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High molecular weight plant DNA extraction for PacBio HiFi sequencing

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ABSTRACT

High molecular weight and high quality DNA is mandatory for successful long-read sequencing. In addition, PacBio HiFi SMRTBell library construction requires omission of traditional DNA extraction chemicals such as guanidinium, chloroform and others. We developed a DNA extraction protocol working well for the recalcitrant plant *Streptocarpus*, and the extracted DNA was successfully used for PacBio HiFi sequencing.

GUIDELINES

This is a DNA extraction protocol designed for *Streptocarpus* PacBio HiFi sequencing on the Sequel II system.

MATERIALS

Chemicals & Reagents

Nuclear isolation base (NIB) buffer (final concentrations):

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

*To prepare 1L NIB buffer:

Tris-HCl, pH 8.0, 1M stock 10.0 ml EDTA, pH 8.0, 500 mM stock 20.0 ml Sucrose 171.2 g KCl 7.5 g

Protocol status: Working We use this protocol and it's working

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70494

Keywords: PacBio HiFi, SMRTBell library, high molecular weight DNA extraction, plant genome, long-read sequencing Add deionized water to make up 1 L.

Mix chemicals in the glass bottle and autoclave. Once opened, store at 4 °C.

• Nuclear isolation (NI) buffer (final concentrations):

Tris-HCl, pH 8.0, 10 mM

EDTA, pH 8.0, 10 mM

Sucrose, 500 mM

KCI, 100 mM

Spermidine, 4 mM

Spermine, 1 mM

β-mercaptoethanol, 0.1 %

*To prepare 400 ml NI buffer

NIB buffer 400.0 ml Spermidine 4M stock 400.0 μ l Spermine 1M stock 400.0 μ l β -mercaptoethanol 400.0 μ l

Mix chemicals on the day of DNA extraction, in glass bottle or 500 ml glass beaker, and leave on ice.

■ 10% Triton X-100/NIB:

*To prepare, dilute 5 ml Triton X-100 with 45 ml NIB buffer in a 50 ml Falcon tube. Heat in water bath to ca. 30 - 50 °C and once Triton X-100 is dissolved store at 4 °C.

Sorbitol buffer base solution (final concentrations):

Tris-HCl, pH 8.0, 100 mM

EDTA, pH 8.0, 5 mM

Sorbitol, 700 mM

*To prepare 1L sorbitol buffer base solution

Tris-HCl, pH 8.0, 1 M stock 100.0 ml EDTA, pH 8.0, 500 mM stock 10.0 ml Sorbitol 127.5 g Add deionized water to make up 1 L.

Mix chemicals in glass bottle and autoclave. Once opened, store at 4 °C.

NOTE: Original sorbitol buffer base uses 350 mM sorbitol (Souza et al. 2012. Genet Mol Res 11: 756-64), but is here increased to 700 mM.

Sorbitol buffer (final concentrations):

Tris-HCl, pH 8.0, 100 mM

EDTA, pH 8.0, 5 mM

Sorbitol, 700 mM

PVP40, 1%

 β -mercaptoethanol, 0.2%

*To prepare 100 ml sorbitol buffer

Sorbitol buffer base solution 100.0 ml

PVP40 1.0 g (ca. half a dispensing spoon)

β-mercaptoethanol 200.0 μl

Prepare sorbitol buffer on day of DNA extraction. Mix chemicals in two of 50 ml Falcon tubes. Mix well.

CTAB lysis buffer (final concentrations):

Tris-HCl, pH 8.0, 100 mM

EDTA, pH 8.0, 20 mM

NaCl, 1.4 M

CTAB, 2%

*To prepare 1L CTAB lysis buffer

Tris-HCl, pH 8.0, 1M stock 100.0 ml EDTA, pH 8.0, 500 mM stock 40.0 ml NaCl 81.8 g CTAB 20.0 g

Add deionized water to make up 1 L.

Mix chemicals, stir well to dissolve CTAB and autoclave. Store at room temperature.

■ 0.25N HCI:

4 M spermidine:

*Dissolve spermidine in Milli-Q or Sigma water. Divide small aliquots into 2 ml tubes and store at -20 °C. Avoid repeated thawing and freezing.

■ 1 M spermine:

*Dissolve spermine in Milli-Q or Sigma water. Divide to a small amount into 2 ml tubes and store at -20 °C. Avoid repeated thawing and freezing.

■ RNase A (e.g. RNase A 100 mg/ml, Qiagen):

*It is recommended to incubate RNase A at 95 °C for 5 minutes in a thermocycler to deactivate DNase contamination. This is mandatory for old stocks.

■ 3M NaOAc (pH 5.2):

*Use of this chemical is optional when DNA needs to be concentrated by precipitation.

■ Low (0.1 ×) TE buffer (final concentrations):

Tris-HCl, pH 8.0, 1.0 mM

EDTA, pH 8.0, 0.1 mM

^{*}Dilute 5N HCl with Milli-Q water in 50 ml Falcon tube. Store at room temperature.

^{*}Prepare in Milli-Q water and autoclave before use.

- Tris-HCl, pH 8.0, 1 M stock solution
- EDTA, pH 8.0, 500 mM stock solution
- Liquid Nitrogen
- PVPP (Polyvinyl polypyrrolidone)
- Isopropanol
- 70% ethanol (molecular grade)
- Proteinase K Qiagen #19131
- Genomic-tip 100/G Qiagen #10243
- Buffer G2 Qiagen #1014636
- Buffer QBT Qiagen #19054
- Buffer QC Qiagen #19055
- Buffer QF Qiagen #19056
- pH indicator strips to check pH range 7.0 8.0

Plastics

- Falcon tubes: 50 ml
- Eppendorf tubes: 1.5 ml, 2 ml
- Eppendorf LoBind tubes: 1.5 ml
- Pipetman tips: 1ml, 1 ml wide-bore, 200 μl, 20 μl
- Nylon mesh 100 μm pore size or Corning cell strainer 100 μm pore size #431752

Equipment

- Fume bench
- Water bath
- Heat block
- Centrifuge for 50 ml Falcon tubes
- Centrifuge for 1.5 ml and 2.0 ml Eppendorf tubes
- Pipetman: 1 ml, 200 μl, 20 μl
- Liquid nitrogen container
- 500 ml glass beakers
- Pestle and mortar (20 30 sets)
- Spectrophotometer: Nanodrop (Thermo Fisher Scientific) or DeNovix DS-11 (DeNovix Inc.)
- Fluorometer: Qubit (Thermo Fisher Scientific) or DeNovix DS-11
- Fragment analyser: TapeStation (Agilent) or Femto Pulse (Agilent)



- Buffers containing β-mercaptoethanol should be handled with great care and appropriate personal protective equipment (PPE; e.g., gloves and lab coat), and work has to be carried out in the fume hood or on the fume bench.
- Liquid nitrogen may cause cold burns, frostbite, and eye damage and needs to be handled with great care with PPE such as cryogenic gloves and safety goggles.

BEFORE START INSTRUCTIONS

- Prepare and check plastic and chemicals listed under section "Materials".
- Ready-to-use nuclei isolation (NI) buffer and sorbitol buffer containing β mercaptoethanol, spermidine, spermine, and PVP40 should be prepared on the
 day of DNA extraction.
- Leave NI buffer and Triton X-100/NIB on ice for precooling.
- Affix 100 μm pore size nylon mesh on top of a 500 ml glass beaker with string or tape. Mesh can be replaced with Corning cell strainers (100 μm pore size) and 50 ml Falcon tubes.
- Depending on plant species and if possible, it is recommended to cultivate > 100 g plant material to allow protocol optimization.

Before starting DNA extraction

1 Prepare 400 ml NI buffer in a glass beaker and leave on ice.



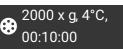
2 Prepare 100 ml sorbitol buffer in two 50 ml Falcon tubes.



- Affix nylon mesh on top of one empty 500 ml glass beaker with string or tape (or place Corning cell strainer on 50 ml Falcon tube).
- 4 Arrange liquid nitrogen and mortar and pestles on fume bench.

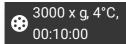
Tissue grinding

- Grind fresh leaf tissue in liquid nitrogen and mortar and pestle 3 times to a fine powder and add ground tissue sample to NI buffer prepared at step 1. Grind 1-2 grams leaf tissue at a time and in total approx. 30 grams in this protocol.
 - On ice
- 6 Filter sample-NI buffer mix through 100 μm pore size nylon mesh/beaker prepared at step 3. Keep all solutions on ice during filtering.
 - ₿ On ice
- 7 Divide filtrate equally to ten 50 ml Falcon tubes kept on ice.
 - On ice
- 8 Add 1/20th volume of 10% Triton X-100/NIB to tubes prepared at step 7. Gently mix by inverting tubes.
 - On ice
- **9** Centrifuge tubes at $2,000 \times g$ for 10 minutes at 4 °C.



- 10 Discard supernatant gently by decantation, without disturbing or losing pellet.
- 11 Add 10 ml sorbitol buffer to each tube and mix gently.

10m



- Discard supernatant by decantation (Optionally, repeat sorbitol buffer wash until supernatant is clear).
- To remove sorbitol buffer completely, invert tubes on dry tissue briefly but take care not to lose pellet. Pellet of nuclei and small cells remaining in tubes can now be **frozen in liquid nitrogen** and stored at -80 °C.



[SAFE STOP POINT for at least a few days]

CTAB lysis

Add 3 ml CTAB lysis buffer directly to frozen pellet, a pinch of PVPP, and 12 µl RNase A to each tube and mix well by gently pipetting with wide-bore tips. Incubate at 58 °C for 20 minutes.

₿ 58 °C

Add 60 μ l proteinase K to each tube. Incubate for more than 3 hours, but less than 5 hours, at 58 °C. Occasionally shake tubes gently.

₿ 58 °C

17 Centrifuge at $4,400 \times g$ for 10 minutes at room temperature. Collect clear lysate to new 50 ml Falcon tube avoiding any cell debris.

4400 x g, Room temperature, 00:10:00

To maximize lysate recovery, move remained debris/lysate to 2 ml tubes and centrifuge at 11,000 rpm for 5 minutes. Move clear lysate to same tube at step 17. In total approx. 30 ml lysate can be obtained.

11000 rpm, Room temperature, 00:05:00

Adjust lysate with 0.25 N HCl to between pH 7.0 - 7.5. Check pH with pH indicator strips. Add 1 ml

10m

5m

or less 0.25N HCl at a time and check with pH paper each time.

20 Divide lysate to two 50 ml Falcon tubes. Add equal volume of Milli-Q water.

NOTE: Do not centrifuge tubes once water is added. Low salt condition tends to promote formation of a CTAB-DNA solidified complex.

Qiagen Genomic-tip 100/G DNA extraction

- Proceed with Qiagen Genomic-tip 100/G following the manufacturer's protocol. Set up six empty 50 ml Falcon tubes, labelled "QBT", "Sample", "QC1", "QC2", "QC3", "Final DNA". Set up three sets of each.
- 22 Set water bath to 50 °C and prewarm buffer QF.

\$ 50 °C

- Place Genomic-tip 100/G column on 50 ml Falcon tube labelled "QBT". Equilibrate Genomic-tip 100/G column with 4 ml buffer QBT. Allow buffer to flow through column completely by gravity.
- Move Genomic-tip 100/G to next Falcon tube labelled "Sample". Load one third of lysate (approx. 20 ml) obtained at step 20 to one Genomic-tip 100/G. Allow lysate to flow through column completely by gravity.
- Move Genomic-tip 100/G to tube labelled "QC1". Load 7.5 ml buffer QC onto column. Allow buffer to flow through column completely by gravity.
- Repeat buffer QC step two more times, on tubes labelled "QC2" and "QC3". In total, DNA in Genomic-tip 100/G column should be washed three times with buffer QC.

For final DNA elution, apply 5 ml QF buffer prewarmed to 50 °C to each Genomic-tip 100/G column.

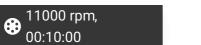


Divide eluted DNA in 1 ml aliquots to 2 ml tubes. Add 0.7 volume (0.7 ml) ice-cold isopropanol. Gently invert and mix and leave tubes at -20 °C overnight.



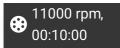
[SAFE STOP POINT for at least a few days]

29 Centrifuge tubes at 11,000 rpm for 10 minutes.



30 Discard supernatant and add 1 ml 70% ethanol.

31 Centrifuge at 11,000 rpm for 10 minutes.



Discard supernatant and air-dry pellet by inverting tubes on clean tissue. Warming tubes at 37 °C for 10 minutes speeds up evaporation of ethanol, but do not over-dry.

33 Add 15-20 μ l Low (0.1 \times) TE buffer.

34 Incubate tubes at 50 °C, and 300 rpm for 1 hour. Collect eluted DNA to 1.5 ml LoBind Eppendorf tube.



35 Add 15-20 μ l Low (0.1 ×) TE buffer for 2nd elution.

10m

10m

Incubate tubes at 50 °C, and 300 rpm for 1 hour. Collect DNA elute to 1.5 ml LoBind Eppendorf tube. Keeping 1st and 2nd DNA elution in separate tubes is recommended.

₿ 50 °C

DNA quality control (QC)

Proceed to DNA quality control. DNA quantification with Qubit and Nanodrop. For DNA quality, obtain A260/A280 and A260/A230 values with Nanodrop. Examine DNA fragment size distribution with TapeStation or FemtoPulse.