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High-molecular weight DNA extraction from challenging fungi using CTAB for lysis and precipitation V.3

Ashley Jones[1], Benjamin Schwessinger[1]

[1]Australian National University

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High molecular weight DNA extraction from all kingdoms
Tech. support email: See@each.protocol

Ashley Jones
Australian National University

ABSTRACT

Extracting pure high-molecular weight DNA from fungi is often 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, making them difficult to remove from solution. Some contaminants may even be undetected by spectrophotometers or fluorometric devices, however they still substantially interferes with long-read DNA sequencing. In attempt to resolve these challenges, a protocol is presented that utilses cetrimonium bromide (CTAB) for lysis and precipitation. CTAB in the lysis solution acts as a cationic detergent to dissolve cell membranes and remove polysaccharides by the formation of CTAB-polysaccharide complexes that co-precipitate during chloroform phase separation. Using CTAB for DNA precipitation under low salt, low ionic conditions, CTAB-DNA complexes form while leaving polysaccharides in solution, resulting in significantly less co-precipitation than alcohol methods. The presented protocol also includes some updates to current strategies and incorporates two different options for DNA clean-up and size selection. Using this protocol, we have been successfully sequencing various fungi with a MinION (Oxford Nanopore Technologies). For wheat stripe rust *Puccinia striiformis* and wheat leaf rust *Puccinia triticina,* we performed the presented method followed by size selection with an automated gel purification system, with sequencing yielding 5.88 Gbp with an N50 of 34.79 kb and 4.88 Gbp with an N50 of 27.61 kb, respectively for the two fungi. These fungi are notorious for being recalcitrant and these results have been positive improvements on many other methods. To increase sequencing output for these samples, more work is needed to identify and remove the elusive contaminants. Other fungi, such as sorghum rot fungus *Macrophomina phaseolina*, we chose an alternative size selection method, precipitation with a polymer and salt solution. This sequencing yielded 13.71 Gbp with an N50 of 21.75 kb, while another strain yielded 9.72 Gbp with an N50 of 43.50 kb.

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GUIDELINES

This protocol builds on previous trials by Ramawater Nagar and Anna Sharp; a warm thanks for their contributions.

This protocol is based on the following publications. When citing, please also include the original publications below.

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 TEXT

Reagents
AMPure XP beads (Agencourt) (or equivalent)
Cetrimonium bromide (CTAB)
Chloroform: isoamyl alcohol (24:1)
Ethylenediaminetetraacetic acid (EDTA)
Ethanol (80%, freshly prepared)
Sodium chloride (NaCl)
Sodium hydroxide (NaOH) 1 M
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 (ideal, can be substituted)
Acid-washed autoclaved sand (fine)
Centrifuge for 50 mL Falcon tubes (up to 16,000 rcf)
Incubator at 60°C that rotates or shakes. Alternatively a water wath.
Liquid Nitrogen
Mortar and pestle

DNA clean-up and size selection (two options are given)
For option 1: precipitation with polymers: see associated protocol
For option 2: gel purification by pippin prep:
AMPure XP beads (Agencourt) (or equivalent)
Magnetic rack for Eppendorf tubes
PippinHT (Sage Science) or equivalent gel-purification system
PippinHT 0.75% agarose casette and 15-20 kb kit (Sage Science)

PREPARATION

1 Grow fungus and collect ~500 mg of sample (spores for rust fungi). Keep frozen at -80°C or liquid nitrogen until ready.

Ideal for fungi that have a small genome, approximately 50 Mb. Take into consideration cell density and genome size; low cell density and/or smaller genomes may need to be prepped twice, larger genomes may not need many

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spores.

2 Set a rotating/shaking incubator (e.g. hybridisation oven) or alternatively a water bath to 60°C. This will be used to dissolve CTAB solutions and later to preciptate DNA with CTAB (best results if rotating or gently shaking).

- *DNA precipitation with CTAB will be optimal if rotating or gently shaking. A water bath will still be suitable, but can produce a lower DNA yield due to a lack of CTAB-DNA complexes forming.*
- *Temperature was originally 55°C, 60°C appears to give better results and not shear the DNA any further. This may beneficial to both lysis and precipitation steps, but has not been extensively tested.*

3 Freshly prepare lysis and precipitation buffers.

Lysis buffer (pH 8)

- 15 mL of lysis buffer per sample (~500 mg).
- Prepare in a 50 mL Falcon tube
- Before adding NaOH: pH 7.4. After adding NaOH: pH 8.

A	B	C	D	E
Reagent	**Target concentration**	**Molecular weight**	**Stock concentration**	**From stock**
CTAB	4% (w/v)	364.45	powder	0.60 g
Tris-HCl (pH 8)	100 mM	157.60	1 M	1.50 mL
EDTA (pH 8)	20 mM	292.24	0.5 M	0.60 mL
NaCl	1.2 M	58.44	5 M	3.60 mL
PVP-40	1%	40,000	10%	1.50 mL
Milli-Q water	-	-	-	~6.95 mL
NaOH	Adjust pH to 8	40.00	1 M	~0.25 mL
-	-	-	-	**Total 15 mL**

Precipitation buffer (pH 8)

- 30 mL of lysis buffer per sample (~500 mg).
- Prepare in a 50 mL Falcon tube
- Before adding NaOH: pH 7.6. After adding NaOH: pH 8.

A	B	C	D	E
Reagent	**Target concentration**	**Molecular weight**	**Stock concentration**	**From stock**
CTAB	2% (w/v)	364.45	powder	0.60 g
Tris-HCl (pH 8)	100 mM	157.60	1 M	3 mL
EDTA (pH 8)	20 mM	292.24	0.5 M	1.20 mL
Milli-Q water	-	-	-	~24.90 mL
NaOH	Adjust pH to 8	40.00	1 M	~0.30 mL
-	-	-	-	**Total 30 mL**

- *The precipitation buffer contains no NaCl. When 2x volume is added to the sample at later stages, the NaCl in*

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the initial lysis buffer is diluted from 1.2 M to ≤ 0.4 M. This triggers DNA precipitation by the formation of CTAB-DNA nucleation complexes

- *CTAB was originally 2% in the lysis buffer. Increasing CTAB to 4% in lysis buffer appears to have beneficial effects on DNA yield and no negative consequence have been observed.*
- *Originally, pH was not adjusted to pH 8 in either lysis or precipitation buffers. Adjusting pH to 8 appears to have beneficial effects on DNA yield and no negative consequence have been observed.*
- *The DNA extraction process still worked when the lysis and precipitation buffers were not adjusted to pH 8. Note Tris-HCl and EDTA stock solutions are already at pH 8.*
- *Original publication (Arseneau et al., 2017) used 500 µL of lysis buffer per 25 mg of mollusc tissue. Amount of lysis buffer per sample could be scaled down.*
- *EDTA is a metal ion chelator. By binding to Mg^{2+}, DNase activity is stopped as it is dependent on Mg^{2+}.*

4 Vortex lysis and precipitation buffers, then place both in the 60°C incubator or water bath. Leave buffers at 60°C until needed at later steps.

Necessary to dissolve the CTAB and may take 20 min or longer. Further vortexing or inverting may be also be required.

5 Collect liquid nitrogen and the sample. Place sample in liquid nitrogen until ready.

SAMPLE LYSIS

6 Place a sterile mortar and pestle into an insulated container. Pour liquid nitrogen into the container to chill the mortar and pestle.

The lid of an ice box can be used. Ensure the container will not crack and spill liquid nitrogen.

7 Add approximately 1 g of acid-washed autoclaved sand to the mortar.

Can be increased to 2 g if necessary to help grind the sample or add volume.

8 Add sample to the mortar and grind throughly (approximately 2-3 min). Keep the mortar and pestle chilled by having liquid nitrogen within the surrounding container.

Avoid pouring liquid nitrogen directly into the mortar or onto the sample, as the sample will disperse all over the pestle and potentially the table.

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9 Transfer the ground sample and sand to a tube of lysis buffer.

10 Add RNAse A and Proteinase K to the lysis buffer:

A	B	C	D
Enzyme	**Target concentration**	**Stock concentration**	**From stock**
RNAse A	200 µg/mL	20 mg/mL	150 µL
Proteinase K	200 µg/mL	20 m/mL	150 µL

- *RNAse A was added earlier than Arseneau et al. (2017) and concentration increased from 50 µg/mL to 200 µg/mL, which is more suitable when adding to a lysis buffer opposed to purified DNA. With less RNAse A, RNA was still present after extraction. Athough RNAse A concentration is generally recommended 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 some), but was less effective at degrading fungal RNA and more costly.*
- *Arseneau et al. (2017) included Proteinase K at a final concentration of 1,130 µg/mL to break down mollusc tissue. Concentrations this high have not been tested with fungi.*
- *RNAse A is still active in EDTA as it is not Mg^{2+} dependent and doesn't appear to require other metal ions as cofactors. Similarily, Proteinase K is still active, as the two binding sites for Ca^{2+} are not directly involved in the proteolysis catalytic mechanism.*
- *RNAse A and Proteinase K 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).*

11 Mix thoroughly by vortexing and inverting the solution. Incubate at 60°C for 60-90 min

Ideal to be rotating or gently shaking, but this is not as critical as CTAB-DNA precipitation at later steps.

CHLOROFORM CLEAN-UP

12 Briefly place at room temperature or on ice, to bring the temperature down.

13 Sample should be in a 50 mL Falcon tube, if not and the sample with lysis buffer occupies more than 50% of the tube capacity, move to a bigger tube or split the sample evenly across two appropriately sized tubes.

14 Add an equal volume of chloroform: isoamyl alcohol (24:1, v/v) and mix by inverting 10-15 times.

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- *Ensure the organic and aqueous phases become mixed at least temporarily.*
- *Solution may appear opaque, but this is not a concern.*

15 Separate the phases by centrifuging at 5,000 rcf for 10 min at 20°C (or room temperature).

Chloroform is denser than water, it 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.

16 Transfer the upper aqueous phase to a new 50 mL Falcon tube.

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

DNA PRECIPITATION

18 Add 2 volumes of precipitation buffer (30 mL to ~15 mL chloroform cleaned sample). Mix by swirling and inverting the tube several times.

19 Incubate at 60°C in a rotating/shaking incubator for 2.5 h or until white crystals of CTAB-DNA complex can be observed floating inside the tubes (incubate at least 1 h).

- *This step is critical for DNA precipitation, as CTAB will nucleate around the DNA slowly and works best with movement (e.g. 150 rpm). Increasing the speed helps.*
- *It is very difficult to see any crystals and likely depends on the sample. For samples with large yields over 77 µg, I did not observe crystals.*
- *No further yield was seen incubating longer than 2.5 h (including overnight incubation). The ideal time may be 1.5-2 h but this needs further testing.*
- *The extended incubation time does not appear to shear the DNA compared to shorter incubation times that result in significantly lower yields.*
- *Precipitation may also be influenced by DNA concentration, which has not been tested. This would require reducing the lysis buffer voume or increasing sample input.*
- *As the lysis buffer has now increased CTAB from 2% to 4% (w/v), it is anticipiated this is also helping the precipitation, but has not been extensively tested to confirm this hypothesis.*

20 Centrifuge at 16,000 rcf for 20 minutes at 20°C (or room temperature) to pellet crystals.

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- *Was originally 10 min, Arseneau et al. (2017) does 3 min. More pellet has been observed the longer the centrifugation.*
- *Centrifuging 2x 15 min, the second spin collected another 8% yield.*
- *Extensive centrifugation at 16,000 rcf could lead to DNA shearing. However 30 mins appears fine with most DNA being 20-50 kb, with a tail of ultra long fragments up to 200 kb.*

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

It is good practice to save the supernatent in another tube, in case the DNA has not precipitated or the pellet has been unknowingly dislodged.

22 Add approximately 10-15 mL freshly prepared 80% ethanol, enough to cover the pellet. Gently swirl and invert the tube.

23 Let the pellet soak for 15 min at room temperature to dissolve excess salts and CTAB.

24 Centrifuge at 16,000 rcf for 5 min.

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

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

27 Pipette 100-200 µL of 10 mM Tris-HCl pH 8 (or nuclease-free water) onto the DNA pellet. Repeatedly pipette the same 100-200 µL onto the pellet until it disloges from the tube wall and dissolves. Proceed with care to avoid pipette mixing as much as possible, to prevent DNA shearing.

- *Elution volume is based on pellet size and how the sample will be size selected later.*
- *Gentle flicking of the tube is ideal but not possible here if using 50 mL Falcon tube.*
- *Wide-bore pipette tips are also an option.*

28 Transfer DNA to a 1.5 mL DNA LoBind Eppendorf tube.

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29 Incubate DNA at room temperature for 5-10 min to ensure the pellet has completely hydrated then place on ice.

30 Quantify the DNA on a Nanodrop and a Qubit fluorometer (dsDNA broad-range assay) (both instruments from Thermo Fisher Scientific). The DNA will be somewhat crude, containing some degraded RNA and potentially residual protein or other contaminants, which will be removed later.

- *For Nanodrop, use 1 µL. As degraded RNA may still be present, Nanodrop results may give a large over-estimation of concentration.*
- *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.*
- *For pure DNA, Nanodrop:Qubit is 1:1, 260/280 is 1.8 and 260/230 is 2.0.*

31 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 extended periods.

DNA CLEAN-UP AND SIZE SELECTION OPTION 1: PRECIPITATION WITH POLYMERS

32 If the DNA is suspected to have a large amount of degraded RNA, first perform a bead clean with AMPure XP magnetic beads (or equivalent). Use 1x volume of beads and follow the manufacturer's instructions (example bead clean workflow is presented in next section).

Removing many small degraded fragments will increase the performance of the following more stringent size selection.

33 Utilise a polymer and salt solution listed in the following protocol (or commercial option such as Short-Read Elimator kit from Circulomics). The size selection section is all that is required.

DNA clean-up and size selection for long-read sequencing v4

Jones, A., Purushotham, N., Nasim, J., and Schwessinger, B. (2021). DNA clean-up and size selection for long-read sequencing. Protocols.io v4, dx.doi.org/10.17504/protocols.io.bwkdpcs6.

34 If some residual degraded RNA is still present, this can be ignored as it will be removed during bead clean-ups during the sequencing library preparation.

DNA CLEAN-UP AND SIZE SELECTION OPTION 2: GEL PURIFICATION BY PIPPIN PREP

35 Using a PippinHT (Sage Science) or similar automated electrophoresis system, gel purify 20-30 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 with 5 µL of loading buffer (200 µL of total sample). The manufacturer recommends a maximum 1.5 µg per lane (15 µg total per cassette), however, can be successfully

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overloaded to 3 µg per lane (perhaps more). The size selection will still be very accurate.

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

37 Collect the contents of all elution wells into a 2 mL or 1.5 mL Eppendorf tube (~250-300 µL).

38 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 Eppendorf tube (additional ~300 µL, tube total is ~600 µL).

39 Add at least 1x volume of AMPure XP beads (~600 µL). Incubate at room temperature for 5 min.

If using 2% Sera-Mag beads in the lab, alternatively fill ~90% of the tube capacity with binding buffer (will be > 2x volume for 2 mL tubes) and add 100 µL of 2% Sera-Mag beads.

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

41 Keeping the tube on the magnetic rack, add 1 mL of freshly prepared 80% ethanol. Discard the ethanol and repeat for a second ethanol wash.

42 Remove all traces of ethanol and let the beads air dry for 1-4 min.

Important! *Do not let the beads dry completely, they will crack and significantly reduce DNA recovery.*

43 Remove the tube from the magnetic rack and elute with 50 µL of 10 mM Tris-HCl pH 8 (or nuclease-free water). Incubate for 10 min at room temperature.

A maximum DNA volume of 48 µL is used in an Oxford Nanopore ligation preparation.

44 Place on a magnetic rack for 5 min, or until the solution becomes clear. Transfer eluted DNA to a 1.5 mL DNA LoBind Eppendorf tube.

45 Perform an additional, second elution on the beads, remove from magnet, resuspend (another 50 µL), incubate, magnetise and transfer to another 1.5 mL DNA LoBind Eppendorf tube.

Save this second elution for other applications such as running a pulse field gel or creating and Illumina short-read

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library.

46 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.

Expect 10-30% recovery relative to total input.

47 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 extended periods.

48 EXPECTED RESULTS

Using the protocol described, we performed DNA extractions for wheat stripe rust *Puccinia striiformis* and wheat leaf rust *Puccinia triticina,* followed by size selection with an automated gel purification system (PippinHT). We have been obtaining clean high-molecular weight DNA (Table 1, Figure 1). Nuclear DNA fragment sizes appear above 10 kb on agarose gels, with mitochondrial DNA and degraded RNA still present until size selection was performed (Figure 2). For sequencing, we adopted the portable MinION sequencer from Oxford Nanopore Technologies. During sequencing, we can obtain 5.88 Gbp with an N50 of 34.79 kb and 4.88 Gbp with an N50 of 27.61 kb, respectively for the two fungi (Table 2, Figure 3). This includes quality reads over 100 kb in length (> Q7, Phred scale). These fungi are notorious for being recalcitrant and these results have been positive improvements on many other methods. To increase sequencing output for these samples, more work is needed to identify and remove the elusive contaminants that are negatively affecting sequencing. Other fungi, such as sorghum rot fungus *Macrophomina phaseolina*, we chose an alternative size selection method, precipitation with a polymer and salt solution. This sequencing yielded 13.71 Gbp with an N50 of 21.75 kb, while another strain yielded 9.72 Gbp with an N50 of 43.50 kb. These results can be found in the relevant DNA clean-up protocol mentioned. A troubleshooting guide for MinION sequencing is presented in Figure 4, illustrating the most common problems researchers have.

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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	47.60	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	49.00	1.92	2.24	50	2.53

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

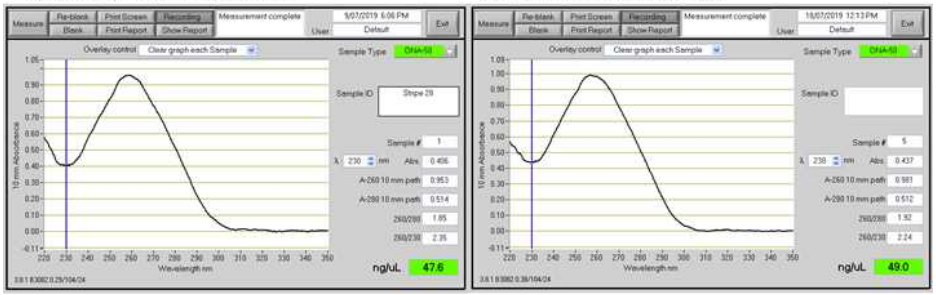

Figure 1: Spectrophotometer results of purified DNA for two recalcitrant fungal species. Readings taken using 1 µL on a Thermo Scientific Nanodrop 1000.

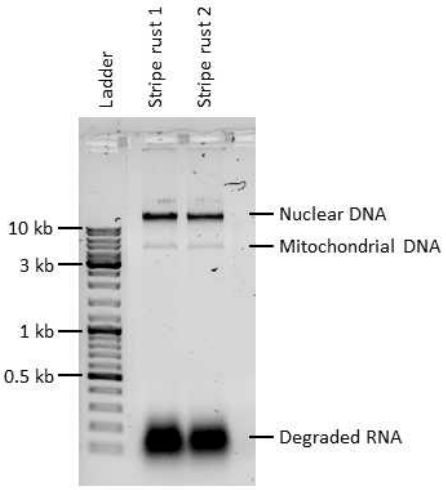

Figure 2: Gel electrophoresis analysis of DNA quality prior to gel purification. Stripe rust *Puccinia striiformis* is shown as a representative example. 100 ng of DNA separated on a 1% agarose gel. Ladder: 500 ng of GeneRuler™ DNA ladder mix (Thermo Scientific). Mitochondrial DNA and degraded RNA are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

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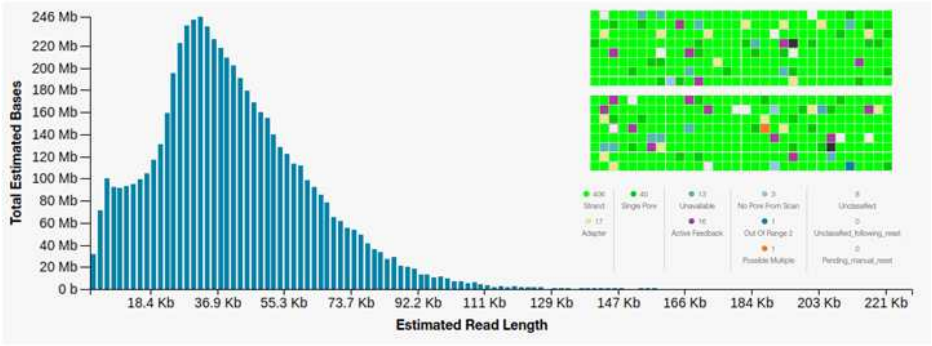
Table 2: Sequencing results with a single MinION flow cell per sample (FLO-MIN 106 R9.4.1 revD).

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.

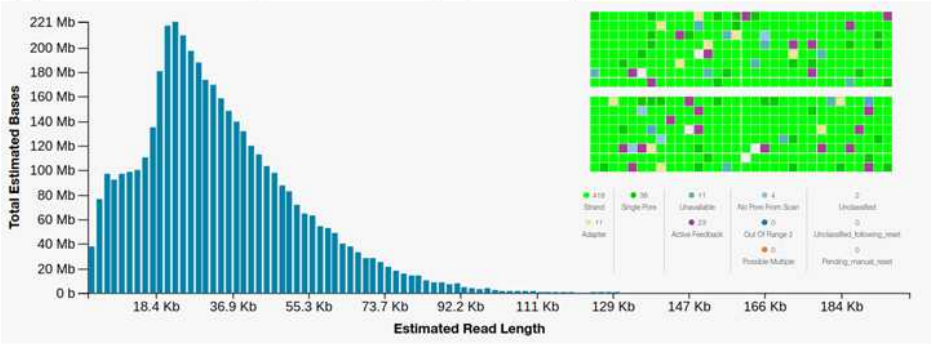


Figure 3: Expected read length histograms on MinKNOW. Both stripe and leaf rust DNA extractions were size selected for 20 kb and above then processed with an end ligation library preparation (SQK-LSK109). Inserts show pore usage, light green indicates pore is active and sequencing is occurring.

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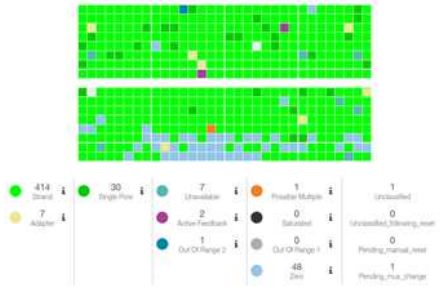
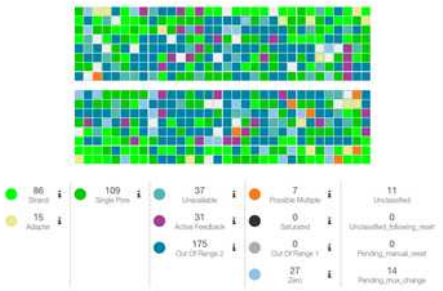
(A)



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.

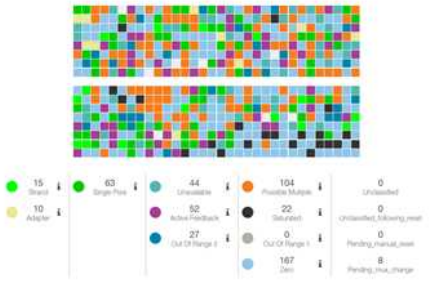
(B)



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.

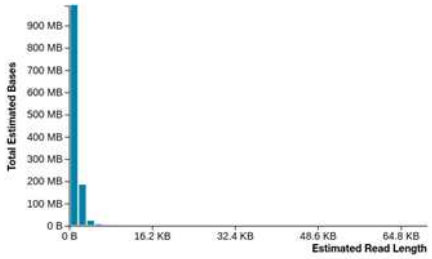
(C)



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.

Citation: Ashley Jones, Benjamin Schwessinger (07/23/2021). High-molecular weight DNA extraction from challenging fungi using CTAB for lysis and precipitation. https://dx.doi.org/10.17504/protocols.io.bwr8pd9w