Bioplatforms Australia Genomics for Australian Plants Initiative
Activity: Reference genomes
Species: *Telopea speciosissima*
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GENOMIC DNA EXTRACTION FOR LONG/LINKED READ SEQUENCING

P. Lu-Irving, Evolutionary Ecology, Royal Botanic Gardens Sydney, 2019

Starting material

Ideally, fresh young leaf tissue which is immediately frozen in liquid nitrogen upon collection from plant. Time from collection to preservation affects molecular weight of recovered DNA.

General handling (Ramaciotti sample submission guidelines 2017)
In general, the following precautions should be taken when handling genomic DNA:

- Avoid over drying of genomic DNA. Allow DNA to air dry and do not use heat.
- When resuspending DNA be gentle. Either carefully invert the tube several times after adding buffer and/or tap the tube gently. Alternatively, allow the DNA to stand in buffer overnight at room temperature (20-22°C) to resuspend.
- Avoid vortexing and harsh pipetting as it can shear genomic DNA. Use wide-bore pipette tips and pipette gently when transferring DNA in solution.
- DNA should be eluted in neutral, buffered solution (e.g. 10 mM Tris Acetate or Tris-HCl, pH 8). Avoid buffers containing EDTA (e.g. TE).
- DNA should be stored at 4°C (short-term) or –20°C / –80°C (long-term).
- Avoid repeated freezing and thawing of genomic DNA, as this will lead to DNA shearing.

Target quantity and quality

Ramaciotti sample submission guidelines 2017
Purity: OD260/280 = 1.8 - 2.0; OD260/230 = ~2.0; no RNA contamination.

Application	Quantity	Min. concentration
PacBio 1kb	2ug	75ng/ul x 30ul
PacBio 2kb	2ug	75ng/ul x 30ul
PacBio 5kb	5ug	75ng/ul x 70ul
PacBio 10kb low input	3ug	75ng/ul x 45ul
PacBio 10kb (AMPure kit)	15ug	250ng/ul x 65ul
PacBio 20kb (AMPure kit)	20ug	250ng/ul x 85ul
PacBio 20kb (BluePippin kit)	20ug	250ng/ul x 85ul
10x Chromium (BluePippin >50kb size selection)	5ug	100ng/ul x 55ul
10x Chromium (no size selection)*	400ng	15ng/ul x 30ul

***** DNA must be fully intact and >50kb (as assessed by pulsed field gel)

AGRF correspondence re: PromethION (Nanopore) sample requirements 2018

"With regards to processing samples, we require ≥ 10 µg of high molecular weight DNA (ideally ≥60Kb). Along with integrity, efficient removal of polysaccharides is an important consideration for you DNA extraction protocol, as they can inhibit the nanopore sequencing."

- ➔ Use higher salt buffer and sorbitol pre-wash to facilitate removal of polysaccharides per Thermo Fisher Application Note 52645, Inglis et al 2018 doi: 10.1371/journal.pone.0206085
- ➔ Aim for A260/230 ratios >1.9

To meet requirements for high DNA yield (long read sequencing), it may be necessary to perform multiple replicate extractions (multiple extraction tubes per sample).

Bulk CTAB extraction (adapted from S. Rutherford and Inglis et al. 2018)

Sorbitol wash buffer (store at 4°C for up to 6 months)

Ingredient	**Concentration**	**for 500mL**	**For 250mL**
Sorbitol	0.35 M	13.66g	6.83g
PVP-40	1 %	5 g	2.5 g
Tris-HCl pH 8 (1M)	100 mM	50 mL	25 mL
EDTA pH 8 (0.5M)	5 mM	5 mL	2.5 mL
β-mercaptoethanol	1 %	(add before use)	(add before use)

Extraction buffer (store at RT for up to 6 months)

Ingredient	**Concentration**	**for 500mL**	**For 250mL**
NaCl	3 M	87.66 g	43.83 g
PVP-40	1 %	5 g	2.5 g
CTAB	3 %	15 g	7.5 g
Tris-HCl pH 8 (1M)	100 mM	50 mL	25 mL
EDTA pH 8 (0.5M)	20 mM	5 mL	2.5 mL
β-mercaptoethanol	1 %	(add before use)	(add before use)

Add 100uL β-mercaptoethanol per 10mL buffer immediately prior to use.

Extraction protocol

Day 1

1. Make up chloroform:isoamyl mixture (24:1) and 77 % ethanol. Place 77 % ethanol in freezer. Heat fresh extraction buffer to 65°C. Add β-mercaptoethanol to wash and extraction buffers.
2. Grind ~2 g leaf material in mortar and pestle under liquid nitrogen to fine powder. Make sure material does not thaw. Transfer to 15 mL tubes.

3. Add 10mL sorbitol wash containing β-me to each tube and mix by inversion for 2 min.
4. Centrifuge tubes at 3,000 x g for 5 min. Discard supernatant.
5. Add 6mL pre-warmed extraction buffer containing β-me to each tube, and resuspend pellets by inverting/tapping tubes.
6. Add 30 uL of proteinase K (20mg/mL) to each tube and mix by inversion.
7. Incubate tubes at 65°C for 1 to 1.5 hours, invert tubes every 10-20 min.
8. Cool down tubes for at least 5 minutes.
9. Add 6 mL of chloroform:isoamyl alcohol (24:1) and mix by inversion for 2 min.
10. Spin at 3000 x g RT for 20 min.
11. Transfer water phase to fresh tube, record the volume transferred.
12. Add 5µL of RNAse A (10 mg/mL) and incubate at 37°C for 15 minutes, gently inverting the tubes periodically.
13. Add 1 volume of chloroform:isoamyl alcohol and mix by inversion for 2 min.
14. Centrifuge tubes at 3000 x g for 20 min.
15. Transfer water phase to fresh tube, record the volumes transferred, and add 1 volume room temperature isopropanol. Mix by inversion for 2 min.
16. Leave tubes in fridge overnight.

Day 2

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17. Centrifuge tubes at 3,000 x g for 30 min.
18. Discard supernatant, pipette off residual isOH, and allow pellet to air-dry on bench (take care not to over-dry).
19. Add 1.5 mL ice cold 77 % ethanol. Gently dislodge pellet from bottom of tube, and pour etOH + pellet into new 1.5 mL tube. Leave in fridge for 20 min.
20. Centrifuge tubes at 14,000 x g for 20 min.
21. Discard supernatant. Add 1.5 mL ice cold 77 % ethanol, gently dislodge pellet by inverting/tapping, and leave in fridge overnight.

Day 3

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22. Centrifuge tubes at 14,000 x g for 20 min.
23. Discard supernatant. Add 1.5 mL of 77 % ethanol. Gently dislodge pellet by inverting/tapping, and leave in fridge for 20 minutes.
24. Centrifuge tubes at 14,000 x g for 20 minutes.
25. Discard supernatant, and allow pellet to air-dry on bench (take care not to over-dry).
26. Re-suspend pellet in 100 µL 10mM Tris-HCl pH 8.
27. Evaluate DNA quantity (yield) using Qubit and evaluate DNA purity and integrity via OD260/280, 260/230 (protein, phenol, carbohydrate contamination) and agarose gel (qualitative size, RNA contamination).
28. If DNA appears pure and intact, store DNA to be sequenced at 4°C and proceed ASAP with sample submission. Store DNA to be archived at -20°C or -80°C.

29. If DNA is intact but requires further purification, dilute as necessary for bead clean-up (concentrations of DNA >~100ng/uL may affect bead binding/elution) and store DNA at 4°C before proceeding ASAP with clean-up.

DNA purification using SPRI beads
(adapted from Schalamun et al. 2018 doi: 10.1111/1755-0998.12938)
Note: expect loss of yield of up to ~75% during bead clean-up.

Wash beads before use:

1. Thoroughly resuspend beads.
2. Pipette 500ul of beads into a 1.5mL microtube.
3. Spin down 1 minute at maximum speed in a benchtop microcentrifuge.
4. Place tube on magnet rack.
5. Remove and save the supernatant.
6. Add 1mL molecular biology grade water to the beads, and vortex to resuspend completely.
7. Spin down 1 minute at maximum speed in a benchtop microcentrifuge.
8. Place tube on magnet rack.
9. Remove and discard the supernatant.
10. Repeat water wash (steps 6-8) four times.
11. Add 1mL 10mM Tris-HCl, pH 8 to the beads, and vortex to resuspend completely.
12. Spin down 1 minute at maximum speed in a benchtop microcentrifuge.
13. Place tube on magnet rack.
14. Remove and discard the supernatant.
15. Resuspend the beads in the original supernatant reserved in step 5, vortexing if necessary.

Washed beads can be stored at 4°C for at least 3 months.

Clean up DNA:

1. Preheat 10mM Tris-HCl, pH 8 to 50°C. Make fresh 70% EtOH.
2. Add 1x volume of washed beads to 1x volume of DNA.
3. Incubate 10 minutes at room temperature, gently inverting the tubes periodically.
4. Touch spin in desktop centrifuge to draw bead suspension to bottom of tube.
5. Place tube on magnet rack and wait until bead pellet forms on side of tube.
6. Remove and discard supernatant.
7. Add 1mL fresh 70% EtOH and slowly rotate the tubes 360° in the magnet rack to wash bead pellet.
8. Remove and discard supernatant.
9. Repeat EtOH wash (steps 6-7) once.
10. Touch spin in desktop centrifuge to draw residual EtOH to bottom of tube, and remove and discard residual EtOH by pipetting, taking care to avoid disturbing the bead pellet.
11. Allow beads to dry briefly (less than 1 minute), then add 50-100uL (depending on expected/desired DNA concentration) 10mM Tris-HCl, pH 8 (preheated to 50°C).

12. Incubate at least 10 minutes at room temperature to elute. If elution is difficult due to high concentration of high molecular weight DNA (thick, fluffy bead pellet), incubate overnight at 37°C.
13. Place tube on magnet rack and wait until bead pellet forms on side of tube.
14. Transfer supernatant to new tube.
15. Evaluate DNA quantity (yield) using Qubit and adjust concentration as necessary for sample submission guidelines. Evaluate DNA purity and integrity via OD260/280, 260/230 (protein, phenol, carbohydrate contamination) and agarose gel (qualitative size, RNA contamination).
16. Store DNA to be sequenced at 4°C and ship ASAP on ice to sequencing facility. Store DNA to be archived at -20°C or -80°C.