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🌐 Scalable high-molecular weight DNA extraction for long-read sequencing

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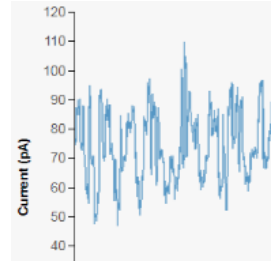
High molecular weight D...

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Protocol status: Working

Used routinely across multiple kingdoms



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Abstract

With rapid advances in long-read DNA sequencing 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 native plants, crops and fungi. To resolve this, we optimised a gentle magnetic bead based high-molecular weight DNA extraction free of columns and high-centrifugation, to limit DNA fragmentation. A protocol that is scalable based on tissue input is presented, that can be used on many species of plants, fungi, reptiles, insects and bacteria. An optional sorbitol wash is listed and is highly recommended for plant tissues. To remove any remaining contaminants such as phenols and polysaccharides, two optional DNA clean-up and size selection strategies are given. Sequencing with Oxford Nanopore Technologies MinION, we can approximately obtain over 15-30 Gbp of sequencing from a single MinION flow cell with N50 values 30-50 kb. This has been routinely achieved with eucalypts, acacias, rice, thymus, wheat, wheat rusts, various other fungi, geckos, skinks, ticks, ladybird beetles, caterpillars and *E. coli*.

Guidelines

This protocol is based on the following publication. When citing, please also note the original publication below.

Mayjonade, B., Gouzy, J., Donnadieu, C., Pouilly, N., Marande, W., Callot, C., Langlade, N., and Munos, S. (2016). Extraction of high-molecular-weight genomic DNA for long-read sequencing of single molecules. *BioTechniques* **61**, 203-205.

Materials

Chemicals for lysis buffer

- Ethylene-bis(oxyethylenenitrilo)tetraacetic acid (EGTA) (optional; necessary for wheat)
- Ethylenediaminetetraacetic acid (EDTA)
- Polyvinylpyrrolidone 40 (PVP-40)
- Sodium chloride (NaCl)
- Sodium dodecyl sulfate (SDS)
- Sodium metabisulfite
- Tris(hydroxymethyl)aminomethane hydrochloride (TRIS-HCl)
- Water (Milli-Q)

Other solutions

- Ethanol (100% and 70%)
- Lysozyme (bacterial culture preps only)
- Nuclease-free water (UltraPure™ Thermo Fisher Scientific 10977015)
- 5 M Potassium acetate
- Proteinase K (20 mg/mL 2×1 mL NEB P8107S)
- RNase A (PureLink 20 mg/mL 25 mL, Thermo Fisher Scientific 12091039)
- 10 mM TRIS-HCl pH 8

2% Sera-Mag beads solution

- Sera-Mag SpeedBead Carboxylate-Modified Magnetic Particles (Hydrophobic), 15 mL (GE Healthcare /Cytiva /Thermo Fisher Scientific product 65152105050250).
- First prepare buffer without the beads. Let the Sera-Mag beads come to room temperature.

Component	Target concentration	MW	Stock concentration	From stock (10 mL)	From stock (50 mL)
Polyethylene glycol (PEG) 8,000	18%	8,000	25%	7.2 mL	36 mL
NaCl	1 M	58.44	5 M	2 mL	10 mL
TRIS-HCl pH 8.0	10 mM	121.14	1 M	100 µL	500 µL
EDTA pH 8.0	1 mM	292.24	0.5 M	20 µL	100 µL
Tween 20	0.05%	1,227.54	10%	50 µL	250 µL



- Homogenise Sera-Mag beads thoroughly by shaking and swirling.
- Prepare 2% Sera-Mag beads (200 μ L for 10 mL buffer) by washing 4 times with water to remove sodium azide. Magnetise, remove supernatant, add 1 mL H₂O, flick tube, repeat.
- Resuspend the clean Sera-Mag beads in 600 μ L nuclease-free water. Transfer into the buffer prepared.
- Store at 4°C for up to 6 months.

Binding buffer solution

- Mix until the solution becomes clear. If PEG 8,000 is not dissolved, it can lead to a poor yield as PEG 8,000 makes DNA to bind to the beads.

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

Special equipment

- Liquid nitrogen
- Mortar and pestle (medium and large preps)
- Ball bearings and TissueLyser (Qiagen) or equivalent (small prep only)
- Magnetic rack (for 2 mL Eppendorf tubes and/or 15 mL and/or 50 mL Falcon Tubes)
- 2 mL Eppendorf tubes (small preps)
- 50 mL Falcon tubes (medium preps)
- 250 mL Schott bottle (large prep only)
- 1.5 mL DNA LoBind Eppendorf tubes (e.g. 022431021) (optional but optimal)
- 200 μ L wide-bore pipette tips (e.g. Vertex 4290-00) (optional)
- Sieve or mesh with a funnel, 300 μ m (e.g. Kartell disk membrane; ART 844) (large prep only)



PREPARATION

- 1 Set a water bath (or equivalent alternative) to 55°C (temperature range 50–60°C)

Note

Will be used to dissolve lysis buffer and also used during lysis (30–90 min).

- 2 Prepare fresh lysis buffer based on the prep size and heat at 55°C in water bath until ready to use.

Prep size	Tissue input	Amount needed per sample
Small (2 mL tube prep)	100–200 mg	750 µL
Medium (50 mL Falcon tube prep)	1–3 g	20 mL
Large (250 mL Schott bottle prep)	≤ 30 g	100 mL

Reagent	Target concentration	MW	Stock	From stock (10 mL)
Polyvinylpyrrolidone 40 (PVP-40)	1%	40,000	10%	1 mL
Sodium metabisulfite	1%	190.11	10%	1 mL
Sodium chloride (NaCl)	0.5 M	58.44	5 M	1 mL
TRIS-HCl pH 8.0	100 mM	121.14	1 M	1 mL
EDTA pH 8.0	50 mM	292.24	0.5 M	1 mL
Sodium dodecyl sulfate (SDS)	2%	288.37	20%	1 mL
Water (Milli-Q)	NA	-	-	4 mL
EGTA pH 8 (optional, see below)	6 mM	380.35	0.5 M	120 µL

**Note**

- *Prepare lysis buffer fresh on the day of use for optimal results.*
- *The solution should be clear before use.*
- *EGTA is recommended for wheat and very soft plant tissues that wilt fast and/or have high amounts of endogenous DNases.*
- *EDTA has a high-affinity for Mg^{2+} , EGTA has a high-affinity for Ca^{2+} .*
- *Prokaryotic DNases are dependent on Mg^{2+} , plant DNases Ca^{2+} .*

- 3 Ensure there is enough 5 M potassium acetate, binding buffer and Sera-Mag beads to carry out the prep size and quantity (listed in Materials).

HOMOGENISATION

- 4 [Bacteria cultures only] Pellet cells in a 2 mL tube or 50 mL Falcon tube by centrifuging at 5,000 rcf for 10 min at room temperature and discard growth medium. Skip next step (grinding) and proceed to adding lysis buffer.
- 5 Grind tissue to a fine powder, keeping frozen with liquid nitrogen.

	Prep size	Tissue input	Recommendation
	Small (2 mL tube prep)	100-200 mg	1-3 ball bearings in 2 mL tube, place in TissueLyser (Qiagen) for 2 min at 25 Hz.
	Medium (50 mL Falcon tube prep)	1-3 g	Mortar and pestle, transfer to a 50 mL Falcon tube.
	Large (250 mL Schott bottle prep)	≤ 30 g	Mortar and pestle, or blender. Transfer to 250 mL Schott bottle.

SORBITOL WASH (OPTIONAL)

- 6 [Optional] If the sample is likely to have a high amount of sugars, oils and/or other endogenous chemicals present (e.g. plant tissue), perform a sorbitol wash. See the following protocol:

Sorbitol washing complex homogenate for improved DNA extractions

Jones, A., and Schwessinger, B. (2020). Sorbitol washing complex homogenate for improved DNA extractions. Protocols.io v1, [dx.doi.org/10.17504/protocols.io.beuvjew17506](https://doi.org/10.17504/protocols.io.beuvjew17506).



CELL LYSIS

- 7 Add lysis buffer to tissue. Vortex or shake vigorously to mix.

Prep size	Lysis buffer to add
Small (2 mL tube prep)	750 μ L
Medium (50 mL Falcon tube prep)	Bring volume to 20 mL
Large (250 mL Schott bottle prep)	Bring volume to 100 mL

Note

Lysis buffer should be 1-2x the approximate volume of the ground tissue.

- 8 [Bacteria cultures only] Add a small scoop of lysozyme to the lysis reaction, approximately 2-4 mg/mL.

- 9 Add RNase A and Proteinase K to the solution. Mix by swirling and inverting.

[Bacteria cultures only] Do not add Proteinase K yet.

Prep size	Lysis volume	RNase A (20 mg/mL stock)	Proteinase K (20 mg/mL stock)
Small (2 mL tube prep)	750 μ L	8 μ L (213 μ g/mL final)	4 μ L (107 μ g/mL final)
Medium (Falcon tube prep)	20 mL	200 μ L (200 μ g/mL final)	100 μ L (100 μ g/mL final)
Large (Schott bottle prep)	100 mL	400 μ L (80 μ g/mL final)	200 μ L (40 μ g/mL final)

Note

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 temperatures 15-70°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.*
- *Lysozyme activity varies based on where it was purified from. Generally, active at temperatures 20-55°C (optimal at 30-50°C), pH 6-9 (optimal pH 6.2).*

- 10 Incubate the samples at 55°C, shaking at ~450 rpm if possible. Recommended time:

	Prep size	Incubation time
	Small (2 mL tube prep)	60 min
	Medium (50 mL Falcon tube prep)	60 min
	Large (250 mL Schott bottle prep)	90-120 min
	[Bacteria cultures only]	30 min

Note

Higher temperatures and longer incubations lead to DNA damage.

- 11 [Bacteria cultures only] Add Proteinase K (see previous table) and incubate at 55°C for an additional 30 min.

CONTAMINANT REMOVAL

- 12 [Large prep only] Filter the homogenate using a sieve (or disk membrane mesh placed in a funnel) into a new 250 mL Schott bottle. Forcibly squeeze out as much residual homogenate from the debris as possible. Then split the homogenate into 50 mL Falcon tubes, bringing the volume to 20 mL for each.



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

	Prep size	Potassium acetate
	Small (2 mL tube prep)	~250 μ L
	Medium (50 mL Falcon tube prep)	~7 mL
	Large (250 mL Schott bottle prep)	~7 mL per 50 mL Falcon tube

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

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

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

- 17 Transfer supernatant to a new tube.

DNA BINDING AND WASH

- 18 Add approximately an equal volume of binding buffer or more. Utilise the capacity of the tube, leaving only a small space for adding Sera-Mag beads. Approximate amounts:

	Prep size	Sample volume	Binding buffer
	Small (2 mL tube prep)	~950 μ L	Bring volume to 1.90 mL
	Medium (50 mL Falcon tube prep)	~25 mL	Bring volume to 50 mL
	Large (250 mL Schott bottle prep)	~25 mL per 50 mL Falcon tube	Bring volume to 50 mL

Note

Adding more buffer can increase to yield, especially if the sample has a lot of contaminants. More shorter fragments will also be recovered, but can be size selected against later.



- 19 Thoroughly mix the 2% Sera-Mag beads and add them to the sample:

Prep size	Sera-Mag beads
Small (2 mL tube prep)	100 μ L
Medium (50 mL Falcon tube prep)	1 mL
Large (250 mL Schott bottle prep)	1 mL per Falcon or 10 mL overall

- 20 Mix by inverting the tube 20 times. Incubate with gentle mixing using a rotator or a shaker platform at room temperature.

Prep size	Recommended time
Small (2 mL tube prep)	≥ 10 min
Medium (50 mL Falcon tube prep)	≥ 60 min or overnight
Large (250 mL Schott bottle prep)	≥ 120 min or overnight

- 21 Place the tube in a magnetic rack until the solution becomes clear.

Prep size	Recommended time
Small (2 mL tube prep)	≥ 10 min
Medium (50 mL Falcon tube prep)	≥ 30 min
Large (250 mL Schott bottle prep)	≥ 120 min or overnight

- 22 Remove the supernatant without disturbing the beads, keeping the tube on the magnetic rack.

Note

Small preps can be done by pipetting, larger preps by decanting.

- 23 Wash the beads by filling the tube with 70% ethanol, let beads settle if disturbed, and decant out the ethanol.
- 24 Repeat the ethanol wash another 2 times, or until satisfied the beads are clean.

**Note**

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

- 25 For medium and large preps, transfer the beads into a 2 mL Eppendorf tube. This can be done by taking the Falcon tube off the rack, adding 1 mL of 70% ethanol to dislodge the beads, transfer suspension to an Eppendorf tube. Repeat with another 1 mL to ensure all beads are transferred. Place the 2 mL Eppendorf tube on the magnetic rack, remove supernatant and repeat the process with other Falcon tubes until all beads are in the same eppendorf tube.

Note

Do not let the beads dry during the process, keep the beads wet with ethanol and perform the process quickly. Larger preps can be done at the sink to discard ethanol easily.

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

Note

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

- 27 Remove tube from magnetic rack and add 10 mM TRIS-HCl pH 8 (or nuclease-free water) to the beads, gently resuspending (use wide-bore pipette tip if available). Gently tapping the tube is also suitable.

If the solution is very thick and cloudy white, there is a large yield; consider slowly increasing the elution volume (in some cases, double or triple the volume, e.g. large genomes, high cell density).

	Prep size	Recommended elution
	Small (2 mL tube prep)	50 µL
	Medium (50 mL Falcon tube prep)	200 µL
	Large (250 mL Schott bottle prep)	500 µL

Note

Larger volume is based on size selection protocols, e.g. PippinHT input across x1 whole cassette or a DNA clean-up protocol.

28 Incubate at room temperature for at least 10 min.

29 Place the tubes in the magnetic rack until the solution becomes clear.

Note

Highly concentrated DNA will take a long time. The tube can be left on the magnetic rack overnight in the fridge, or increase the elution volume.

30 Transfer the supernatant (contains DNA) to a 1.5 mL Eppendorf tube, avoiding any carry-over of beads. Use DNA LoBind tube if available.

31 Perform an additional, second elution on the beads (same volume as previous), remove from magnet, resuspend, incubate, magnetise and transfer to another 1.5 mL Eppendorf tube. Use DNA LoBind tube if available.

Note

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

32 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.

Note

- *For Nanodrop, use 1 μ L. As the DNA is crude, it 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 μ L when sample is plentiful. Using 1 μ L is prone to pipetting errors.*
- *For pure DNA, Nanodrop:Qubit is 1:1, 260/280 is 1.8 and 260/230 is 2.0.*
- *DNA yield can be 20-500 μ g.*

33 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 12 months.

- 34 For some species, the DNA may be pure enough to proceed to sequencing. If the DNA is crude (likely for plant samples), consider DNA clean-up and size selection options below.

DNA CLEAN-UP AND SIZE SELECTION OPTION 1: SHORT-READ ELIMINATOR

- 35 If the DNA is clear, has small quantities of impurities, consider utilising a Short-Read Eliminator kit from Circulomics.

If the DNA is discoloured with visible impurities, it is best to perform a DNA clean-up first before using the Short-Read Eliminator kit. See the following protocol:

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

Jones, A., Purushotham, N., Nasim, J., and Schwessinger, B. (2020). DNA clean-up and size selection for long-read sequencing. Protocols.io v3, [dx.doi.org/10.17504/protocols.io.betdjei17506](https://doi.org/10.17504/protocols.io.betdjei17506).

Note

It has been observed that the Short-Read Eliminator kit can result in and unexpected increased absorbance/fluorescence from Nanodrop/Qubit results. If this is seen, consider using more DNA input into the sequencing library prep than recommended. Perhaps the DNA double helix becomes altered or unwound. Alternatively, what is traditionally measured as DNA concentration has always been an under-estimate.

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

- 36 For most recalcitrant plants, the DNA will still have a high quantity of impurities and DNA fragmentation is inevitable. Gel purification is an ideal solution to both problems. Proceed to gel purification by Pippin Prep (Sage Science) or equivalent.
- 37 If a precipitant is noticeable in the DNA solution, briefly spin down and take the upper aqueous layer to gel purification below.

Note

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.

- 38 Using a PippinHT (Sage Science) or similar automated electrophoresis product, gel purify approximately 30 µg, following the manufacturer's instructions. A 20 kb high pass separation is recommended, however if DNA is limited, 15 kb high pass is 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 safely overloaded to 3 µg per lane (30 µg total) without noticeable consequences. Overloading further slows the migration of longer fragments and shorter fragments are eluted as the size selection will not be precise.

Note

If the sample is very dirty and may not electrophorese correctly, consider a chloroform: isoamyl alcohol 24:1 clean-up before using the PippinHT.

- 39 After separation, wait at least 45 min (hours or overnight is suitable), to aid elution and recovery.
- 40 Collect the contents of all elution wells into a 1.5 mL Eppendorf tube (approx. 300 µL). Use DNA LoBind tube if available.
- 41 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 Eppendorf tube (another 300 µL, tube total is approx. 600 µL).
- 42 Add 1.2x binding buffer (approx. 720 µL), and 100 µL of 2% Sera-Mag beads to the 1.5 mL Eppendorf tube. Incubate for 5-10 min at room temperature.
- 43 Place on a magnetic rack for 5 min, or until the solution becomes clear. Discard the supernatant.
- 44 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.
- 45 Remove all traces of ethanol and let the beads air dry for 1-4 min.

Note

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

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

Note

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

- 47 Place on a magnetic rack for 5 min, or until the solution becomes clear. Transfer eluted DNA to a new 1.5 mL Eppendorf tube. Use DNA LoBind tube if available. Use a wide-bore pipette tip if available.
- 48 Perform an additional, second elution on the beads (same volume as previous), remove from magnet, resuspend, incubate, magnetise and transfer to another 1.5 mL Eppendorf tube. Use DNA LoBind tube if available.

Note

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

- 49 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 μ L.
- Qubit fluorometer is highly dependent on the accuracy of the amount pipetted. Use 2 μ L when sample is plentiful. Using 1 μ L is prone to pipetting errors.
- For pure DNA, Nanodrop:Qubit is 1:1, 260/280 is 1.8 and 260/230 is 2.0.
- Expect 20-30% recovery relative to total input (~6-9 μ g out of 30 μ g).

- 50 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 12 months.