Bioplatforms Australia Genomics for Australian Plants Initiative
Activity: Reference genomes
Species: *Acacia pycnantha*
Contact: Todd McLay, todd.mclay@gmail.com

High quality and high yields of DNA for long read sequencing using a sorbitol prewash and CTAB or SDS

This protocol is heavily based on Inglis et al 2018 'Fast and inexpensive protocols for consistent extraction of high-quality DNA and RNA from challenging plant and fungal samples for high-throughput SNP genotyping and sequencing applications (https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0206085)', with a few modifications, and extended for 50 mL preps. in falcon tubes. There are two different extraction buffers, using CTAB or SDS, which may help remove different secondary compounds from recalcitrant plants. I tried both with *Acacia pycnantha*, and both extraction buffers yielded similar DNA quality and quantity. Tissue selection is extremely important, I didn't have much success until I used young phyllodes (and I removed a lot of the midvein and extra-floral nectary).

The numbers here are rough so you will have to judge the volumes based on how much tissue you are using. I started with about 10-15 grams of tissue and ended up using six 50 mL falcon tubes. I would probably just start with the assumption you are going to use 6 Falcons, at worst it just means a little more fiddling at the end but at least all the chemicals will be in excess. The two extraction buffers use the same protocol until the extraction step. CTAB uses two chloroform:isoamyl (24:1; CIa) steps whereas SDS uses 5 M potassium acetate to precipitate the SDS, followed by a CIA step. Not vortexing, using cutoff tips and just general care should be taken to reduce DNA fragmentation.

Other useful resources:
https://www.protocols.io/view/nuclear-dna-purification-from-recalcitrant-plant-s-vmee43e
https://www.protocols.io/view/plant-nuclear-genomic-dna-preps-rncd5aw

Equipment

- 250 and 500 mL Schott bottles
- Centrifuge with rotor for 50 mL falcon tubes
    - Our model is an Eppendorf Centrifuge 5430R, with the F-35-6-30 rotor
- Fume hood
- NutriBullet (or blender, or your favourite way of grinding tissue)
- 500 mL measuring cylinders
- 50 mL Falcons
- 1.5 mL Eppendorfs
- 5 mL pipette (or other high volume will be helpful but not essential).

Buffers to premake

Sorbitol buffer: mix on stir plate, can keep for 1 months

- You need ~ 50 mL per falcon tube.

	Start conc. M	**Final conc. M**	**For 400 mL**
D-Sorbitol	*powder*	0.35 M	25.52 g
PVP-40	*powder*	1%	4 g
EDTA	0.5 M	5 mM	4 mL
TrisHCl	1 M	0.1 M	40 mL
Water			To 400 mL

CTAB extraction buffer (200mL): mix on stir plate, ideally make fresh. This is quite goopy at room temp. but becomes less viscous when heated at 60degC.

- You will need ~25 mL per falcon tube.

	Start conc. M	**Final conc. M**	**For 200 mL solution**
CTAB	*Powder*	3%	6 g
Sodium metabisulfite	*Powder*	1%	2 g
PVP-40	*powder*	2%	4 g
TrisHCl pH8	1 M	100 mM	20 mL
NaCl	5 M	2.8 M	112 mL
EDTA ph8	0.5 M	50 mM	20 mL of 0.5 M
Water			To 200 mL

SDS extraction buffer (200 mL): mix on stir plate, ideally make fresh. From Ash Jones' protocol: https://www.protocols.io/view/nuclear-dna-purification-from-recalcitrant-plant-s-vmee43e

- You will need ~25 mL per falcon tube.

	Start conc. M	**Final conc. M**	**For 200 mL solution**
PVP-40	*Powder*	1%	2 g
Sodium metabisulfite	*Powder*	1%	2 g
NaCl	5 M	1 M	40mL
TrisHCl pH8	1 M	100 mM	20 mL
EDTA ph8	0.5 M	5 mM	20 mL
Sodium dodecyl sulfate (SDS)	20%	3%	30 mL
Water			To 200 (~90 mL)

1X TE

9.88 ml H2O
0.1 mL (100 uL) 1 M Tris
0.02 mL (20 uL) 0.5 MEDTA

BSA:NaCl (1:5)

- 5M NaCl in a 5:1 ratio to 100x BSA
- BSA 100X is 4% powder in H20
- Need 2.5 mL per 25 mL of CTAB used.

Serapure or Ampure beads.

- Need approximately 1 mL per falcon tube of sample.
- https://ethanomics.files.wordpress.com/2012/08/serapure_v2-2.pdf
- a magnet for 1.5 mL tubes.

You also need

- BME (~6 mL),
- Proteinase K (Qiagen, 20 mg/mL)
- RNase-A (Stratec, 10 mg/mL)
- 2.4 M sodium acetate
- 5 M potassium acetate (if using SDS extraction buffer)
- chloroform:isoamyl 24:1 (or just chloroform will do, the isoamyl makes the interphase after spinning more robust)
- ethanol
- method for checking DNA quantity (Qubit etc.), quality (i.e. Nanodrop), lengths (can be approximated using a gel with a HMW ladder, better to use something like a TapeStation, Bioanalyser, or Fragment analyser).

DNA extraction protocol

Tissue grinding and Sorbitol pre-wash

1. Add 180 mL extraction buffer (30 mL per falcon tube; CTAB or SDS) in 250 mL Schott bottle and place in 60degC heat-bath.

2. Place tissue into Nutribullet. Add **sorbitol buffer** to 'fill line' (~250 mL). Blitz for 20-30 seconds in 5 second bursts or until all the tissue appears to be well macerated.

3. Pour tissue+buffer into a 500 mL measuring cyclinder for decanting into falcons.

4. Decant into 50 mL falcon tubes. This can be a little tricky as you want to get an even amount of tissue into each tube for centrifuge balance and the tissue sits at the bottom of the cylinder. I decanted, then weighed all the tubes, and tried to even them out from there. At this stage the tissue+buffer can be a bit bubbly, so it might be useful to wait before decanting, but I fiddled around until the weights were even enough to spin.

5. To each tube add 500 uL BME and invert several times to mix.

6. Spin at 4500 rpm (1134 g) for 10 mins. In fumehood, discard supernatant by pouring off into a beaker (this has BME in it so should be disposed of appropriately).
   - NB. At this point I saw two very obviously different layers of solid in the tube: a light green sludge that I assume is polysaccharides, and dark green tissue (Figure 1). I went on with the extraction, but I do wonder if removing the sludge might improve things.

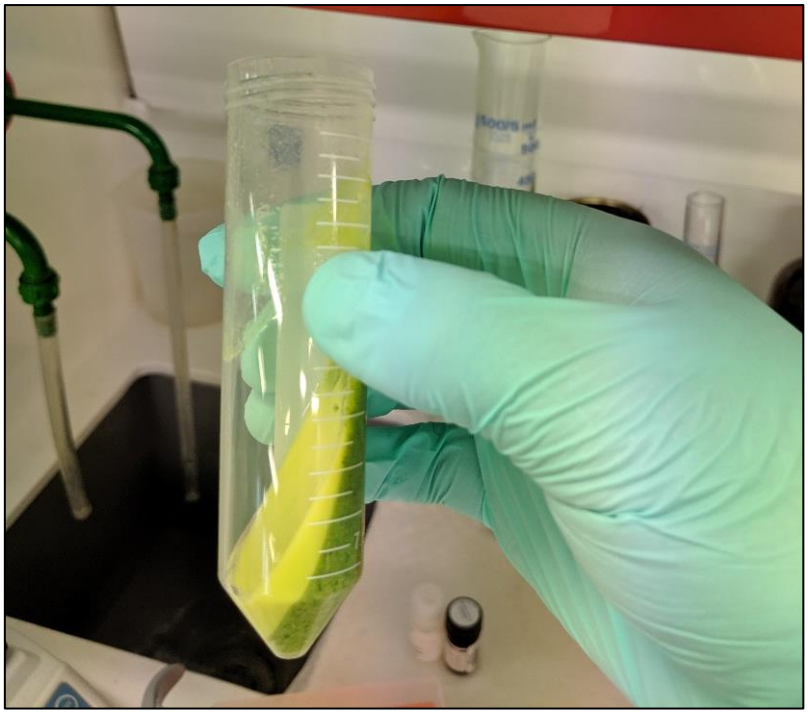


Figure 1: Two distinct layers after sorbitol prewash and centrifugation.

Adding extraction buffer

6. In the fumehood, add pre-warmed (60degC) **Extraction buffer** (CTAB or SDS) to each tube to bring volume to 25 mL (approx. 19-20 mL). Invert to mix. Add 500 uL of BME (2% v/v), 100 uL RNAse (~0.4 mg/mL), and 100 uL Proteinase K (~0.8 mg/mL). Invert to mix.

9. Add 2.5 mL **BSA:NaCl (1:5)** solution to each tube, invert again, and incubate at 60degC for 1-2 hrs, mixing several times during the incubation by inversion (or incubate on a gentle shaker).

For CTAB buffer extraction

10a. Add chloroform:isoamyl to each tube to fill to 50 mL (~25 mL, you might have to use less to fit it into the tube). Invert gently to mix (5-10 times), and open tube to release gas. Spin for 5 min at max speed (7830 rpm, 3400 g).

11a. Transfer top aqueous phase from each tube to new tube (~20-25 mL). Add equal parts chloroform:isoamyl, invert to mix and open tube to release gas build up, and spin at max for 5 min.

12a. Transfer top aqueous phase from both tubes to new tube (~20-25 mL).

For SDS buffer extraction

10b. Add equal volume of 5M postassium acetate to each tube (~20-25 mL). Invert gently to mix (5-10 times). Spin for 5 min at max speed (7830 rpm, 3400 g).

- NB. The KAc will precipitate the SDS as a white-grey substance, and the spin

should pull most of it to the bottom of the tube. However, some particles might still be floating in the supernatant, and can't be avoided when transferring. The CIa will deal with these so you don't need to worry too much about transferring them. You can also repeat step 10b a few times if you think not all the SDS is precipitated

11b. Transfer top aqueous phase from each tube to new tube (~20-25 mL). Add equal parts chloroform:isoamyl, invert to mix and open tube to release gas build up, and spin at max for 5 min.

12b. Transfer top aqueous phase from both tubes to new tube (~20-25 mL).

DNA precipitation with isopropanol

13. Add ~0.15 volume 2.4 M sodium acetate (~3 mL), and an equal volume of isopropanol (~20-25 mL). Invert 5-10 times to mix.

14. Leave at room temp for 15 mins for the DNA to precipitate. You may see strings of DNA forming, but do not worry if you don't.

15. Spin at max speed for 10-15 minutes. A pellet should be visible, either white or clear (Figure 2).

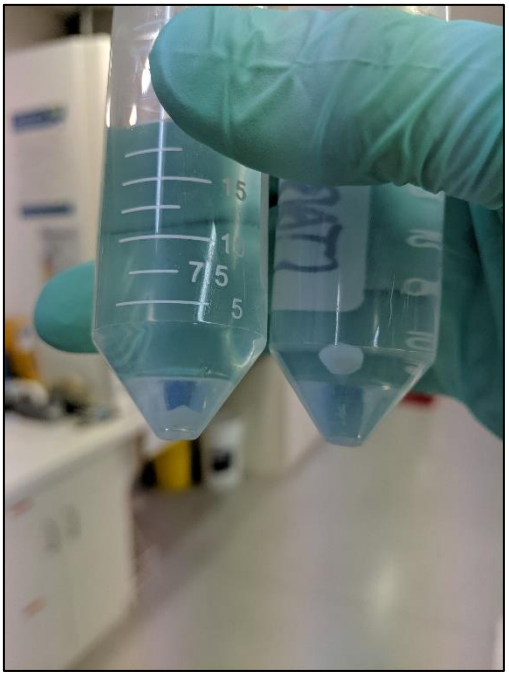


Figure 2: Pellets of DNA after the isopropanol spin. The pellet on the left-hand side has slid down to the bottom of the tube.

16. Carefully pour off liquid. You must be quite careful here as the pellet doesn't stick to the tube as well as it would in an Eppendorf.

17. Add 45 mL of 80% ethanol (I make mine fresh in a measuring cylinder). Invert gently, it's

ok if the pellet comes off the edge of the tube.

18. Spin at max speed for 5 mins.

19. Very carefully pour off the liquid as the pellet might be mobile here. You want to remove as much ethanol as possible (I used a kimwipe taped onto the end of a spatula, but avoid touching the pellet directly).

20. Cover tubes with a kimwipe and let the pellet dry in the fumehood for 30-60 mins until most of the ethanol is removed.

21. Resuspend pellet using 1 mL of TE or 10 mM TrisHCl ph8. If the pellet hasn't moved down to the bottom of the tube try and make that happen, or, make sure the liquid is in contact with the pellet by tilting the tube. Let the resuspension occur for at least an hour (or until the pellet is no longer visible).

22. After you are satisfied the pellet has resuspended, spin briefly, and transfer the DNA in solution to a 1.5 mL (or 2 mL) Eppendorf tube.

Bead precipitation, cleaning, and very small fragment removal

23. Add 500 uL of Serapure/Ampure bead to tube. Invert gently several times to mix solutions. Incubate at RT for 15-30 mins.

24. Place on magnet for 30 mins (or until solution becomes clear).

25. Remove the supernatant, taking care to not disrupt the beads.

26. Add 1.5 mL of freshly made 80% ethanol. Let sit for 1-2 mins.

27. Remove ethanol. This step can be repeated multiple times if you think the DNA needs lots of cleaning. I had some trouble with the DNA+bead pellet being very goopy and not sticking to the magnet very well (maybe there was too much DNA in the tube?). Just work carefully.

28. After your final ethanol wash, remove the tubes from the magnet and let air dry for 1-2 mins (don't leave this too long as the beads will dry, crack, and the DNA won't be easily eluted; less is more here).

29. Add 100-200 uL (depending on pellet size) of 10 mM TrisHCl ph8. Invert the tube to mix the beads into solution. Let sit for at 30-60 mins at RT (a heat block at 37deg can quicken this step).

30. Place tubes back on the magnet for 30 mins (or until solution becomes clear).

31. Remove the supernatant into new tubes. This is your final DNA in solution!

32. Check each tube using nanodrop and Qubit to get quantity and quality information. If you are happy with the concentration and quality of all six tubes you can combine them (one of mine didn't have DNA because I think I tipped it out during the ethanol clean). If you want to increase the concentration combine tubes and concentrate using beads (i.e. elute in less Tris.). In the end I had 500 uL of DNA at 150 ng/uL, a 260/280 of 1.9, and a 260/230 of 2.34 (Figure 3). TapeStation showed that 55% of fragments were 20kb or greater (Figure 4). This can be size selected for using a PippinHT.

33. Store the DNA in the fridge.

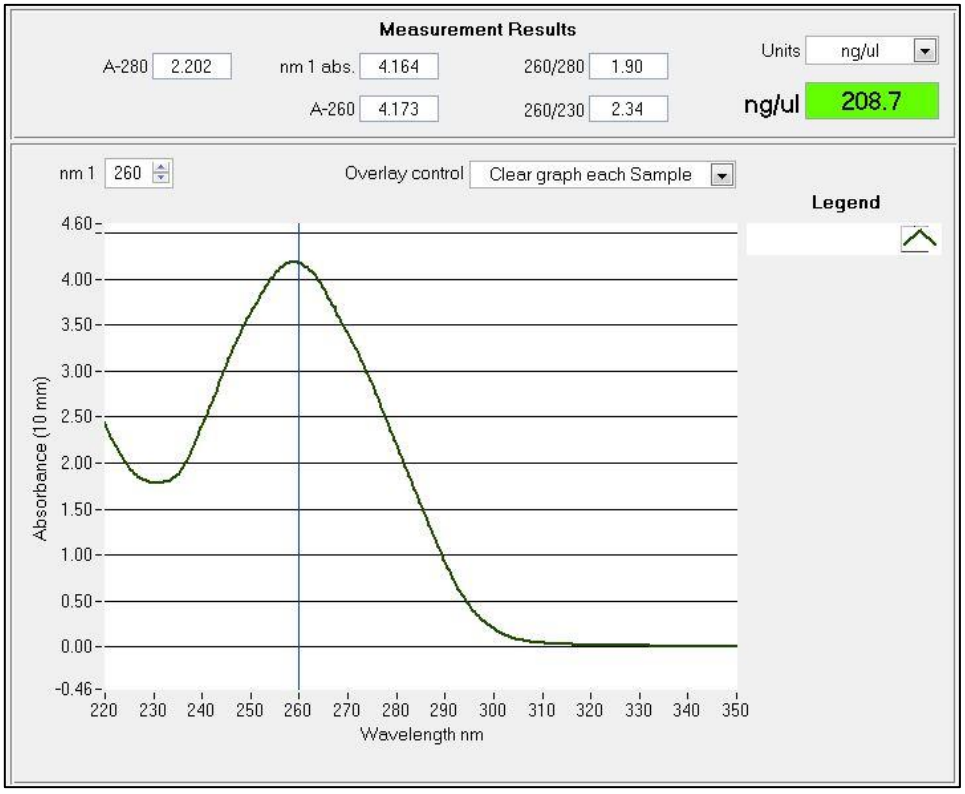
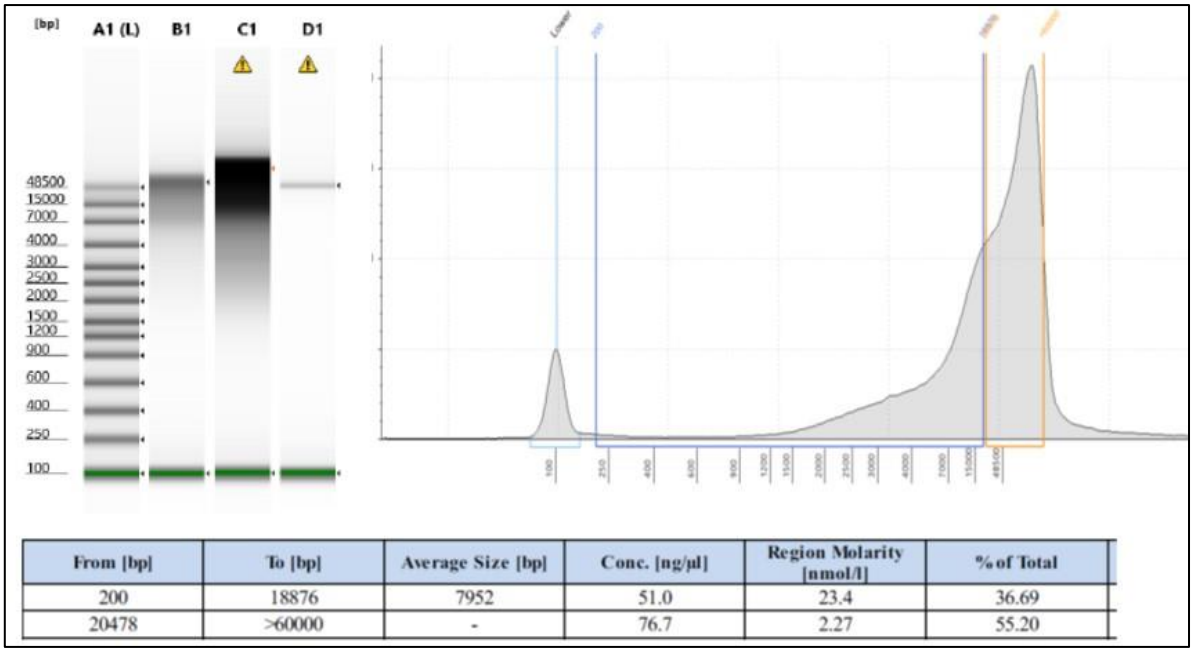


Figure 3: DNA quality measured using spectrophotometry (Nanodrop). The concentration according to Qubit was 150 ng/uL.



From [bp]	To [bp]	Average Size [bp]	Conc. [ng/µl]	Region Molarity [nmol/l]	% of Total
200	18876	7952	51.0	23.4	36.69
20478	>60000	-	76.7	2.27	55.20

Figure 4: TapeStation output, showing DNA lengths and concentrations in two size ranges: 200-20000 bp, and 20000 bp +.