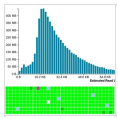

Jul 25, 2019

Nuclear DNA purification from recalcitrant plant species for long-read sequencing V.3

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1 Works for me dx.doi.org/10.17504/protocols.io.28bghsn

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 based nuclei extraction to remove cytoplasmic secondary metabolites, phenols and 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, 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 can approximately obtain over 10 gigabases of sequencing from a single MinION revC flow cell and over 15 gigabases with new revD flow cells, up to 23 gigabases. This includes quality reads over 200 kb in length, average N50 values over 20 kb and some N50 values exceeding 45 kb. Such ultra-long reads assist the assembly of high quality genomes, from telomere to telomere.

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, x11 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 2 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 $MgCl_2$	95.21	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
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	182.65	powder	36.53 g
pH 6 equilibration with HCl	36.46	5 M	5 mL
1 mM Dithiothreitol (DTT)*	154.25	powder	0.154 g

*DTT is unstable, having a very short half-life. Add fresh on the day.

Nuclei wash buffer (2 L for two samples)

- Prepare two lots of 1 L for two samples.
- Adjust to pH 7 (add NaOH; estimate listed below).

Component	MW	Stock	Quantity (1 L) (make twice)
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 $MgCl_2$	95.21	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
0.5% (v/v) Triton X-100	647	10%	50 mL
200 mM L-lysine	182.65	powder	36.53
pH 7 equilibration with NaOH	40.00	5 M	3 mL
1 mM Dithiothreitol (DTT)*	154.25	powder	0.154 g

*DTT is unstable, having a very short half-life. Add fresh on the day.

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)	Quantity (50 mL)
18% Polyethylene glycol (PEG) 8,000	8,000	25%	7.2 mL	36 mL
1 M NaCl	58.44	5 M	2 mL	10 mL
10 mM TRIS-HCl pH 8.0	121.14	1 M	100 µL	500 µL
1 mM EDTA pH 8.0	292.24	0.5 M	20 µL	100 µL
0.05% Tween 20	1,227.54	10%	50 µL	250 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 1 mL H_2O, flick tube, repeat.
- Resuspend the clean Sera-Mag beads in 600 µL nuclease-free water. Transfer into 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)

- 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.

Component	MW	Stock	Quantity (50 mL)	Quantity (1 L)
20% Polyethylene glycol (PEG) 8,000	8,000	powder	10 g	200 g
3 M NaCl	58.44	powder	8.75 g	175 g

Other solutions

- Triton X-100 (ideally 10% solution)
- Ethanol (100% and 70%)
- RNAse A (20 mg/mL Invitrogen PureLink)
- Proteinase K (20 mg/mL NEB P8107S)
- 5 M Potassium acetate
- Nuclease-free water (UltraPure™ ThermoFisher 10977015)

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)

- Magnetic rack (for 15 mL Falcon and 1.5 mL 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)

SKIPPING NUCLEI: ALTERNATIVE LYSIS (optimistic)

1 For soft tissue with with a high cell density, approximately 1 g (0.5-1.5 g) of tissue can be ground in a mortar and pestle, keeping frozen with liquid nitrogen. Then proceed to 'DNA EXTRACTION FROM NUCLEI', using the ground tissue instead of a nuclei pellet. For recalcitrant species, purify nuclei as detailed below from 'BLENDER HOMOGENISATION'.

This has been trialled with wild and cultivated rice, being hilghly successful (400 and 900 Mb genomes). Eucalyptus will not have a sufficient yield and will be highly contaminated.

BLENDER HOMOGENISATION (4°C cold room)

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

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

3 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).

4 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)

5 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.

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

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

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

8 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

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

9

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

Use this time to clean the blender and space used for filtering. Cool the the centrifuge to 4°C.

11 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.

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

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

13 Discard the supernatant and add up to 450 mL of nuclei wash buffer per Nalgene bottle (save 100 mL).

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

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

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

16 Discard supernatant and repeat with another ~450 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.

2 L of nuclei wash buffer is suitable for x2 samples to be washed x2 times with 450 mL each, then a final resuspension with 100 mL each.

17 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.

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

19 Transfer supernatant into new 50 mL Falcon tubes and centrifuge at 1,000 rcf and 4°C for 30 minutes.

Optional. Recovers a small pellet that can be added to the larger pellet or used for other applications like HiC library preps.

20 The nuclei pellets can now be stored -80°C.

DNA EXTRACTION FROM NUCLEI

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

The solution should be clear before use.

22 To a 3-5 mL nuclei pellet, add 10 mL of lysis buffer. Vortex vigorously for 1 min.

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

23 Add RNAse A and Proteinase K to the solution. Mix by swirling and inverting.

Enzyme	Stock	Quantity (10 mL lysis buffer)
400 µg/mL RNAse A	20 mg/mL	200 µL
100 µg/mL Proteinase K	20 mg/mL	50 µL

Proteinase K and RNAse A can co-exist in the same solution, even in the presence of EDTA.

- *RNAse A is highly resistant to proteolysis by Proteinase K.*
- *Activity of RNAse A and Proteinase K is not dependent on ion cofactors, EDTA has no effect.*
- *Both are recommended to be 50-100 µg/mL.*
- *Qiagen DNeasy plant kits use as much as 2,000 µg/mL RNAse A.*
- *RNAse T has less DNase activity (still present), but was less effective at degrading RNA.*
- *RNAse A is active at temperatures15-70°C (optimal at 60°C), pH 6-10 (optimal pH 7.6).*
- *Proteinase K is active at temperatures 20-60°C (optimal 50-60°C), pH 4-12 (optimal pH 8).*
- *Proteinase K degrades proteins in the presence of detergents, including SDS.*
- *Proteinase K activity is stimulated if up to 2% SDS or 4 M urea is present in the reaction.*

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

Higher temperatures and longer incubations lead to DNA damage.

25 Add 1/3 volume (4-5 mL) of 5 M Potassium Acetate and mix by inverting to precipitate the proteins and the polysaccharides that will complex with SDS.

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

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

28 Transfer supernatant to a new tube (15 mL Falcon), centrifuge again at 5,000 rcf for 10 min at 4°C.

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

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

Beads are in excess and could be reduced.

31 Mix by inverting the tube 20 times. Incubate with gentle agitation using a rotator or a shaker platform for 1 h at room temperature.

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

33 Remove the supernatant without disturbing the beads.

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

35 Repeat the ethanol wash another 2 times, or until satisfied the beads are clean.

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.

36 Transfer the beads into a 2 mL Eppendorf tube. This can be done by taking a Falcon tube off the rack, adding 1 mL of ethanol to dislodge the beads, transfer suspension to an Eppendorf tube, repeat with another 1 mL. Repeat proces with the other Falcon tube into another Eppendorf tube. Place the 2 mL Eppendorf tubes on the magnetic rack, remove supernatant and repeat the process until all beads are in the same tube.

37 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.

38 Using a wide-bore pipette tip, add 220 µL of nuclease-free water to the beads, gently resuspending. Gently tapping the tube is also suitable. Incubate at room temperature for at least 10 min.

Larger volume based on PippinHT input across x1 whole cassette.

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

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

40 Transfer 200-220 µL of supernatant (contains DNA) to a DNA LoBind eppendorf tube, avoiding any carry-over of beads.

41 Add an additional 100 µL of nuclease-free water to the beads, remove from magnet, resuspend, incubate, magnetise and transfer to a new 1.5 mL Eppendorf tube.

Save this second elution for other applications such as running a pulse field gel.

42 Quantify the DNA on a Nanodrop and a Qubit fluorometer (dsDNA broad-range assay) (both instruments from Thermo Fisher Scientific). The DNA is still crude and will likely have other contaminants present.

- *For Nanodrop, use 1 µL. However, as the DNA is crude, it will give a large over-estimation of concentreation.*
- *Qubit fluorometer is the most reliable. However, it is highly dependent on the accuracy of the amount pipetted. Use 2 µL when sample is plentiful. Using 1 µL is prone to pipetting errors.*
- *Expect 20-100 µg of DNA.*

43 Store DNA at 4°C to prevent cycles of freeze-thawing that shear the DNA.

No effects on DNA integrity have been noticed for samples stored at 4°C for 3 months.

GEL PURIFICATION: PIPPIN PREP

44 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.

45 If a precipitant is noticebale in the DNA solution, briefly spin down and take the upper aqueous layer to gel purification below.

For some samples, this may be carbohydrates, which will be removed during gel purification. DNA is in the aqueous layer. It is possible 4°C promotes this precipitation.

46 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.

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.

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

48 Collect the contents of all elution wells into a 1.5 mL DNA LoBind Eppendorf tube (approx. 300 µL).

49 Add 30 µL of 0.1% tween in electrophoresis buffer to each elution well (provided in kit). Wait for 5 min and then transfer the contents to the same 1.5 mL DNA LoBind Eppendorf tube (another 300 µL, tube total is approx. 600 µL).

50 Add 1.2x binding buffer (approx. 720 µL), and 100 µL of 2% Sera-Mag beads to the 1.5 mL DNA LoBind Eppendorf tube. Incubate for 5-10 min at room temperature.

51 Place on a magnetic rack for 5 min, or until the solution becomes clear. Discard the supernatant.

52 Keeping the tube on the magnetic rack, add 1 mL of freshly prepared 70% ethanol. Discard the ethanol and repeat for a second ethanol wash.

53 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.

54 Remove the tube from the magnetic rack and elute with 60 µL of nuclease-free water. Incubate for 10 min at room temperature.

A maximum DNA volume of 48 µL is used in an Oxford Nanopore ligation prep (e.g. SQK-LSK109).

55 Place on a magnetic rack for 5 min, or until the solution becomes clear. Transfer eluted DNA to a new 1.5 mL DNA LoBind Eppendorf tube using a wide-bore pipette tip.

56 Add an additional 60 µL of nuclease-free water to the beads, remove from magnet, resuspend, incubate, magnetise and transfer to a new 1.5 mL Eppendorf tube.

Save this second elution for other applications such as running a pulse field gel.

57 Quantify the DNA on a Nanodrop and a Qubit fluorometer (dsDNA broad-range assay) (both instruments from Thermo Fisher Scientific). The DNA should be pure, free of contaminants.

- *For Nanodrop, use 1 µL. Qubit fluorometer is highly dependent on the accuracy of the amount pipetted. Use 2 µL when sample is plentiful. Using 1 µL is prone to pipetting errors.*
- *For pure DNA, Nanodrop: Qubit quantifications are 1:1.*
- *Expect 20-30% recovery relative to total input (~6-9 µg out of 30 µg).*

58 Store DNA at 4°C to prevent cycles of freeze-thawing that shear the DNA.

No effects on DNA integrity have been noticed for samples stored at 4°C for 3 months.

SEQUENCING LIBRARY PREPARATION

59 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 a ligation based method (SQK-LSK109). 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 our sequencing experiences realtive to PippinHT size selection:

Fragment sizes	Rapid SQK-RAD004	Ligation SQK-LSK109
Amplicon or digest (~1 kb)	Not recommended	200 ng
No size selection (~8 kb)	800 ng	2,000 ng
15 kb gel purification	1,000 ng	3,000 ng
20 kb gel purification	1,200 ng	3,000 ng
30 kb gel purification	Not recommended	4,000 ng
40 kb gel purification	Not recommended	5,000 ng

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

Air bubbles destroy nanopores, significantly reducing sequencing yield.

61 **Expected Results**

Using the protocol described, we have been obtaining clean high molecular weight DNA (Figure 1, Table 1). DNA fragment size ranges from 20-200 kb in length (Figure 2). During sequencing, we can approximately obtain over 10 gigabases of sequencing from a single MinION revC flow cell, and over 15 gigabases with new revD flow cells, up to 23 gigabases (Table 2, Figure 3, Figure 4). This includes quality reads over 200 kb in length. N50 values depend on library preparation method, but can reproducibly obtain N50 values > 20 kb using ligation kits, and can exceed N50 of 45 kb. A troubleshooting guide is presented in Figure 5, illustrating the most common problems researchers have.

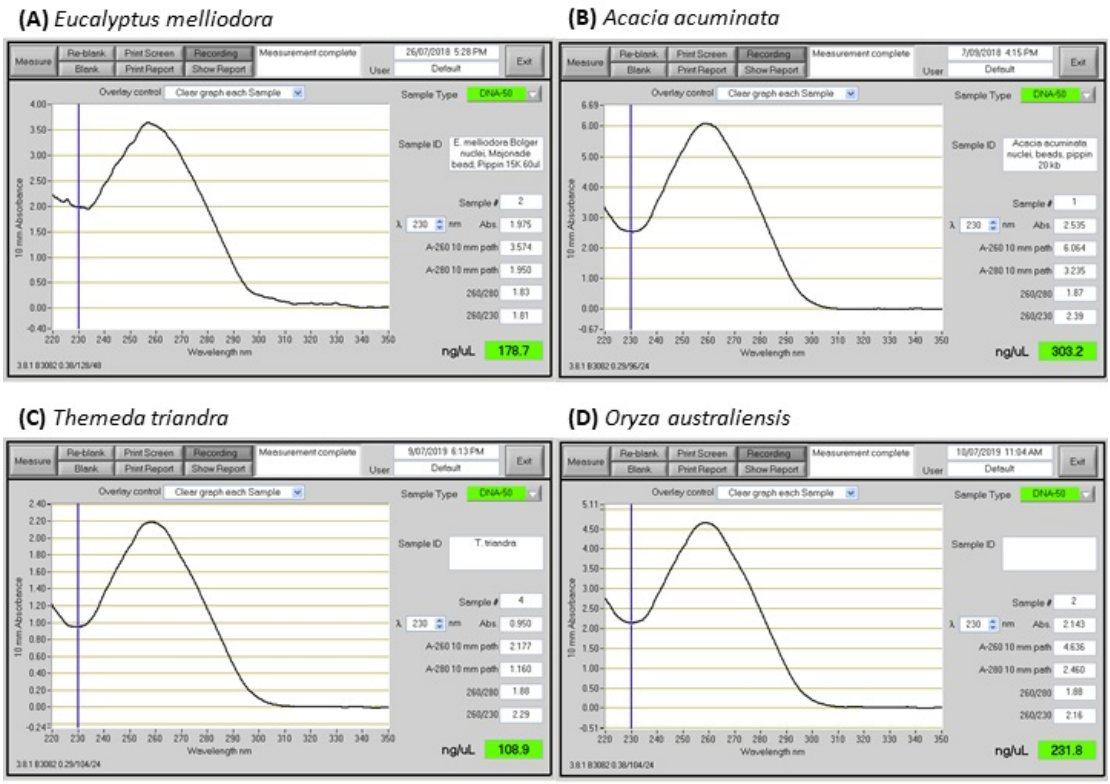


Figure 1: Spectrophotometer results of a nuclear DNA purification performed on four recalcitrant plant species from different genera. Readings taken using 1 µL on a Thermo Scientific Nanodrop 1000.

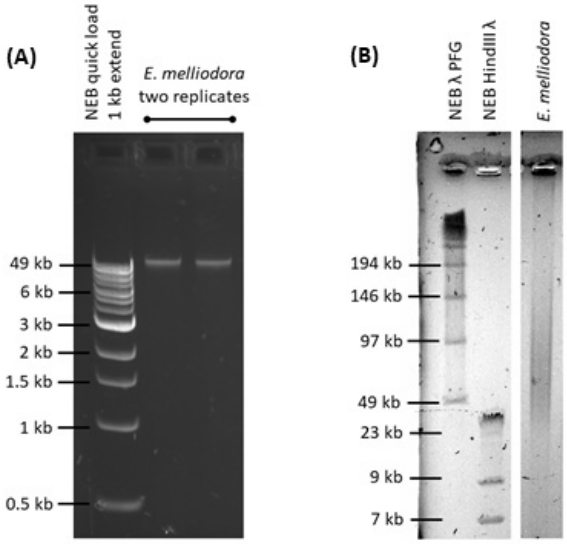


Figure 2: Gel electrophoresis analysis of DNA quality. *Eucalyptus melliodora* is shown as a representative example. (A) 50 ng of DNA separated on a 1% agarose gel. (B) 300 ng of DNA separated by pulsed field gel electrophoresis.

Table 1: Pippin prep and DNA quantification on four different genera. One of two nuclei pellets processed.

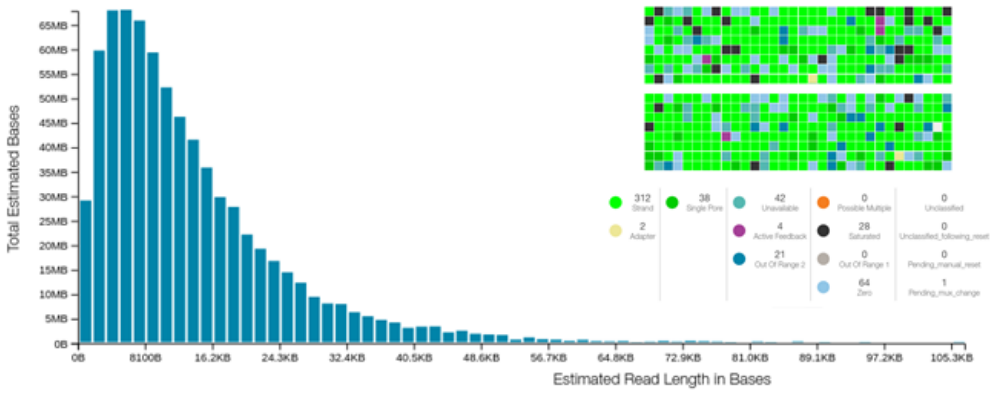
Sample	Approx DNA µg	Pippin input	Pippin setting	Qubit ng/µL	Nano ng/µL	260/ 280	260/ 230	Recovery µg
E. melliodora	36	18	15 kb	134	178.7	1.83	1.81	8.0 (44%)
A. acuminata	98	39	20 kb	312	303	1.87	2.39	19 (19%)
T. triandra	60	30	30 kb	110	109	1.88	2.29	5.5 (18%)
O. australiensis	35	35	20 kb	240	232	1.88	2.16	12 (34%)

Table 2: Sequencing results with a single MinION flow cell per sample (FLO-MIN 106 R9.4.1 revC or revD). For library input with ligation kits, first number is the initial input, second number is the recovery after all cleans (amount loaded on flow cell array).

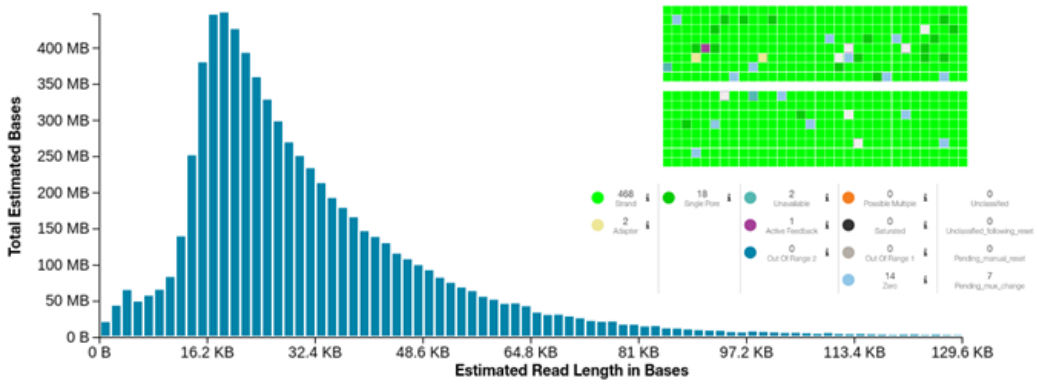
Sample	Pippin prep ≥	Library input µg	Library preparation	Cell	Pores	Output Gb	Base call Gb	N50 kb
E. melliodora	15 kb	4/1.1	Ligation SQK-LSK109	revC	1,289	9.39	7.99	22.05
E. melliodora	15 kb	0.8	Rapid SQK-RAD004	revC	1,313	10.20	9.37	8.05
E. sideroxylon	15 kb	1	Rapid SQK-RAD004	revC	1,584	13.15	11.26	7.66
E. marginata	20 kb	1	Rapid SQK-RAD004	revC	1,384	10.10	9.33	11.24
E. albens	20 kb	4/1.1	Ligation SQK-LSK109	revC	1,279	12.50	9.82	24.00
E. globulus	20 kb	4/1.7	Ligation SQK-LSK109	revC	1,446	12.1	9.52	25.69
E. viminalis	20 kb	4/1.7	Ligation SQK-LSK109	revC	1,432	12.27	10.38	22.74
E.camaldulensis	20 kb	3/1.5	Ligation SQK-LSK109	revD	1,258	12.79	11.40	24.43
E. microcorys	20 kb	3/1.0	Ligation SQK-LSK109	revD	1,556	22.99	19.9	23.98
E. cloezania	20 kb	1.5/0.8	Ligation SQK-LSK109	revD	1,381	10.23	9.51	25.15
E. erythrocorys	20 kb	3/1.1	Ligation SQK-LSK109	revD	1,589	12.78	10.90	36.60
E. salubris	20 kb	1.2/0.9	Ligation SQK-LSK109	revD	1,167	14.01	12.12	29.05
E. decipiens	20 kb	3/1.4	Ligation SQK-LSK109	revD	1,106	9.03	8.52	29.61
E. virginea	20 kb	3/1.1	Ligation SQK-LSK109	revD	1,059	12.87	11.03	29.46
E. brandiana	20 kb	2.3/1.1	Ligation SQK-LSK109	revD	1,182	18.64	16.22	30.55
E. tenuipes	40 kb	7/2.0	Ligation SQK-LSK109	revD	1,634	9.99	9.24	45.32

A. floribunda	20 kb	2.4/0.7	Ligation SQK-LSK109	revD	1,383	16.54	13.61	26.63
C. maculata	20 kb	2.9/1	Ligation SQK-LSK109	revD	1,448	16.50	13.18	31.63
C. calophylla	15 kb	3/1.2	Ligation SQK-LSK109	revD	1,380	16.64	13.52	24.06
A. acuminata	20 kb	4/1.4	Ligation SQK-LSK109	revC	1,239	15.85	12.50	13.05
T. triandra	30 kb	1.8/0.8	Ligation SQK-LSK109	revD	1,309	10.20	9.60	32.8
O. sativa	20 kb	3/1.23	Ligation SQK-LSK109	revD	1393	18.68	13.95	11.6
O. meridionalis	20 kb	3/1.4	Ligation SQK-LSK109	revD	1359	11.34	10.89	34.39
O. australiensis	20 kb	3/1.1	Ligation SQK-LSK109	revD	1466	17.19	16.39	45.05

(A) *Eucalyptus melliodora;* 15 kb size selection, transposase prep (SQK-RAD004). Output: 10.20 Gb, N50 8 kb.



(B) *Eucalyptus melliodora;* 15 kb size selection, ligation prep (SQK-LSK109). Output: 9.39 Gb, N50 22 kb.



(C) *Themeda triandra;* 30 kb size selection, ligation prep (SQK-LSK109). Output: 10.20 Gb, N50 33 kb .

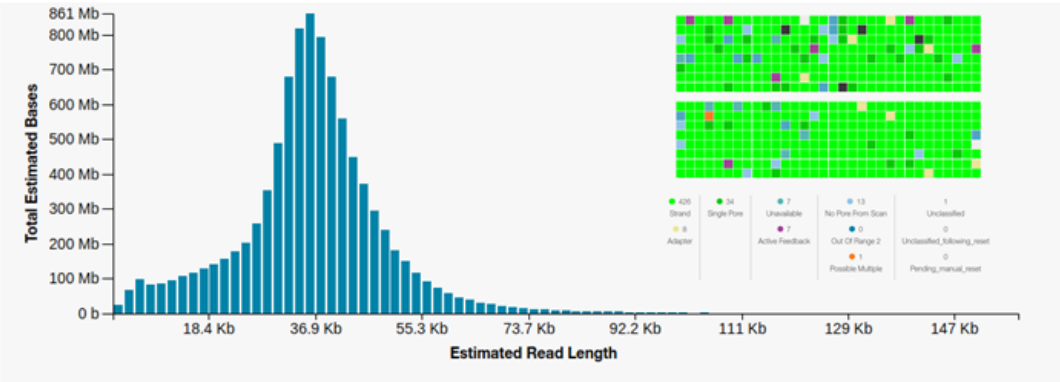
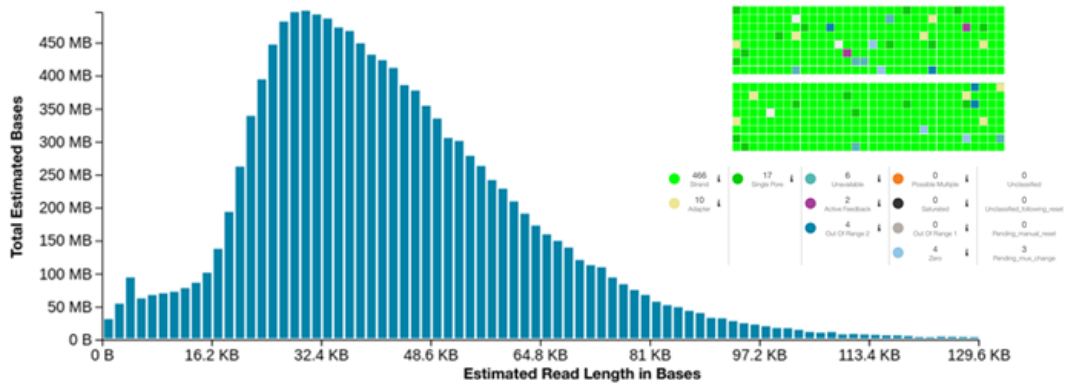
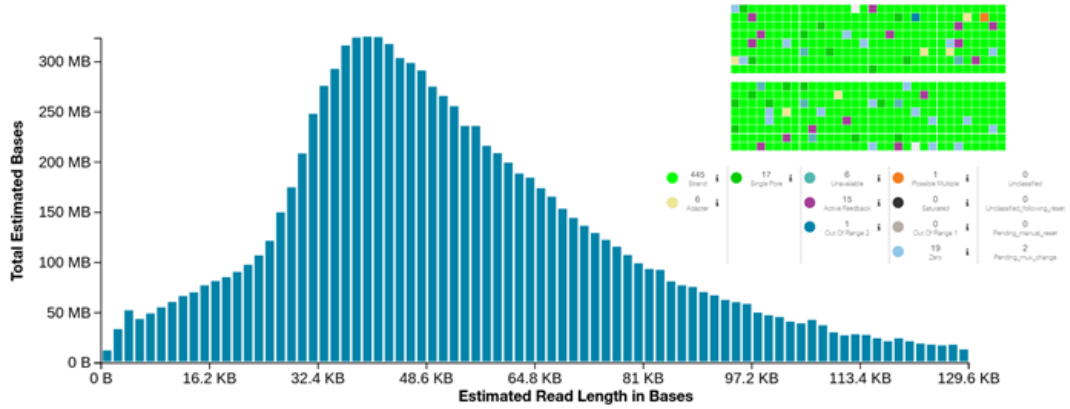


Figure 3: Expected read length histograms on MinKNOW 2.0. An *Eucalyptus melliodora* DNA prep was size selected for 15 kb and above then processed with a rapid transposase (A) and end ligation (B) library preps. (C) *Themeda triandra* size selected for 30 kb and above, then processed by a ligation library prep. Inserts show pore usage, light green indicates pore is active and sequencing is occurring.

(A) *Corymbia maculata;* 20 kb size selection, ligation prep (SQK-LSK109). Output: 16.50 Gb, N50 32 kb.



(B) *Eucalyptus tenuipes;* 40 kb size selection, ligation prep (SQK-LSK109). Output: 9.99 Gb, N50 45 kb.



(C) *Oryza australiensis;* 20 kb size selection, ligation prep (SQK-LSK109). Output: 17.19 Gb, N50 45 kb.

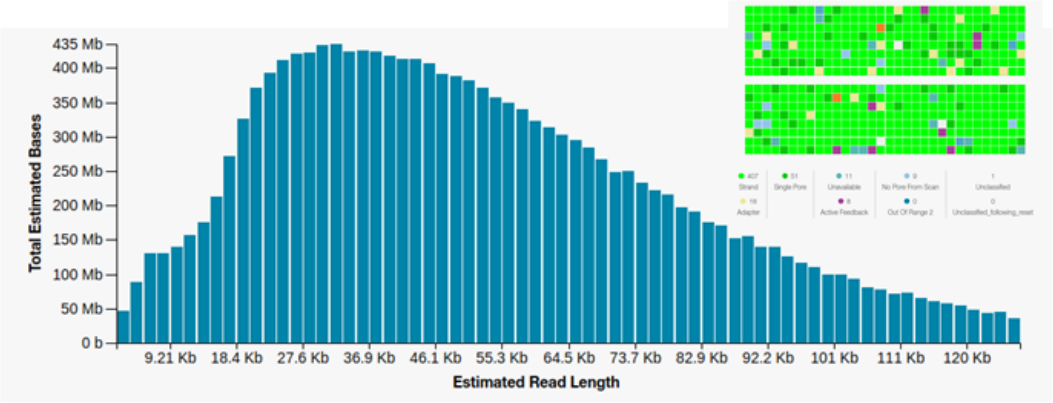
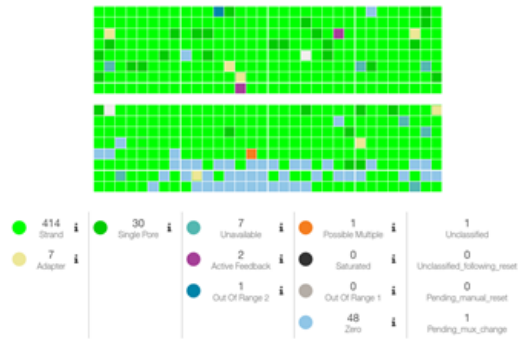


Figure 4: Optimal read length histograms on MinKNOW 2.0. (A) *Corymbia maculata,* size selected for 20 kb, yielding a high output and higher than expected N50. (B) *Eucalyptus tenuipes,* size selected for 40 kb and above, highly increased N50 however decreased overall yield. (C) *Oryza australiensis,* size selected for 20 kb, yielding high output and high N50. Inserts show pore usage, light green indicates pore is active.

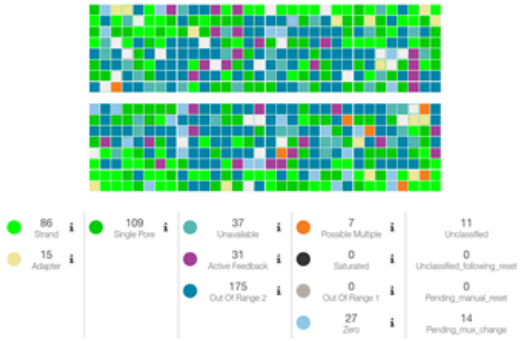
(A)



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.

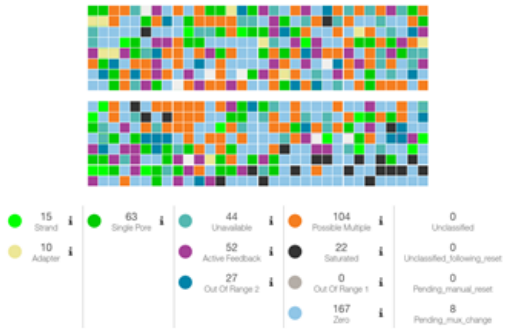
(B)



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.

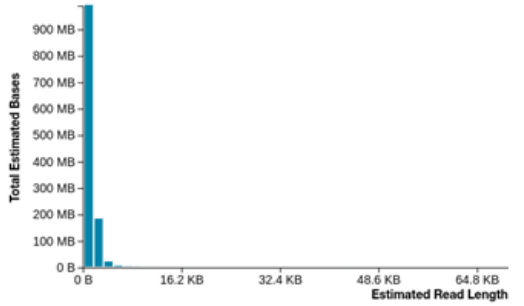
(C)



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 5: 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.