 Add 100 µl QIAGEN Proteinase K to the tube.
Swirl the tubes to mix well.

28 Incubate the tubes at 50 °C in the water bath, for > 5 hours or overnight.
50 °C

Genomic-tip

29 Set up for QIAGEN Genomic-tip 100/G.

Note

Following the protocol from page 49 of the QIAGEN Genomic DNA Handbook 06/2015.

At this stage in the protocol you will have approx. 30 ml of lysate (six tubes of approx. 5 ml lysate, obtained from 30 g of fresh leaf material ground in 200 ml NIB). It is better not to apply more than 7 ml of lysate to a Genomic-tip 100/G column, since the lysate also contains contaminants and thus an excess amount of contaminants can result in poor DNA quality. Therefore, there are options, to either a) re-using the Genomic-tip column to process multiples of 5 ml lysate from the same sample, or b) use a separate Genomic-tip column for each subsample. For speed and cost savings we use a combination of the two options– two Genomic-tips per sample, reused twice.

29.1 Set up Genomic-tip kit: Label 5 × 50 ml Falcon tubes without lids as follows: **1.** QBT, **2.** Sample, **3.** QC 1, **4.** QC 2,
5. Final sample.

Note

Use a new Falcon tube for '**5.** Final sample', the others can be washed, reused tubes.

Note

Optional: Save all the crude extracts and washed-through filtrates and analyze them by electrophoresis to assess the performance of each Genomic-tip step.

29.2 Pre-heat Buffer QF to 50 °C in the water bath.

50 °C

30 To obtain the clear lysate, centrifuge the 50 ml Falcon tubes containing nuclei and cell lysate from step 28 at 3,000 × *g* for 10 minutes.

3000 x g, 00:10:00

31

Equilibrating a Genomic-tip 100/G column.

31.1 Place the column mounted in the column holder on top of the tube labelled '**1.** QBT' (see Fig.4).



Fig. 4. Samples applied to a QIAGEN Genomic-tip 100/G column

31.2 Add 4 ml Buffer QBT to the column.
Allow the column to empty by gravity flow, do not force the remaining buffer through.

32 Move the column to the '**2.** Sample' tube and apply 5 ml of the clear lysate (supernatant obtained at step 30) to the equilibrated Genomic-tip.
Allow the column to empty by gravity flow.

33 Wash Genomic-tip 100/G columns with Buffer QC .

33.1 Move the column to the '**3.** QC 1' tube and add 7.5 ml of Buffer QC.
Allow Buffer QC to move through the column by gravity flow.

33.2 Move the column to the '**4.** QC 2' tube and add 7.5 ml Buffer QC.
Allow Buffer QC to move through the column by gravity flow.

Note

Traces of Buffer QC in the column will not affect the elution step.

34 Eluting the genomic DNA from the Genomic-tip 100/G column.

34.1 Move the column to the '**5.** Final Sample' tube and add 5 ml of Buffer QF pre-warmed to 50 °C.

34.2 Allow Buffer QF to flow through the column by gravity.
Store the eluted DNA sample on ice.

Note

The Genomic-tip columns can be reused for the same sample. Simply repeat from step 31– step 34 with the used column. The columns are stable for up to six hours from first equilibration. Do not dry columns.

DNA precipitation & elution

35 Transfer the DNA sample obtained at step 34 to a 2 ml Eppendorf tube.
Add 1 ml sample per tube (totaling 30 tubes from 30 ml elution buffer [5 ml Buffer QF × 6 tubes] at the step 34 in our example).

36 Add 700 µl of ice-cold isopropanol to each tube and gently invert the tubes to mix.

37 Leave samples at −20 °C for > 2 hours or overnight.
-20 °C

38 Centrifuge tubes at 11,000 rpm for 10 minutes.
11000 rpm, 00:10:00

39 Discard the supernatant.

Note

It is recommended to retain the supernatant until the safe recovery of the DNA pellet has been verified.

40 Add 400 µl ice cold 70% ethanol to the tubes.

41 Centrifuge the tubes at 11,000 rpm for 10 minutes.
11000 rpm, 00:10:00

42 Discard the supernatant by tipping the tube, with the pellet on the upper side, over a clean glass beaker or a Falcon tube.

Note

It is recommended to retain the supernatant until the safe recovery of the DNA pellet has been verified.

Note

Isopropanol pellets have a glassy appearance and may be difficult to see – often they appear (if at all!) as very fine small lines on the side of the tube. To better locate the pellet on the hinge side of the tube, place the tube with the hinge facing outwards in the centrifuge.
Isopropanol pellets are also more loosely attached to the side of the tube, so care should be taken when removing the supernatant. Pure HMW DNA is transparent.

43 Invert tubes on tissue to remove residual ethanol.
Air dry pellet for approximately 10 – 20 minutes to remove all trace of ethanol.

Note

Do not overdry the pellet as this will make the DNA difficult to dissolve and can also induce nicked-DNA damage.

44 Add 50 µl TE buffer (or any desired buffer for the downstream application) to the each tube.

Note

If the pellet is not visible, apply the elution buffer to the side of the tube where the pellet is expected to be. Gently flick the tube to rinse the elution buffer over the wall of the tube. Do not pipet or vortex the DNA sample at this stage.

45 Leave at 50 °C for 1 hour.

50 °C

Note

High molecular weight DNA should be dissolved very gently to avoid shearing. We incubate the samples at 50 °C in a ThermoMixer with gentle agitation at 300 rpm.

46 Collect the DNA in two 2ml tubes; i.e., from the 30 tubes with 50 µl DNA sample in each (see step 44), 1.5 ml of DNA sample is obtained, and thus add 750 µl of DNA sample to each tube.

Note

Optional: Check the concentration with NanoDrop to estimate the final concentration. If the concentration is high enough for the downstream application, you can stop here and proceed to the Quality Control steps.

47 2nd Precipitation to concentrate DNA:

47.1 Add 1/10th volume (75 µl) of 3 M NaOAc and an equal volume (825 µl)of ice-cold isopropanol to each tube.

47.2 Leave the tubes at −20 °C, for more than 1 hour to overnight.

-20 °C

47.3 Centrifuge the tubes at 11,000 rpm for 10 minutes.

11000 rpm, 00:10:00

47.4 Discard the supernatant and Invert tubes on tissue to remove residual ethanol. Air-dry pellet for 10 – 20 minutes. Do not overdry the DNA samples.

48 Elute the DNA pellet in an appropriate volume of elution buffer depending on the downstream application.

Note

For PacBio genome sequencing, a DNA concentration of > 100 ng/µl is recommended by Kazusa DNA Research Institute (Chiba, Japan). We recommend to add a small volume of elution buffer (*e.g.* 50 µl) to achieve high concentrations.

49 Leave the tubes at 50 °C, 300 rpm in a ThermoMixer for 1 hour.

50 °C

Quality Control

50 Assessing DNA integrity: Perform quality checks for DNA integrity by 0.8% agarose gel electrophoresis or Agilent Technologies TapeStation Genomic DNA ScreenTape.

51 Assessing DNA quality: Perform a DNA quality assessment for OD values with a spectrophotometer such as NanoDrop or DeNovix.

52 Assessing DNA quantity: For NGS applications, DNA quantity must be assessed using a fluorometer such as the Qubit or DeNovix.

Note

The DNA concentration values from spectrophotometer and fluorometer assays match in good quality DNA. If they are greatly differ, the DNA quality may be compromised.

Note

Optional: The presence of residual RNA can be measured by Qubit RNA HS assay.