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High-molecular weight DNA extraction from challenging fungi using CTAB and gel purification V.2

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High molecular weight DNA extraction from all kingdoms
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We use this protocol and it's working

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Abstract

Extracting pure high-molecular weight DNA from some fungal species is difficult due to the presence of polysaccharides and potentially other compounds which biochemically mimic DNA or interfere with the DNA extraction process. Such compounds can co-elute with DNA in many extraction methods, being difficult to separate fom the DNA. Although the contaminant may not be detected by spectrophotometers or fluorometric devices, it substantially interferes with long-read DNA sequencing, such as Oxford Nanopore Technologies. To partially resolve this, a protocol is presented with some updates to current strategies and incorporates a gel purification with a Pippin Prep (Sage Science). Using this protocol, we have been successfully sequencing the wheat stripe rust *Puccinia striiformis* and leaf rust *Puccinia triticina* with a MinION (Oxford Nanopore Technologies). Sequencing yields have surpassed 4 gigabases with an N50 of approximately 30 kb. To increase sequencing output, more work is needed to identify and remove the elusive contaminants.

Guidelines

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

This protocol is based on the following protocols below.

Nagar, R. and Schwessinger, B. (2018). Multi-step high purity high molecular weight DNA extraction protocol from challenging fungal tissues. *Protocols.io*

Arseneau et al. (2017). Modified low-salt CTAB extraction of high-quality DNA from contaminant-rich tissues. *Molecular Ecology Resources* **17**(4), 686-693.

Xin, Z. and Chen, J. (2012). A high throughput DNA extraction method with high yield and quality. *Plant Methods* **8**, 26.

Materials

Reagents

AMPure XP beads (Agencourt) (or equivalent)
Cetrimonium bromide (CTAB)
Chloroform: isoamyl alcohol (24:1)
Ethylenediaminetetraacetic acid (EDTA)
Ethanol (70%)
Sodium chloride (NaCl)
Proteinase K (20 mg/mL)
Polyvinylpyrrolidone (PVP) 40,000
RNAse A (20 mg/mL)
Trisaminomethane hydrochloride (Tris-HCl) pH 8
Water, high-purity (e.g. Milli-Q system)
Water, nuclease-free

Special Equipment

1.5 mL DNA LoBind Eppendorf tube (optional)
Acid-washed autoclaved sand (fine)
Centrifuge for 50 mL Falcon tubes (up to 16,000 rcf)
Magnetic rack for 1.5 mL Eppendorf tubes
Mortar and pestle
PippinHT (Sage Science) or equivalent gel-purification system
PippinHT 0.75% agarose casette and 15-20 kb kit (Sage Science)
Water bath

PREPARATION

1 Prepare up to 600 mg of fungal spores, taking into consideration the genome size (larger genome would need less spores). Keep frozen in liquid nitrogen.

2 Set a water bath to 55°C. This will be used to dissolve 2% CTAB solutions and to preciptate DNA with CTAB.

3 Freshly prepare lysis and precipitation buffers.

Lysis buffer

- 5 mL per 100 mg of sample.
- 600 mg of sample is recommended for challenging fungi with a small genome (~50 MB).
- Therefore a total 30 mL of lysis buffer is required.

	Component	MW	Stock	Quantity (5 mL)	Quantity (30 mL)
	2% CTAB (w/v)	364.45	powder	0.10 g	0.60 g
	100 mM Tris-HCl (pH 8)	157.60	1 M	0.50 mL	3 mL
	20 mM EDTA (pH 8)	292.24	0.5 M	0.20 mL	1.20 mL
	1.2 M NaCl	58.44	5 M	1.20 mL	7.20 mL
	1% PVP	40,000	10%	0.50 mL	3 mL
	Milli-Q water	-	-	2.60 mL	15.60 mL

Note

- *EDTA is a metal ion chelator. By binding to Mg^{2+}, DNase activity is stopped as it is dependent on Mg2+.*

Precipitation buffer

- 10 mL per 100 mg of sample.

- 60 mL is recommended to process 600 mg of sample.
- Prepare 30 mL twice in two separate 50 mL Falcon tubes.

	Component	MW	Stock	Quantity (10 mL)	Quantity (30 mL) (prepare twice)
	2% CTAB (w/v)	364.45	powder	0.20 g	0.60 g
	100 mM Tris-HCl (pH 8)	157.60	1 M	1 mL	3 mL
	20 mM EDTA (pH 8)	292.24	0.5 M	0.40 mL	1.20 mL
	Milli-Q water	-	-	8.60 mL	25.80 mL

Note

The DNA extraction process appeared unaffected when the lysis and precipitation buffers were not adjusted to pH 8. Note Tris-HCl and EDTA stock solutions are already at pH 8.

4 Vortex lysis and precipitation buffers, then place both in the 55°C water bath to dissolve CTAB. Further vortexing or inverting may be needed to dissolve all CTAB. Leave buffers at 55°C until needed.

SAMPLE LYSIS

5 Place a sterile mortar and pestle into an insulated container. Pour liquid nitrogen into the container to chill the mortar and pestle. Add 1-2 g of acid-washed autoclaved sand to the mortar.

Note

- *The lid of an ice box can be used. If liquid nitrogen is later poured directly onto the spores, spores disperse all over the pestle and potentially the table.*
- *For less than 500 mg of spores, add 1 g of sand . For 500 mg or more spores, use 2 g of sand.*

6 Add spores to the mortar and grind for approximately 1-2 min. Keep the mortar and pestle chilled by having liquid nitrogen within the surrounding container.

7 Transfer the ground spores and sand to the lysis buffer.

8 Add RNAse A and Proteinase K to the solution:

	Enzyme	Stock	Quantity (5 mL lysis buffer)	Quantity (30 mL lysis buffer)
	100 µg/mL RNAse A	20 mg/mL	25 µL	150 µL
	100 µg/mL Proteinase K	20 mg/mL	25 µL	150 µL

Note

- *RNAse A was added earlier than previous protocols and concentration doubled as RNA has been still present after extraction. Athough RNAse A concentration is generally reccomended to be 1-100 µg/mL, Qiagen DNeasy plant kits use as much as 2,000 µg/mL. RNAse T has less DNase activity (still present), but was less effective at degrading fungal RNA.*
- *RNAse A activity is not Mg^{2+} dependent and doesn't appear to require metal ions as cofactors. Similarily, Proteinase K is still active in EDTA, as the two binding sites for Ca^{2+} are not directly involved in the proteolysis catalytic mechanism.*
- *Proteinase K and RNAse A can co-exist in the same solution. RNAse A is highly resistant to proteolysis by Proteinase K. Both are recommended to be 50-100 µg/mL. 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).*

9 Vortex and invert the solution. Incubate at 55°C for at least 1 h.

CHLOROFORM CLEAN-UP

10 If the sample occupies more than 50% of the tube capacity, split the sample evenly across two 50 mL Falcon tubes (or appropriate sized tube).

11 Add an equal volume of chloroform: isoamyl alcohol (24:1, v/v) and mix by inverting 10-15 times. Ensure the organic and aqueous phases become mixed at least temporarily.

Note

Approximately 5 mL per 100 mg of sample processed.

12 Separate the phases by centrifuging at 5,000 rcf for 10 min at 20°C. Transfer the upper aqueous phase to a new 50 mL Falcon tube.

Note

Chloroform is denser than water, will mix with the oragnic phase which settles to the bottom and the aqueous phase is at the top. Some protocols recommend centrifuging at 12,000 rpm. Not tested, however if centrifugation hasn't been sufficient the interphase will be cloud-like and poorly compacted, with oraganic substances still in the aqueous phase.

13 Repeat the chloroform: isoamyl alcohol clean (equal volume).

DNA PRECIPITATION

14 Add 2 volumes of precipitation buffer, mix by inverting. Incubate at 55°C for 1 h or until white crystals of CTAB-DNA complex can be observed floating inside the tubes.

15 Centrifuge at 16,000 rcf for 10 minutes at 20°C to pellet crystals.

Note

Could spin at 5,000 rcf for 5 min to pellet only high-molecular weight DNA.

16 Carefully decant the supernatant as soon as possible, without disturbing the pellet. Care must be taken as the pellet is fragile, being easily dislodged.

17 Add approximately 10-15 mL freshly prepared 70% ethanol, enough to cover the pellet. Let the pellet soak for 15 min at room temperature to dissolve excess salts and CTAB.

18 Centrifuge at 16,000 rcf for 5 min. Carefully decant the supernatant as soon as possible, without disturbing the pellet.

19 Air-dry the pellet for 10-15 min, or until all ethanol has evaporated.

20 Dissolve DNA with a maximum of 200 µL nuclease-free water. If the sample was previously split across two 50 mL Falcon tubes, add 100 µL to each. Avoid pipette mixing as much as possible to prevent DNA shearing. Gentle flicking of the tube and incubating at room temperature is ideal. Wide-bore pipette tips are also an option.

Note

- *Volume is based on loading the sample across a whole PippinHT (Sage Science) casette at later steps.*
- *If planning to deviate from this protocol and perform other clean-ups or enzymatic digestions, it is recommended to dissolve the pellet in 50-500 µL of 10 mM Tris (pH 8.0). This keeps the DNA buffered and in solution.*

21 Transfer DNA to a 1.5 mL DNA LoBind Eppendorf tube. Combine any samples that were previously split.

22 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 RNA and other contaminants present.

Note

For Nanodrop, use 1 µL. However, Qubit fluorometer accuracy 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.

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

Note

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

GEL PURIFICATION: PIPPIN PREP

24 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.

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

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

27 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).

28 Add 1.2x volume of AMPure XP beads (approx. 720 µL). Incubate at room temperature for 5 min.

Note

Alternatively, add 1.2x binding buffer (approx. 720 µL), and 100 µL of 2% Sera-Mag beads.

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

30 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.

31 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.

32 Remove the tube from the magnetic rack and elute with 50 µL of nuclease-free water. Incubate for 10 min at room temperature.

Note

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

33 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.

34 Add an additional 50 µL of nuclease-free water to the beads, remove from magnet, resuspend, incubate, magnetise and transfer to a new 1.5 mL Eppendorf tube.

Note

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

35 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.

Note

- *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 10-30% recovery relative to total input.*

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

Note

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

SEQUENCING LIBRARY PREPARATION

37 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

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

Note

Air bubbles destroy nanopores, significantly reducing sequencing yield.

39 **EXPECTED RESULTS**

Using the protocol described, we have been obtaining clean high-molecular weight DNA (Table 1, Figure 1). DNA fragment size ranges are above 10 kb in length (Figure 2). During sequencing, we can approximately obtain over 4 gigabases of sequencing from a single MinION revD flow cell (Table 2, Figure 3). This includes quality reads over 100 kb in length and N50 values of approximately 30 kb. An elusive contaminant is present amongst the DNA, which is affecting sequencing yield. More work is needed to identify and remove the contaminant, to increase sequencing yield. A troubleshooting guide for MinION sequencing is presented in Figure 4, illustrating the most common problems researchers have.

Table 1: DNA quantification before and after gel purification. Approximately 600 mg spores in extraction.

Sample	Pippin input	Pippin setting	Qubit ng/µL	Nano ng/µL	260/280	260/230	Volume µL	Yield µg (Qubit)
Stripe rust crude DNA	NA	NA	59.70	1,753	2.18	2.48	200	11.94
Stripe rust purified DNA	11.00	20 kb	34.60	47.6	1.85	2.35	50	1.73
Leaf rust crude DNA	NA	NA	51.70	1,997	2.19	2.52	200	10.34
Leaf rust purified DNA	10.00	20 kb	50.60	55	1.97	1.67	50	2.53

(A) Stripe rust *Puccinia striiformis* **(B)** Leaf rust *Puccinia triticina*

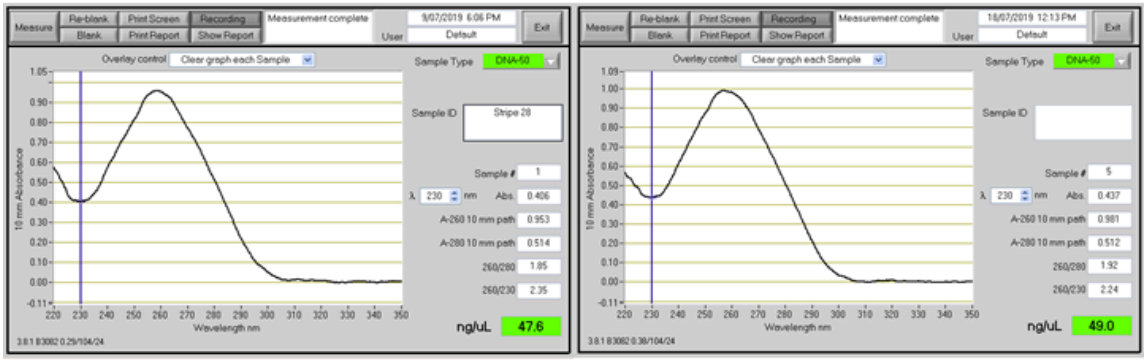


Figure 1: Spectrophotometer results of purified DNA for two recalcitrant fungal species. Readings taken using 1 µL on a Thermo Scientific Nanodrop 1000.

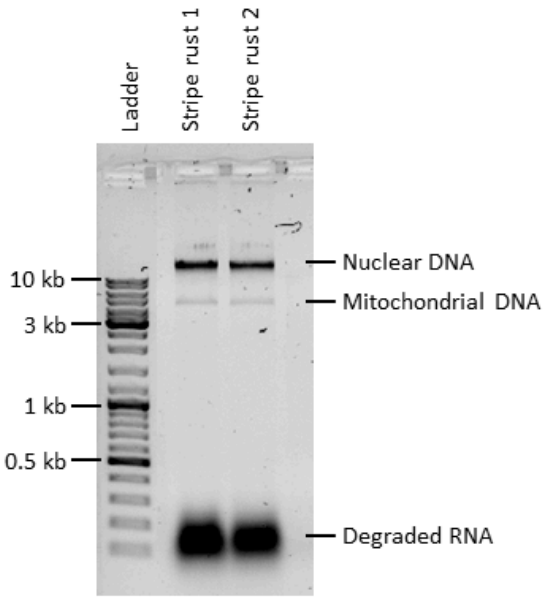
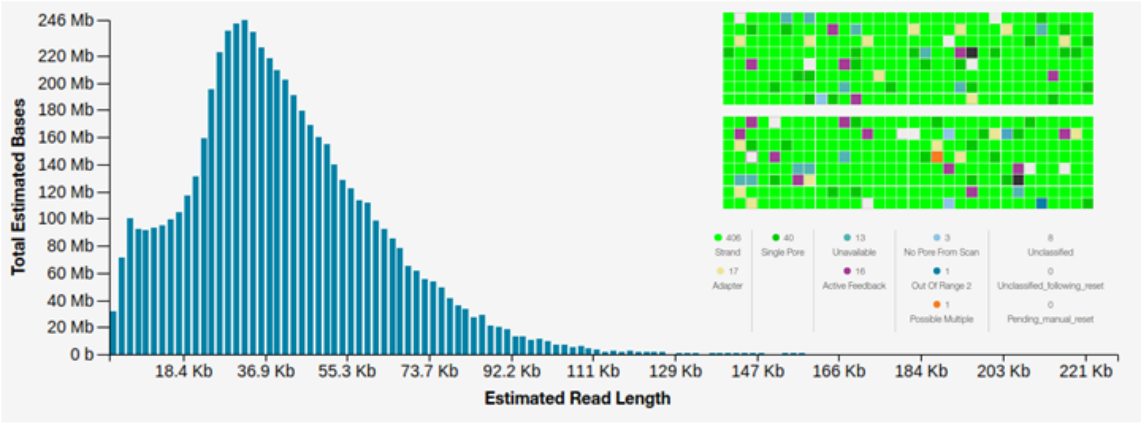


Figure 2: Gel electrophoresis analysis of DNA quality prior to gel purification. Stripe rust *Puccinia striiformis* is shown as a representative example. 100 ng of DNA separated on a 1% agarose gel. Ladder: 500 ng of GeneRuler™ DNA ladder mix (Thermo Scientific). Mitochondrial DNA and degraded RNA are abundant, both are removed during gel purification.

Table 2: Sequencing results with a single MinION flow cell per sample (FLO-MIN 106 R9.4.1 revD).

Sample	Pippin prep ≥	Library input µg	Loaded µg	Library preparation	Pores	Output Gb	Base call Gb	N50 kb
Stripe rust purified DNA	20 kb	1.70	0.61	Ligation SQK-LSK109	1,479	5.88	5.65	34.79
Leaf rust purified DNA	20 kb	2.25	0.88	Ligation SQK-LSK109	1,445	4.31	4.15	27.61

(A) Stripe rust *Puccinia striiformis;* 20 kb size selection, output 5.88 Gb, N50 34.79 kb.



(B) Leaf rust *Puccinia triticina;* 20 kb size selection, output 4.31 Gb, N50 27.61 kb.

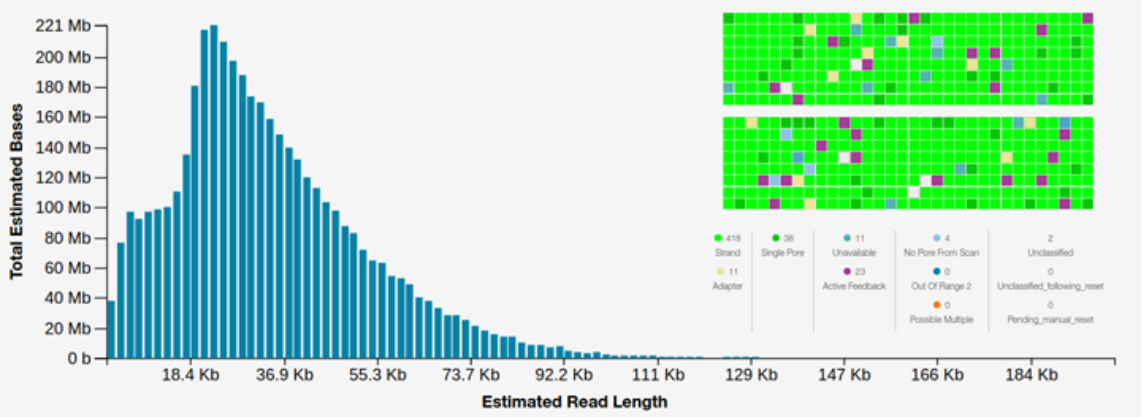
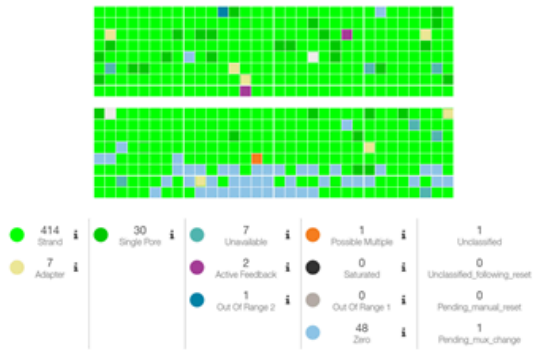


Figure 3: Expected read length histograms on MinKNOW. Both stripe and leaf rust DNA extractions were size selected for 20 kb and above then processed with an end ligation library preparation (SQK-LSK109). Inserts show pore usage, light green indicates pore is active and sequencing is occurring.

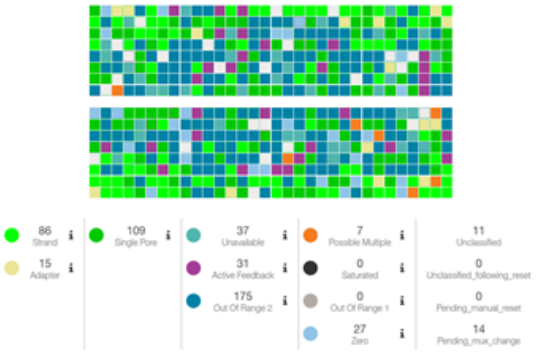
(A)



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.

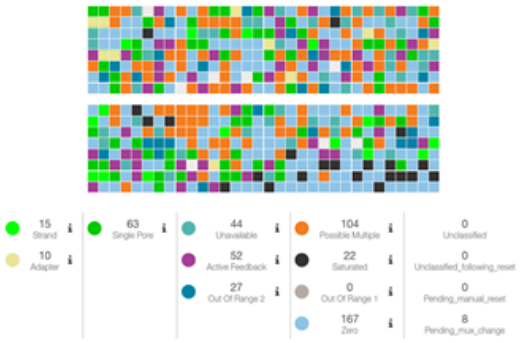
(B)



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.

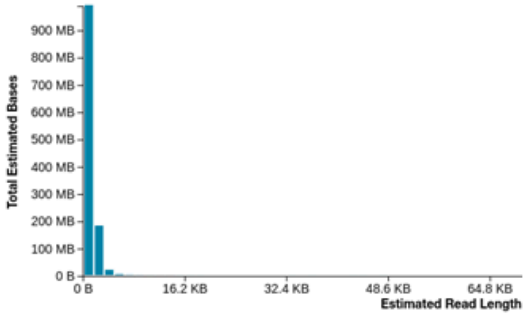
(C)



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 screenshots shown from anonymous colleagues with permission.