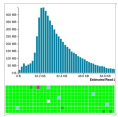

Nuclear DNA purification from recalcitrant plant species for long-read sequencing

Version 2

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dx.doi.org/10.17504/protocols.io.vmee43e

MinION user group for high molecular weight DNA extraction from all kingdoms

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ABSTRACT

Evolution has driven genetic diversity of life on Earth, but also created highly complex genomes that are difficult to sequence. Current draft genomes can have thousands to hundreds of thousands of contigs rather than chromosomes, containing incorrect assemblies, gaps and errors. With rapid advances in long-read technologies, it is becoming possible to resolve complex genomes, including repetitive, polyploid plant genomes. Despite the technology being available, a challenge persists: the extraction of pure high molecular weight DNA suitable for long-read sequencing. This is particularly true of recalcitrant native Australian trees such as Eucalypts and Acacias. To resolve this, firstly we optimised a density gradient and detergent based nuclei extraction to limit reads from high copy count plastid genomes. Secondly, we optimised a gentle high molecular weight DNA extraction free of columns and high centrifugation, to limit DNA fragmentation. Finally, the DNA was purified and size selected by gel electrophoresis. For sequencing, we adopted the portable MinION sequencer from Oxford Nanopore Technologies. Using these approaches, we have been obtaining >9 gigabases of sequencing from a single MinION flow cell, including reads over 100 kb in length. Such ultra-long reads assist the assembly of high quality genomes, from telomere to telomere.

PROTOCOL STATUS

Working

Verified across multiple genera.

GUIDELINES

This protocol is a modified and optimised combination of the following two publications. When citing, please also note the original publications below.

Bolger *et al.* (2014). The genome of the stress-tolerant wild tomato species Solanum pennellii. Nature Genetics **46,** 1034.

Mayjonade *et al.* (2016). Extraction of high-molecular-weight genomic DNA for long-read sequencing of single molecules. BioTechniques **61,** 203-205.

This research builds on the work of Benjamin Schwessinger and Miriam Schalamun; a warm thanks for their contributions.

Eucalyptus and Acacia tissue used in this protocol was kindly provided by the Australian National Botanic Gardens, Canberra, Australia.

BEFORE STARTING

This protocol is designed for two sample nuclei isolations and DNA extractions, although upscaling is possible. Prepare the following for two samples:

- Autoclave x2 500 mL Nalgene bottles, x10 1 L Schott bottles, x2 beakers and x2 funnels.
- Collect a branch of Eucalyptus leaves, harvest and wash leaves with tap water.
- Weigh 50 g of leaves into 50 g takeaway containers.
- Pre-chill the blender in 4°C cold room.
- For two sample extractions, on the day prepare 1 L nuclei isolation buffer and 1 L of nuclei wash buffer, and cool both down to 4°C.
- Prepare lysis buffer fresh on the day of use.
- Prepare all other solutions as listed below, storing at room temperature until use.

Nuclei isolation buffer (1 L for two samples)

- Adjust to pH 6 (add HCl; estimate listed below).
- Cool down to 4°C

Component	MW	Stock	Quantity (1 L)
1 M Hexylene gylcol (2-Methyl-2,4-pentanediol)	118.17	7.51 M (shipped liquid)	133 mL
10 mM PIPES	302.4	0.5 M	20 mL
10 mM $MgCl_2$	203.3	1 M	10 mL
10 mM Sodium metabisulfite	190.11	10%= 0.53 M	20 mL
6 mM Ethylene glycol tetraacetic acid (EGTA) pH 7	380.4	0.5 M	12 mL
pH 6 equilibration with HCl	36.46	5 M	4 mL
0.5% (w/v) Sodium diethyldithiocarbamate	171.26	10%	50 mL
4% (w/v) PVP-10	10,000	powder	20 g
200 mM L-lysine	146.19	powder	36.53 g
1 mM Dithiothreitol (DTT)	154.25	powder	0.154 g

Nuclei wash buffer (1 L for two samples)

- Adjust to pH 7 (add NaOH; estimate listed below).
- Cool down to 4°C

Component	MW	Stock	Quantity (1 L)
0.5 M Hexylene gylcol (2-Methyl-2,4-pentanediol)	118.17	7.51 M (shipped liquid)	66.66 mL
10 mM PIPES	302.4	0.5 M	20 mL
10 mM $MgCl_2$	203.3	1 M	10 mL
10 mM Sodium metabisulfite	190.11	10%= 0.53 M	20 mL
6 mM Ethylene glycol tetraacetic acid (EGTA) pH 7	380.4	0.5 M	12 mL
pH 7 equilibration with NaOH	40.00	5 M	3 mL
0.5% (v/v) Triton X-100	647	10%	50 mL
200 mM L-lysine	182.65	powder	36.53
1 mM Dithiothreitol (DTT)	154.25	powder	0.154 g

Lysis buffer (10 mL per sample)

- Prepare a fresh solution for optimal results. The solution should be clear before use.

Component	MW	Stock	Quantity (10 mL)
1% Polyvinylpyrrolidone 40 (PVP-40)	40,000	10%	1 mL
1% Sodium metabisulfite	190.11	10%	1 mL
0.5 M NaCl	58.44	5 M	1 mL
100 mM TRIS-HCl pH 8.0	121.14	1 M	1 mL
5 mM EDTA pH 8.0	292.24	0.5 M	1 mL
3% Sodium dodecyl sulfate (SDS)	288.37	20%	1.5 mL

Water	-	-	3.5 mL

2% Sera-Mag beads solution (10 mL stock)

- Sera-Mag SpeedBead magnetic carboxylate modified particles (Thermo Scientific 65152105050250).
- Store at 4°C for up to 6 months.
- First prepare buffer without the beads. Let the Sera-Mag beads come to room temperature.

Component	MW	Stock	Quantity (10 mL)
18% Polyethylene glycol (PEG) 8,000	8,000	25%	7,200 µL
1 M NaCl	58.44	5 M	2,000 µL
10 mM TRIS-HCl pH 8.0	121.14	1 M	100 µL
1 mM EDTA pH 8.0	292.24	0.5 M	20 µL
0.05% Tween 20	1,227.54	10%	50 µL
Water	-	-	430 µL

- Homogenise Sera-Mag beads thoroughly by shaking and swirling.
- Prepare 2% Sera-Mag beads (200 µL for 10 mL buffer) by washing 4 times with water to remove sodium azide. Magnetise, remove supernatant, add ~500 µL H_2O, flick tube, magnetise etc.
- Resuspend the clean Sera-Mag beads in the buffer prepared.
- Note: Agencourt AMPure XP (Beckman Coulter) can be used but they must be washed 4 times with water and resuspended in their initial buffer.

Binding buffer solution (10 mL per sample)

- 2 g of PEG 8,000 (or 8 mL of 25% stock) [final = 20%]
- 75 g of sodium chloride [final = 3 M]
- Adjust to 10 mL with molecular biology grade water (approx 2 mL).

Mix until the solution becomes clear. If PEG8000 is not dissolved, it can lead to a poor yield as PEG 8000 makes gDNA to bind to the beads.

Other solutions

- Triton X-100 (ideally 10% solution)
- Ethanol (100% and 70%)
- RNAse A (e.g. 20 mg/mL Invitrogen PureLink)
- 5 M Potassium acetate

Special equipment (for two samples)

- 500 mL Nalgene bottles (x2)
- 1 L Schott bottles (x10)
- Beakers (x2)
- Funnel (preferably glass) (x2)
- Large fine hair paint brushes (x2) (e.g. J.Burrows Mop Goat Hair 24 #9341694325125)
- etic rMack (for Falcon and Eppendorf tubes)
- 15 mL Falcon tubes
- 50 mL Falcon tubes
- DNA LoBind Eppendorf tubes (e.g. 022431021)
- Sieve or mesh, 300 µm (e.g. Kartell disk membrane; ART 844)

- Miracloth or stretcher sheets (e.g Drager 334201)
- 200 µL wide-bore pipette tips (e.g. Vertex 4290-00)

BLENDER LYSIS (4°C cold room)

1 Place 50 g of leaves into a blender with enough nuclei isolation buffer to cover the blades (approximately 150 mL).

NOTE
Optimised for Eucalyptus, a diploid ~500 Mb genome. Larger genomes or polyploid plants can have less input accordingly.

2 Homogenise the tissue on max speed. Transfer to a beaker, repeatedly washing out blender with nuclei isolation buffer (500 mL buffer is allocated per extraction).

3 Clean the blender with tap water, 70% ethanol and then MQW; process the other sample (repeating steps 1 and 2).

FILTRATION (ideally 4°C cold room)

4 Filter homogenate using a sieve (or disk membrane mesh placed in a funnel) into a 1 L Schott bottle. Forcibly squeeze out as much residual homogenate from the leaf debris as possible, maximising nuclei capture.

5 Filter homogenate through 1 layer of Miracloth using a funnel and 1 L Schott bottle. Gently squeeze residual homogenate through the Miracloth.

NOTE
May need to change the layer half way through (very dirty and clogged).

6 Repeat Filtration through 2 layers of Miracloth then 4 layers (using new Schott bottles).

7 Finally, gravity filter through 8 layers of Miracloth (no squeezing) into a 500 mL Nalgene bottle. Repeat steps 4-6 for the second sample.

NUCLEI ISOLATION

8 Add 2.5 mL of 100% Triton X-100, or 25 mL of 10% (final concentration 0.5%).

9 Incubate mixture on an ice bath with gentle rocking for 30 min.

NOTE
Use this time to clean the mess made with the blender and filtering

10 Weigh the Nalgene bottles with contents and lids to ensure they are equal for balancing purposes. Also ensure the Nalgene bottle has an intact o-ring to avoid leakage during centrifugation.

11 Centrifuge at 600 rcf and 4 °C for 20 minutes.

NOTE
4,600 rpm on a Sorvall RC5C using rotor SLA-3000 (00).

12 Discard the supernatant and add 200 mL of nuclei wash buffer.

13 Gently resuspend the pellet using a large fine hair paint brush, soaked in freshly made pre-chilled nuclei wash buffer.

NOTE
Ensure the bottles are balanced and have an o-ring as previously described.

14 Centrifuge at 600 rcf and 4°C for 20 minutes.

15 Discard supernatant and repeat with another 200 mL of nuclei wash buffer. The pellet should become grey-white with no traces of green. If not, make more nuclei wash buffer and repeat the resuspending and washing steps.

NOTE
1 L of nuclei wash buffer is suitable for x2 samples to be washed x2 times with 200 mL each, then a final resuspension with 100 mL each.

16 After the final spin down, discard the supernatant and resuspend the pellet with 50 mL of nuclei wash buffer. Transfer evenly across two 50 mL Falcon tubes. Repeat the resuspension with another 50 mL of buffer and add to the two Falcon tubes.

17 Centrifuge at 600 rcf and 4°C for 30 minutes.

18 Discard the supernatant. The nuclei pellets can now be stored -80°C.

DNA EXTRACTION FROM NUCLEI

19 Prepare 10 mL of fresh lysis buffer per sample. Ideally, pre-heat at 50-65°C.

NOTE
The solution should be clear before use.

20 To a 3-5 mL nuclei pellet, add 10 mL of lysis buffer.

NOTE
Therefore, the SDS will be approximately 2% final concentration. Usually just one of the two Falcon tubes of a nuclei prep per sample are used.

21 Add 200 µL of RNAse A to each sample (20 mg/mL Invitrogen PureLink).

22 Incubate the samples at 50-60°C for 1 h, shaking at 400 - 900 rpm if possible.

NOTE
Higher temperatures and longer incubations lead to DNA damage.

23 Add 1/3 volume of 5 M Potassium Acetate and mix by inverting to precipitate the proteins and the polysaccharides that will complex with SDS.

24 Incubate on ice (4°C) for 10 min (don't rotate, DNA vulnerable).

25 Centrifuge at 5,000 rcf for 5 min at 4°C.

26 Transfer supernatant to a new tube, centrifuge again at 5,000 rcf for 10 min at 4°C.

27 Transfer to a new tube and add 1.2x binding buffer. Ideally, split across x2 15 mL Falcon tubes.

28 Add 1 mL of 2% Sera-Mag beads (500 µL per Falcon tube if split).

NOTE
Beads are in excess and could be reduced.

29 Mix by inverting the tube 20 times. Incubate with gentle agitation using a rotator or a rocker platform for 15 min at room temperature.

30 Place the tube in a magnetic rack for 30 min or more (until the solution becomes clear, can be over an hour).

31 Remove the supernatant without disturbing the beads.

32 Wash the beads by filling the tube with 70% ethanol, let beads settle if disturbed, and pour out ethanol.

33 Repeat the ethanol wash another 3-5 times, until satisfied the beads are clean.

NOTE
If the beads are very dirty, remove the tube from the magnetic rack, resuspend beads by flicking the tube, magnetise until clear, remove supernatant, repeat washing.

34 Transfer the beads to an Eppendorf tube. This can be done in a series of small volume ethanol washes that resuspend the beads, transfer to Eppendorf tube, then place on the magnetic rack, remove supernatant and repeat into the same tube.

35 Remove all traces of ethanol, and let the beads air dry for 1-4 min. Do not let the beads dry completely, they will crack and significantly reduce DNA recovery.

36 Using a wide-bore pipette tip, add 220 µL of ultra-pure H_2O to the beads, gently resuspending. Gently tapping the tube is also suitable.

NOTE
Larger volume based on PippinHT input across x1 whole cassette.

37 Place the tubes in the magnetic rack for ~30 min. Let the solution become clear.

NOTE
Highly concentrated DNA will take a long time. The tube can be left on the magnetic rack overnight, or add more water.

38 Transfer 200 µL of supernatant (contains DNA) to a new tube, avoiding any carry-over of beads.

GEL PURIFICATION: PIPPIN PREP

39 For some species, the DNA may be pure enough for sequencing at this point. However, for most recalcitrant plants, the DNA will still have impurities and DNA fragmentation is inevitable. Gel purification is an ideal solution to both problems.

40 Using a PippinHT (Sage Science) or similar automated electrophoresis product, gel purify approximately 30 µg, following the manufacturer's instructions. A 15 kb high pass separation is recommended, however if DNA is plentiful, 20 kb high pass is more suitable. The PippinHT has 12 lanes, however a lane pair needs be dedicated to an external ladder, leaving 10 lanes for samples. 20 µL of DNA goes into each lane (therefore 200 µL elution in previous section). The manufacturer recommends a maximum 1.5 µg per lane (15 µg total per cassette), however, can be successfully overloaded to 3 µg per lane (perhaps more). The size selection will not be as precise, but is of no concern here.

NOTE

Alternatively, a chloroform: isoamyl alcohol 24:1 clean-up can be performed to remove residual phenols and proteins. Other possibilities (untested), are digestion with proteinase K and another bead clean up. Also would be interesting to experiment with adding PVP to the binding buffer to further reduce polyphenols.

41 After separation, wait at least 45 min (hours or overnight is suitable), to aid elution and recovery.

42 Collect the contents of all elution wells into a DNA LoBind Eppendorf tube (approx. 300 µL).

43 Add 30 µL of 0.1% tween solution to each elution well (provided in kit). Wait at least 10 min and then transfer the contents to the same DNA LoBind Eppendorf tube (another 300 µL, tube total is approx. 600 µL).

44 Add 1.2x binding buffer (approx. 720 µL), and 100 µL of 2% Sera-Mag beads to the DNA LoBind Eppendorf tube. Incubate, magnetise and ethanol wash as previously described in 'DNA extraction from nuclei'.

45 After drying the beads elute with 65 µL of ultra-pure water. Incubate, magnetise and transfer to a new DNA LoBind Eppendorf tube using a wide-bore pipette tip. Expect 20-30% recovery relative to total input (~6-9 µg).

SEQUENCING LIBRARY PREPARATION

46 For sequencing, we adopted the portable MinION sequencer from Oxford Nanopore Technologies. There are two native genomic DNA library preparations available; a rapid transposase based method (SQK-RAD004) and ligation based method (SQK-LSK108 /109). Following the manufacturer's instructions, prepare a library. Note that Oxford Nanopore recommends a mass of DNA optimised for 0.2 pmol (193 fmol). However, this is based on an average length of 8 kb. Therefore, the amount of input DNA needs to be adjusted. The following is recommended based on PippinHT size selection:

Fragment sizes	Rapid SQK-RAD004	Ligation SQK-LSK108 /109
No size selection, ~8 kb	800 ng	4,000 ng
15 kb high pass	1,000 ng	4,000 ng
20 kb high pass	1,200 ng	4,000 ng

47 Load the MinION and perform sequencing according to Oxford Nanopore's instructions. Ensure no bubbles are introduced into the array.

EXPECTED RESULT

Using the protocol described, we have been obtaining clean high molecular weight DNA (Figure 1, Table 1). DNA fragment size has been predominantly 20-140 kb in length (Figure 2). During sequencing, we have been obtaining >9 gigabases from a single MinION flow cell for recalcitrant *Eucalyptus* and *Acacia* species, including reads over 100 kb in length (Table 2, Figure 3). N50 values depend on library preparation method, but can reproducibly obtain N50 values >20 kb using ligation kits. A troubleshooting guide is presented in Figure 4, illustrating the most common problems researchers have.

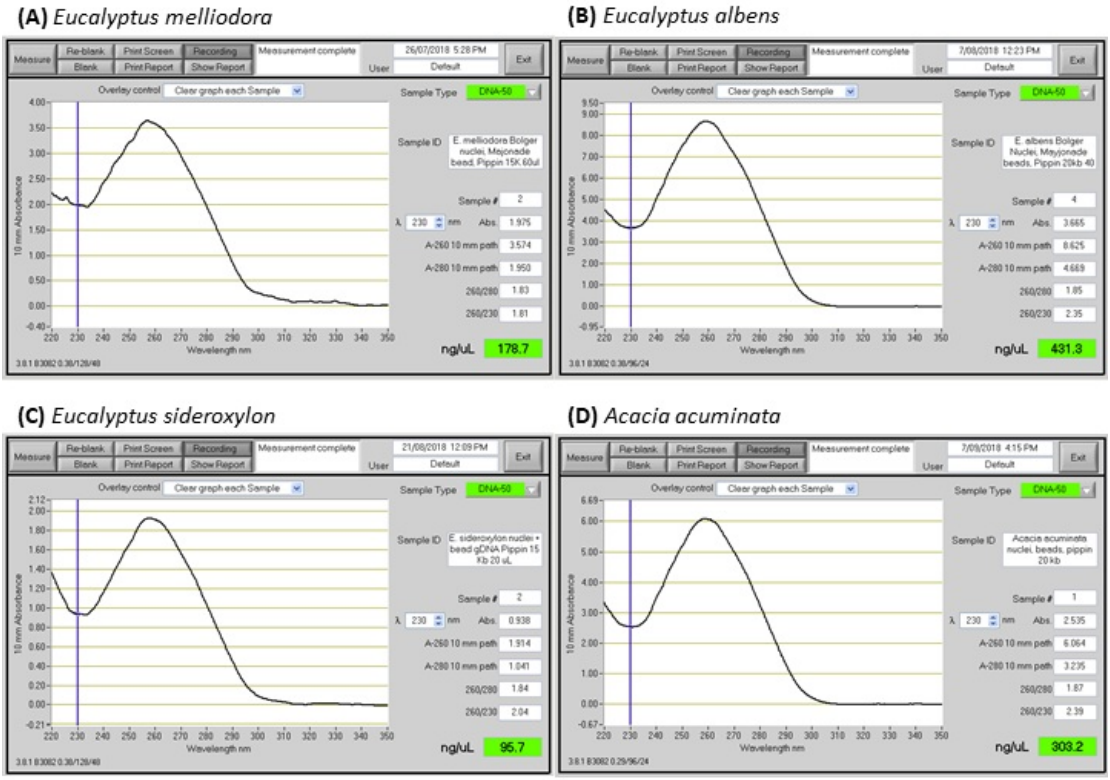


Figure 1: Spectrophotometer results of a nuclear DNA purification performed on recalcitrant *Eucalyptus* and *Acacia* species. Readings taken using 1 µL on a Thermo Scientific Nanodrop 1000.

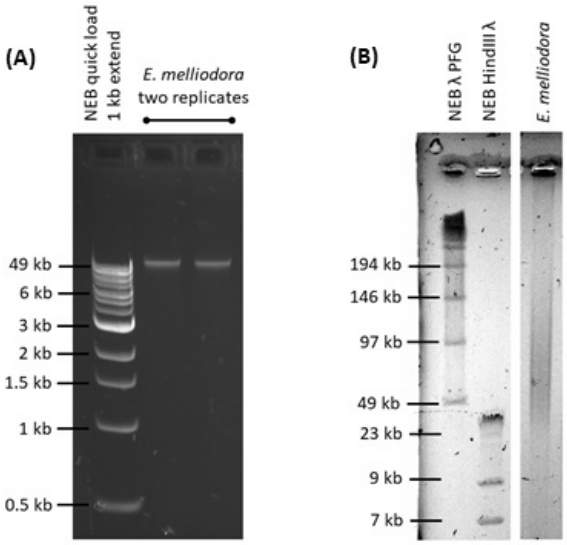


Figure 2: Gel electrophoresis analysis of DNA quality. *Eucalyptus melliodora* is shown as a representative example. (A) 50 ng of DNA separated on a 1% agarose gel. (B) 300 ng of DNA separated by pulsed field gel electrophoresis.

Table 1: DNA quantification and pippin prep. One of two nuclei pellets processed.

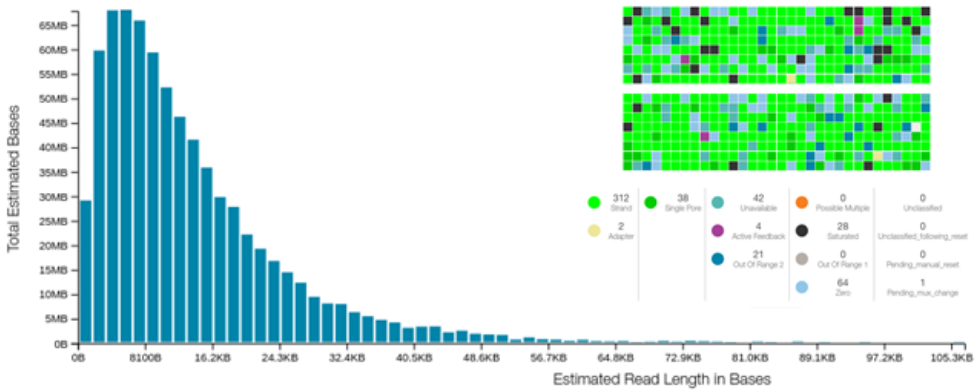
Sample	Approx DNA µg	Pippin input	Pippin setting	Qubit ng/µL	Nano ng/µL	260/280	260/230	Recovery µg
E. melliodora	36	18	15 kb	134	178.7	1.83	1.81	8.0 (44%)
E. marginata	29	29	20 kb	139	170.0	1.88	2.37	8.6 (30%)
E. albens	32	32	20 kb	259	431.3	1.85	2.35	8.8 (28%)
E. sideroxylon	7.6	7.6	15 kb	95.6	95.7	1.84	2.04	1.9 (25%)
A. acuminata	98	39	20 kb	312	303	1.87	2.39	19 (19%)

Table 2: Sequencing results with a MinION flow cell per sample (FLO-MIN 106 R9.4.1 revC), except for the first entry, which shows the results of a PromethION flow cell performed at a sequencing facility. For library input with ligation kits, first number is the initial input, second number is the recovery after all cleans.

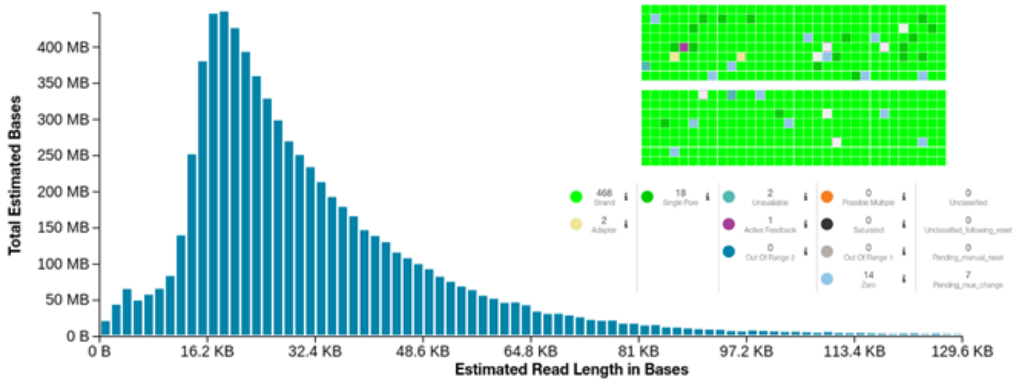
Sample	Pippin prep ≥	Library input µg	Library preparation	Pores	Output Gb	Base call Gb	N50 kb
E. melliodora[1]	20 kb	1/?	PromethION LSK109	2,270	?	34.33	33.01
E. melliodora	15 kb	0.8	Rapid SQK-RAD004	1,313	10.20	9.37	8.05
E. melliodora	15 kb	4/1.1	Ligation SQK-LSK109	1,289	9.39	7.99	22.05
E. marginata	20 kb	1	Rapid SQK-RAD004	1,384	10.10	9.33	11.24
E. marginata	20 kb	4/0.9	Ligation SQK-LSK109	1,325	11.05	9.25	25.86
E. albens[2]	20 kb	1	Rapid SQK-RAD004	1,208	9.26	7.74	9.02
E. albens	20 kb	4/1.1	Ligation SQK-LSK109	1,279	12.50	9.82	24.00
E. sideroxylon	15 kb	1	Rapid SQK-RAD004	1,584	13.15	11.26	7.66
E. sideroxylon[3]	15 kb	0.6	Rapid SQK-RAD004	1,443	6.71	5.95	7.75
A. acuminata	20 kb	4/1.4	Ligation SQK-LSK109	1,229	14.72	10.68	12.23
A. acuminata	20 kb	4/1.4	Ligation SQK-LSK109	1,239	15.85	12.50	13.05

[1]Using 4 µg is recommended (this run was performed externally by sequencing facility staff).
[2]Device disconnected for several hours due to IT issues (lost reads).
[3]Not enough DNA (depleted) but required more coverage. Resulted in lower sequencing yield.

(A) *Eucalyptus melliodora;* rapid transposase library prep (SQK-RAD004). Output: 10.20 Gb.



(B) *Eucalyptus melliodora;* end ligation library prep (SQK-LSK109). Output: 9.39 Gb.



(C) *Eucalyptus albens;* end ligation library prep (SQK-LSK109). Output: 12.50 Gb.

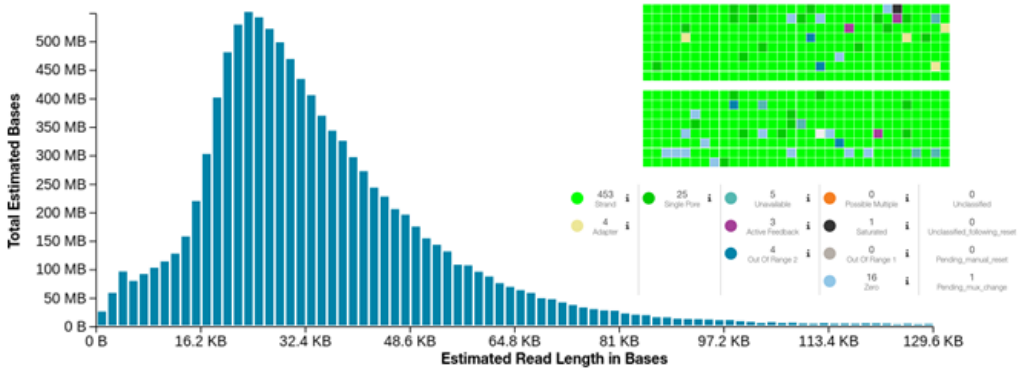
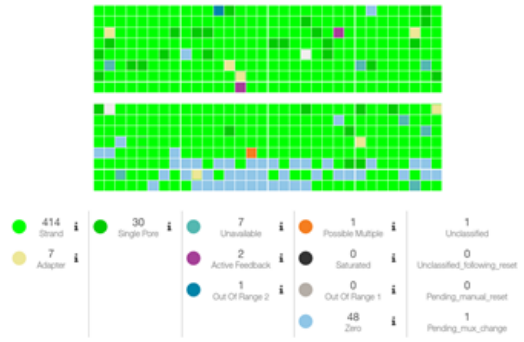


Figure 3: Expected read length histogram on MinKNOW 2.0. An *E. melliodora* DNA prep was processed with rapid transposase (A) and end ligation (B) library preps. (C) *E. albens* processed by ligation library prep. Inserts show pore usage.

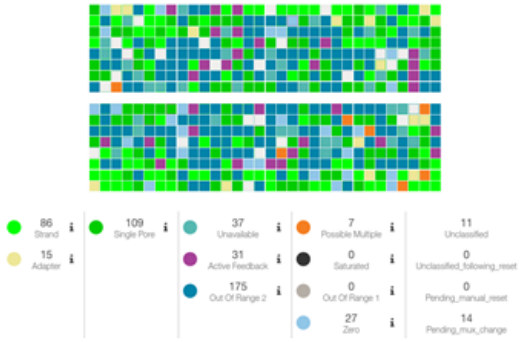
(A)



Air bubble introduced into array during MinION loading. Pipetting all FLB buffer into the priming port is not necessary; ensure no air is introduced. Pull pipette away while dispensing. Gently load SpotON port drop-wise, keeping the pipette tip away from the port.

Expected output: 6-9 Gb.

(B)



More inactive pores (single) than actively sequencing (strand). Check molarity; high number of DNA fragments are necessary to collide with a nanopore. Active feedback and out of range indicates contamination, which may have inhibited the ligation of adapters.

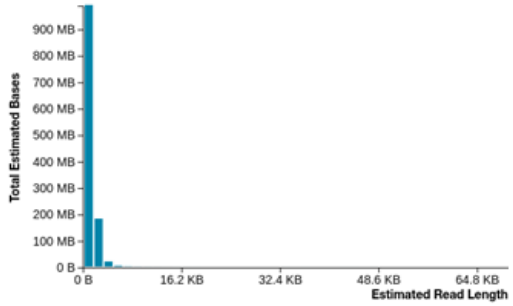
Expected output: 3-6 Gb.

(C)

High amount of contamination, pores are being destroyed. Likely high levels of endogenous phenols and secondary metabolites from plant cells. Check 260/230 value on Nanodrop; should be 2 or higher. Clean DNA further by gel purification or chloroform phase separation.

Expected output: < 1 Gb.

(D)



DNA highly sheared and/or degraded during DNA extraction. All reads < 10 kb. Avoid column-based DNA extractions, vortexing, high concentrations of acids and high temperatures. Beware of contamination with exogenous DNases. There may not be enough adapter to ligate onto too many DNA fragments. Degraded DNA will ligate poorly.

Figure 4: Troubleshooting guide for poorly performing MinION runs. MinION performance is largely dependent on DNA quality, which can inhibit sequencing through the nanopore. Technical issues include air bubbles and undesirable DNA shearing. MinKNOW 2.0 screenshots shown from anonymous colleagues with permission.