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High molecular weight plant DNA extraction for PacBio HiFi sequencing

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Kanae Nishii[1,2], Michael Möller[1], Robert G. Foster[3], Laura L. Forrest[1], Nathan Kelso[1], Sadie Barber[1], Caroline Howard[4], Michelle L. Hart[1]

[1]Royal Botanic Garden Edinburgh; [2]Kanagawa University; [3]Edinburgh Genomics, The University of Edinburgh; [4]Wellcome Sanger Institute

Kanae Nishii
Royal Botanic Garden Edinburgh

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We use this protocol and it's working

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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

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
  KCl, 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 µl
Spermine 1M stock	400.0 µl
β-mercaptoethanol	400.0 µl

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 HCl:

∗Dilute 5N HCl with Milli-Q water in 50 ml Falcon tube. Store at room temperature.

- 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

*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)

Troubleshooting

Safety warnings

- 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

- 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.
On ice

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

3 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

5 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 × *g* for 10 minutes at 4 °C.
10m
2000 x g, 4°C, 00:10:00

10 Discard supernatant gently by decantation, without disturbing or losing pellet.

11 Add 10 ml sorbitol buffer to each tube and mix gently.

12 Centrifuge tubes at 3,000 × *g* for 10 minutes at 4 °C. 10m
3000 x g, 4°C, 00:10:00

13 Discard supernatant by decantation (Optionally, repeat sorbitol buffer wash until supernatant is clear).

14 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.
-80 °C

[SAFE STOP POINT for at least a few days]

CTAB lysis

15 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

16 Add 60 µl 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 × *g* for 10 minutes at room temperature. Collect clear lysate to new 50 ml Falcon tube avoiding any cell debris. 10m
4400 x g, Room temperature, 00:10:00

18 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. 5m
11000 rpm, Room temperature, 00:05:00

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

21 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

23 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.

24 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.

25 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.

26 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.

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

28 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.
-20 °C
[SAFE STOP POINT for at least a few days]

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

30 Discard supernatant and add 1 ml 70% ethanol.

31 Centrifuge at 11,000 rpm for 10 minutes. 10m
11000 rpm, 00:10:00

32 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 µl Low (0.1 ×) TE buffer.

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

35 Add 15-20 µl Low (0.1 ×) TE buffer for 2ndelution.

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

DNA quality control (QC)

37 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.

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