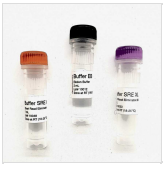

Version 4

Jul 16, 2021

DNA clean-up and size selection for long-read sequencing V.4

In 1 collection

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2 *Works for me* Share dx.doi.org/10.17504/protocols.io.bwkdpcs6

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

Ashley Jones
Australian National University

ABSTRACT

DNA extractions often contain impurities which limit the output of long-read sequencing technologies. Here a protocol is provided which removes impurities and size selects for longer fragments. To remove residual RNA and protein, an additional RNAse A and Proteinase K treatment is performed. A clean-up with chloroform: isoamyl alcohol (24:1) removes these proteins and other hydrophobic organics such as lipids. A low volume ethanol precipitation and wash is used to concentrate the DNA, hopefully also reducing polysaccharides. An optional needle shearing is described which can help create a more uniform DNA length to maximise sequencing output. Finally, polymer and salt based solutions are used to selectively precipitate DNA based on size, which also appears to further clean the DNA. This was trialled for the sorghum rot fungus *Macrophomina phaseolina*, providing highly promising results with an Oxford Nanopore MinION. One strain yielded 13.71 Gbases with an N50 of 21.75 kb, another strain yielded 9.72 Gbases with an N50 of 43.50 kb. Similar results have been obtained with other fungi, plants, reptiles and insects.

DOI

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PROTOCOL CITATION

Ashley Jones, Neeraj Purushotham, Jamila Nasim, Benjamin Schwessinger 2021. DNA clean-up and size selection for long-read sequencing. **protocols.io**
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COLLECTIONS

High-molecular weight DNA extraction, clean-up and size selection for long-read sequencing

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PARENT PROTOCOLS

Part of collection

High-molecular weight DNA extraction, clean-up and size selection for long-read sequencing

GUIDELINES

This protocol is optimsed for crude high-molecular weight DNA. For best results, avoid column based DNA extractions (which shear the DNA) and avoid using phenol (interferes with sequencing, can irreversibly bind to DNA).

The protocol is flexible and different sections may be used or skipped for different applications. If the starting DNA is crude, with suspected RNA, protein and other contaminants (e.g. large concentration discrepency between Qubit and Nanodrop, variable Nanodrop 260/280 and 260/230 values), it is recommended to follow the protocol in full. If the DNA is cleaner and these values are closer to what is expected, performing just the size selection with a polymer and salt solution may be all that is needed for some users.

The use of polymers and salts to selectively precipitate DNA started with early publications such as the following and has since been further developed in the community, including by our valued collaborators.

Lis, J. and Schleif, R. (1975). Size fractionation of double-stranded DNA by precipitation with polyethylene glycol. *Nucleic Acids Research* **2(3)**, 383-389.

MATERIALS TEXT

Chloroform: isoamyl alcohol (24:1 v/v)
1.5 mL Eppendorf DNA LoBind tubes (ideal, can be substituted)
Ethanol (100% and 70%)
Needle (29 or 26 gauge) with syringe (optional, only for specific applications)
Proteinase K (20 mg/mL)
RNAse A (20 mg/mL)
3 M Sodium acetate pH 5.2
10 mM Tris-HCl pH 8 (nuclease-free)
Water (nuclease-free)

Polymer and salt based solution for size selection. Choose one, more details provided in the method.
Note: ideally filter sterilise the solution, although this will be difficult due to viscosity.

- 4% PVP 360,000, 1.2 M KCl, 20mM Tris-HCl pH 8 (>10 kb)
- 3% PVP 360,000, 1.2 M NaCl, 20 mM Tris-HCl pH 8 (> 25 kb)
- 9% PEG 8,000, 1 M NaCl, 10 mM Tris-HCl pH 8 (>10 kb)

Commercial solutions are also available:

- Short-Read Eliminator XS (> 10 kb) (Circulomics SKU SS-100-121-01)
- Short-Read Eliminator (> 25 kb) (Circulomics SKU SS-100-101-01)
- Short-Read Eliminator XL (> 40 kb) (Circulomics SKU SS-100-111-01)

RNA AND PROTEIN REMOVAL

1 Aliquot 10-30 µg of DNA into a 1.5 mL eppendorf tube. Increase volume to 200 µL with 10 mM Tris-HCl pH 8.

- *DNA quantification must be based on Qubit Fluorometer (Thermo Fisher Scientific), or similar device. A high quantity of RNA may be present.*
- *Volume can exceed 200 µL, maximum of 600 µL due to tube capacity at later steps (ethanol precipitation).*

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2 Add RNAse A and Proteinase K

A	B	C	D
Enzyme	**Target concentration**	**Stock concentration**	**Quantity from stock**
RNAse A	100 µg/mL	20 mg/mL	1 µL
Proteinase K	100 µg/mL	20 mg/mL	1 µL

3 Incubate the samples at 55°C (or 50-60°C) for 20 min, shaking at 400-500 rpm if possible.

CHLOROFORM CLEAN

4 Increase the volume to 600 µL with 10 mM Tris-HCl pH 8 (add 400 µL).

5 Add an equal volume of chloroform: isoamyl alcohol (24:1, v/v) (600 µL) and mix by inverting 10-15 times.

Ensure the organic and aqueous phases become mixed at least temporarily.

6 Separate the phases by centrifuging at 16,000 rcf for 1 min at 20°C (or room temperature).

7 Transfer the upper aqueous phase to a new 1.5 mL Eppendorf tube.

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

DNA PRECIPITATION

9 Add 1.5x volume of 100% ethanol (~900 µL) and 0.1x volume of 3 M sodium acetate pH 5.2 (~60 µL). Mix thoroughly by inverting the tube.

Sample volume should be a maximum 600 µL after cleaning with chloroform: isoamyl alcohol.

10 Incubate the sample on ice for 1 min.

11 Centrifuge at 16,000 rcf for 1 min at 20°C (or room temperature).

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High-molecular weight DNA should pellet easily, therefore you could pulse spin for 10 s at maximum speed. If no pellet can be seen, centrifuge for longer. Bringing ethanol concentration to 2x volume is also an option.

12 Carefully decant the supernatant as soon as possible, without disturbing the pellet.

13 Add approximately 700 µL of freshly prepared 70% ethanol, enough to cover the pellet.

14 Let the pellet soak for 1 min at room temperature to dissolve excess salts.

15 Centrifuge at 16,000 rcf for 1 min at 20°C (or room temperature).

16 Carefully decant the supernatant as soon as possible, without disturbing the pellet.

17 Repeat previous steps for a second 70% ethanol wash.

18 Air-dry the pellet by placing the tube upside-down on tissue paper for 5-15 min (until all ethanol has evaporated). Be careful of water touching or dislodging the pellet.

19 Dissolve DNA with 60 µL of 10 mM Tris-HCl pH 8.

20 Quantify the DNA on a Nanodrop and a Qubit fluorometer (dsDNA broad-range assay).

NEEDLE SHEARING (OPTIONAL)

21 Needle shearing is optional and should be skipped in most cases. It can maximise long-read sequencing output at the expense of read length. It is not ideal for genome assembly projects, but it helps provide a more uniform DNA length for specific applications where output is valued more than read length.

For instance, repetitive polyploid plant DNA 100 kb and higher can block nanopores, stopping sequencing prematurely. Also, sequencing adapters may not be effectively ligated to these fragments if secondary structures are forming.

Choose a needle and a syringe; increasing needle gauge (smaller diameter), causes more DNA shearing.

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A	B	C
Needle	**Passes**	**Estimated N50**
29 gauge	5	10-20 kb
26 gauge	5	20-35 kb

- *No difference was found between 5, 10, 15 and 20 passes using the 29 gauge needle with plant DNA. The 26 gauge needle has not been extensivley tested.*
- *The chloroform clean-up and DNA precipitation from previous sections has already caused some DNA shearing.*

22 Aliquot 3-9 µg of DNA into a 1.5 mL Eppendorf DNA LoBind tube. Adjust the volume to exactly 60 µL using 10 mM Tris-HCl (pH 8).

23 Connect the needle to a syringe and perform 5 passes with DNA (aspirate and dispense 5 times).

24 Proceed directly to size selection.

SIZE SELECTION

25 DNA can be size selected by using polymer and salt solutions to preferentially precipitate high-molecular weight DNA, effectively removing shorter fragments. Key solutions are presented here and some commercially available options are also listed. Choose a size selection solution below that suits your experiment, all work on similar principles. These are 2x solutions that will be mixed with an equal volume of DNA at later steps, therefore the final concentrations will be half of what is listed (1x).

A	B	C
Solution (2x)	**Size selection**	**Recovery**
4% PVP 360,000, 1.2 M KCl, 20 mM Tris-HCl pH 8	> 10 kb	50-75%
3% PVP 360,000, 1.2 M NaCl, 20 mM Tris-HCl pH 8	> 25 kb	30-60%
9% PEG 8,000, 1 M NaCl, 10 mM Tris-HCl pH 8	>10 kb	Needs more testing
Short-Read Eliminator XS (Circulomics)	> 10 kb	50-75%
Short-Read Eliminator (Circulomics)	> 25 kb	30-60%
Short-Read Eliminator XL (Circulomics)	> 40 kb	< 40%

Size selection and recovery estimates are based on high-molecular weight DNA from non-model species; results may vary considerably. Expect higher loss if DNA is significantly sheared. If the DNA sample is very contaminated, size selection may perform poorly or the DNA may not precipitate.

26 Aliquot 3-9 µg of DNA into a 1.5 mL Eppendorf DNA LoBind tube. Adjust the volume to exactly 60 µL using 10 mM Tris-HCl pH 8 (alternatively buffer EB if using a Circulomics product).

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- *DNA mass must be measured by Qubit Fluorometer (Thermo Fisher Scientific) or equivalent.*
- *DNA must not contain high levels of salts, polyphenols, polysaccharides or other contaminants.*
- *If you want to add more DNA, increase the volume slightly (also more size selection solution later). For example, 12 µg of DNA in 100 µL (adding 100 µL of size selection solution later).*

27 Add 60 µL (an equal volume) of the chosen size selection solution. Mix thoroughly by gently tapping the tube or by gently pipetting up and down.

28 Centrifuge at 10,000 rcf for 30 min at 20°C (or room temperature). Note where the DNA will pellet, by marking the tube or placing the tubes in a consistent orientation within the rotor.

Recovery will be impacted if centrifugation is performed at low temperature (e.g. 4°C).

29 Carefully remove the supernatant with a pipette as soon as possible, without disturbing the pellet. Care must be taken as the pellet is fragile, being easily dislodged.

- *The DNA pellet may not be visible. Be careful of accidentally losing the pellet; mark the expected spot and proceed as if it was there.*
- *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.*

30 Add 200 µL of freshly prepared 70% ethanol, enough to cover the pellet. Do not tap or mix after adding ethanol.

31 Centrifuge at 10,000 rcf for 2 min at at 20°C (or room temperature).

32 Carefully remove the supernatant with a pipette as soon as possible, without disturbing the pellet.

33 Repeat previous steps for a second 70% ethanol wash.

34 Air-dry the pellet by placing the tube upside-down on tissue paper for 5-15 min at room temperature (until all ethanol has evaporated). Be careful of water touching or dislodging the pellet.

Add 50 µL of 10 mM Tris-HCl pH 8 (alternatively buffer EB if using a Circulomics product). Mix by gently tapping the

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35 tube.

36 Let the pellet hydrate at room temperature, may take 10 min. If it does not hydrate, incubate at 50°C for 10 min.

Circulomics protocols recommend 50°C for 1 h, which seems excessive. DNA concentration was found to increase < 8%. Limit DNA shearing by avoiding high temperatures. Pellet will also hydrate further when stored at 4°C.

37 After incubation, gently tap the tube to ensure that the DNA is properly resuspended and mixed.

38 Quantify the DNA on a Nanodrop and a Qubit fluorometer (dsDNA broad-range assay).

39 Store DNA at 4°C (in the fridge) 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.

40 EXPECTED RESULTS

Fresh mycelia (approximately 2 g) was used to extract crude high-molecular weight DNA using a CTAB method previously described. See Jones *et al.* (2019) High-molecular weight DNA extraction from challenging fungi using CTAB and gel purification, Protocols.io.

The DNA was then cleaned and size selected by following the presented protocol in full, with the final size selection being performed with a Short-Read Eliminator (SRE) (Circulomics, > 25 kb selection). Initially the Qubit:Nanodrop ratio had a large discrepancy (Table 1 and Figure 1). This is due to the presence of RNA, given the 260/280 is above 1.8. Cleaning removed this discrepancy, particularly with the removal of all RNA. With RNA not present, the 260/230 ratio is more representative, despite appearing worse.

For sequencing, we adopted a MinION sequencer from Oxford Nanopore Technologies, creating a native genomic DNA library (SQK-LSK109) following the manufacturer's instructions. Although the DNA was not perfect, a high sequencing output was achieved (Table 2). Size selection with SRE resulted in long-reads and a high N50 (Figure 2).

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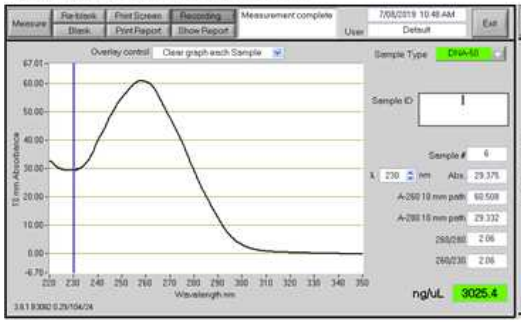
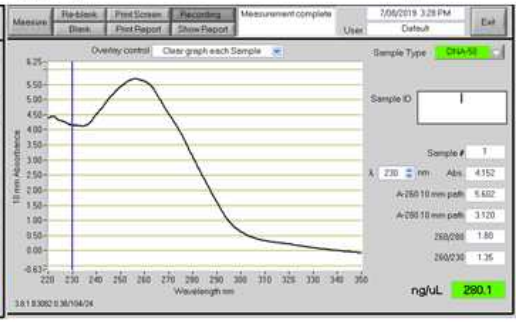
Table 1: Quantification of the crude DNA, DNA after cleaning and after size selection.

Sample	DNA input µg	SRE kit	Qubit ng/µL	Nano ng/µL	Qubit: Nano	260/ 280	260/ 230	Vol µL	Yield µg (Qubit)
Mp strain 1 crude DNA	NA	NA	64.30	526.80	1:8.19	2.05	2.09	300	19.29
Mp strain 1 clean DNA	19.00	NA	271	431.80	1:1.59	1.92	2.16	50	13.55
Mp strain 1 clean + SRE	9.00	25 kb	74.10	97.60	1:1.31	1.88	1.67	50	3.71
Mp strain 2 crude DNA	NA	NA	258	3,025	1:11.72	2.06	2.06	300	77.40
Mp strain 2 clean DNA	19.00	NA	272	280.10	1:1.03	1.80	1.35	50	13.60
Mp strain 2 clean + SRE	9.00	25 kb	94	146.10	1:1.55	1.82	1.29	50	4.70

(A) *M. phaseolina* before clean



(B) *M. phaseolina* after chloroform cleaning



(B) *M. phaseolina* after clean and short read eliminator

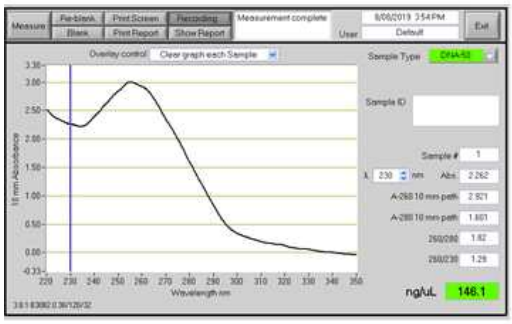


Figure 1: Spectrophotometer results of DNA at multiple stages of the protocol. Strain 2 is shown as an example. (A) Initial DNA before cleaning. (B) DNA after an initial clean with chloroform and ethanol precipitation. (C) DNA after cleaning with chloroform and size selecting with a short read eliminator kit (Circulomics). Readings taken using 1 µL on a Thermo Scientific Nanodrop 1000.

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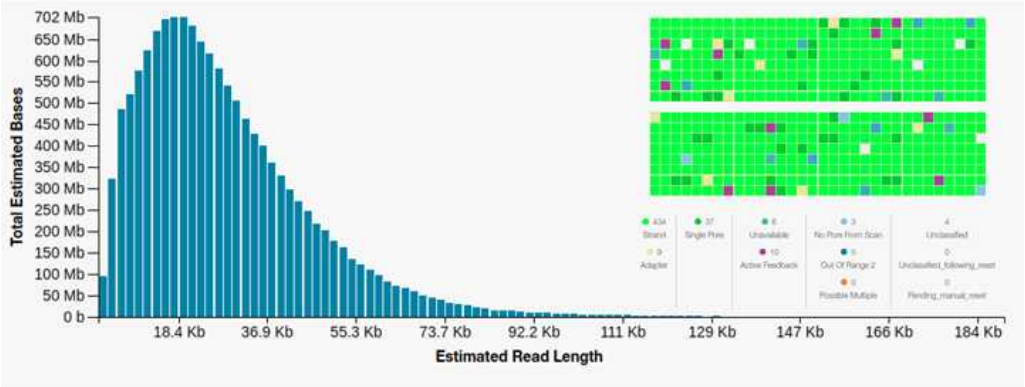
Table 2: Sequencing results with a single MinION flow cell per sample (FLO-MIN 106 R9.4.1 revD).

Sample	SRE ≥	Library input µg	Loaded µg	Library preparation	Pores	Output Gb	Base call Gb	N50 kb
Mp strain 1	25 kb	3.00	1.00	Ligation SQK-LSK109	1,557	13.71	13.29	21.75
Mp strain 2	25 kb	3.00	0.94	Ligation SQK-LSK109	1,362	9.72	9.57	43.50

(A) Sorghum rot *Macrophomina phaseolina* strain 1; short read eliminator 25 kb, 13.71 Gb, N50 21.75 kb.



(B) Sorghum rot *Macrophomina phaseolina* strain 2; short read eliminator 25 kb, 9.72 Gb, N50 43.50 kb.

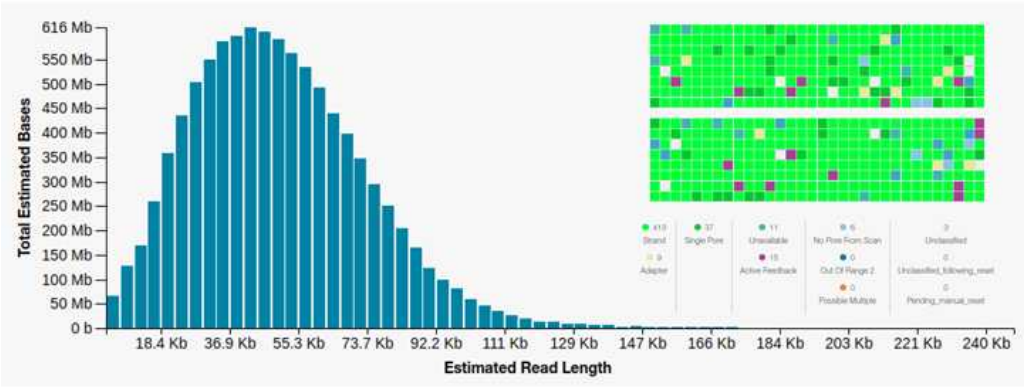


Figure 2: Expected read length histograms on MinKNOW. Both sorghum rot *Macrophomina phaseolina* strains were size selected with a short read eliminator kit (Circulomics) for 25 kb and above. DNA libraries were then prepared with an end ligation kit (Oxford Nanopore SQK-LSK109). Inserts show pore usage, light green indicates pore is active and sequencing is occurring.

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