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High molecular weight gDNA extraction after Mayjonade et al. optimised for eucalyptus for nanopore sequencing V.9

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High molecular weight DNA extraction from all kingdoms
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Abstract

Extraction of high quality DNA for long read sequencing e.g. the Oxford Nanopore
Optimized for DNA extraction from eucalyptus grandis and eucalyptus pauciflora.

This protocol contains an optional Chloroform clean up step which is necessary for eucalyptus but might not be for other tissue.

For long DNA fragments don't vortex the DNA sample.



Guidelines

Modified from the protocol of Baptiste Mayjonade, Jérôme Gouzy, Cécile Donnadieu, Nicolas Pouilly, William Marande, Caroline Callot, Nicolas Langlade and Stéphane Munos, High molecular weight gDNA extraction, Bio Techniques, Vol. 61, No. 4, October 2016, pp. 203-205.

Link to webpage: <http://www.biotechniques.com/BiotechniquesJournal/2016/October/Extraction-of-high-molecular-weight-genomic-DNA-for-long-read-sequencing-of----single-molecules/biotechniques-365135.html>

When citing please make sure to also mention the original Mayjonade et al. protocol as described above.

Thank you Benjamin Schwessinger for the constant support in the lab and outside!

Reagents required (stock solutions)

Lysis buffer:

- 10% PVP 40
- 10% PVP 10
- 5M NaCl
- 1M TRIS pH 8
- 0.5 M EDTA
- 20% SDS
- Sodium metabisulfite (190.1 g/mol)
- Molecular biology grade water

Beads solution (adapted from Philippe Jolivet and Joseph W. Foley, 2015 - Solutions for purifying nucleic acids by solid-phase reversible immobilization (SPRI)) very useful tips about preparing the high concentrated PEG solutions can be found - definitely worth a look!

- Sera-Mag SpeedBeads (GE Healthcare, 65152105050250)
- 50% PEG 8000
- 5 M NaCl
- 1 M Tris-HCl pH 8
- 0.5 M EDTA pH 8
- molecular biology grade water

Other solutions:

- 5M Potassium Acetate pH 7.5
- Ethanol 70%
- TE-Buffer (10 mM Tris, 1 mM EDTA)



Additional for Clean up step:

Chloroform:Isoamylalcohol (24:1)

Natriumacetate 3 M

Ethanol 100 %

Ethanol 70 %

TE-Buffer

Enzymes

RNAse A (1000 U/ml, Thermo Fisher EN0541)

Proteinase K (800U/ml, NEB P81072)

Materials:

TissueLyser II (Qiagen)

Thermomixer

Magnetic rack for Eppendorf tubes

Troubleshooting

Before start

Prepare beads stock solution

This is the same beads stock solution as in the size selection protocol: <https://www.protocols.io/view/dna-size-selection-1kb-and-clean-up-using-an-optim-ir3cd8n>

For 10 mL:

	Final	stock	Input
	10 mM Tris-HCl	1 M	100 μ l
	1 mM EDTA pH 8	0.5 M	20 μ l
	1.6 M NaCl	5 M	3.2 ml
	11% PEG 8000	50% (w/v)	2.2 ml
	0.4% beads (v/v)	100%	40 μ l
	Milliq Water		4.44 ml

**Because there were some questions considering the beads concentration:*

- 0.4% beads final are 40 μ L of the Sera Mag speed beads in a total Volume of 10 mL 'beads stock solution'- thats not much but you definitely should see them in the solution!

- there is no issue in increasing the beads concentration to e.g. 1% (100 μ L in the final 10 mL), there should be no change in size selection, the beads have a binding capacity of 5 μ g/ml anyway. I tested the different beads concentrations and there was no difference between final 0.4% - 2% beads, I just took the 0.4% because I needed a lot of beads solution in total.

-Make sure to use the Sera-Mag SpeedBeads and not the AMPure XP beads for preparing the beads stock solution. The AMPure XP beads are used straight (at 0.45V) without further manipulation.

1. Frist combine only Water, Tris-HCl, EDTA and NaCl in a 50 mL tube.



2. Vortex Sera-Mag SpeedBeads (GE Healthcare, 65152105050250) very well and pipette 40 μ l into a 1.5 ml tube, put it on the magnetic rack and wait until solution has cleared up and all beads have bound to the back of the tube
3. Wash beads by removing supernatant and adding 1.5 ml milliQ water
4. Take tube off the magnet, mix well, spin down in a microcentrifuge and put back on the magnet
5. Wait for beads to assemble at the back of the tube
6. Pipette off and discard supernatant
7. Repeat washing (steps 3 - 6) 3 more times
8. After pipetting of the supernatant the last time take off tube from the magnet and add 40 μ l of the previous (step 1) prepared stock solution, mix well, spin down and pipette everything into the remaining stock solution in the 50 mL tube and mix
9. Now the 2.2 ml 50% PEG can be added to the stock solution, which after vortexing very well is ready for use. Be careful to actually pipette 2.2 ml as solution is very viscous, but the final concentration of PEG is crucial for the clean up to work properly.

Always use fresh lysis buffer and fresh 70 % Ethanol

Clean up steps 26 - 41 are optional and are only necessary in recalcitrant tissue like eucalyptus. The extraction and clean up step can also be done on separate days if DNA is stored at 4°C.

Prepare lysis buffer

- 1 Always prepare fresh lysis buffer before extraction for optimal results (especially the DTT should be added fresh)

For 10 mL Lysis Buffer:

	Final	stock	Input
	1% PVP 40	10%	1 mL
	1% PVP 10	10%	1 mL
	500 mM NaCl	5 M	1 mL
	100 mM TRIS pH 8	1 M	1 mL
	50 mM EDTA	0.5 M	1 mL
	1.25% SDS	20%	625 μ L
	1% (w/v)! Sodium metabisulfite	190.1 g/mol	0.1 g
	5 mM Dithiothreitol (DTT)	1 M	50 μ L
	Milliq Water		4.3 mL

Heat lysis buffer to 64 °C for 30 minutes. (This is important for the DNase heat inactivation)


After cooling down to room temperature add per 1 mL lysis buffer 1 μ L RNase A (in this case 10 μ L)

 00:30:00 Heating to 64 °C

Prepare tissue

- 2 In the meantime prepare 2 mL Eppendorf tubes with 1-2 metal beads (5 mm) and 100 mg of tissue and transfer tubes into liquid nitrogen.


This is the easiest with fresh tissue because freeze thawing is avoided during cutting. But if there is no other way, tissue frozen in liquid nitrogen and making sure tissue stays frozen also works.

 100 mg Tissue

Grinding


- 3 Before grinding make sure tissue is completely frozen in liquid nitrogen and do the grinding steps as quickly as possible to avoid freeze thawing. The grinding rock can also be frozen to ensure that.

Grind tissue using an automated grinder (Qiagen TissueLyzer II) for 40 seconds (actual grinding time may differ from tissue to tissue)


 00:00:40 Grinding

Extraction I

- 4 Add 700 μ L of preheated buffer and mix by inverting tube until no frozen clumps of tissue are left
(This can take up to a few minutes, but it's worth it)


 700 μ L Preheated buffer

- 5 Incubate at 37°C in a thermomixer shaking at 400 rpm (slowly) for 20 minutes

 00:20:00 Incubation at 37°C

- 6 Add 10 μ L Proteinase K per tube and incubate for another 20 - 30 min at 37°C in the thermomixer

 10 μ L Proteinase K

 00:30:00 Incubation at 37°C

- 7 Take the tubes out of the thermomixer and cool down on ice for 5 minutes

 00:05:00 Cool down

- 8 Add 210 μ L (~0.3 volumes) of 5M Potassium Acetate and mix by inverting the tube 20 times and then immediately keep on ice at ~4°C

 210 μ L 5M Potassium Acetate


- 9 Centrifuge at 8000g for 12 minutes at 4°C


 00:12:00 Centrifugation at 4°C

- 10 Transfer the supernatant (~600 μ L) to a new 1.5 mL tube without disturbing the pellet

- 11 Add 1 volume (~600 μ L) of beads solution previously prepared (make sure beads are at room temperature and well homogenized via vortexing for approximately 30 seconds)



 600 µL Beads solution


 00:00:30 Vortex

Note

Only vortex the beads alone before adding to the sample
Do NOT vortex the sample containing the DNA


12 Mix by inversion and then incubate on a rotor for 10 minutes at RT

(In the meantime put TE buffer into waterbath at 50°C so that its preheated later)

 00:10:00 Incubation on rotor


13 Spin down the tube for 1 second


14 Place the tube in a magnetic rack for 5 minutes (until beads are stuck to the wall of the tube and solution becomes clear)

 00:05:00 Magnetic rack

15 Remove the supernatant without disturbing the beads

16 Add 1 mL of fresh 70 % Ethanol and wait for 30 seconds

 1 mL 70 % Ethanol

 00:00:30 Wait

17 Remove supernatant without disturbing the beads

18 Repeat the washing steps 15 - 16 once more

 [go to step #15](#) Repeat washing

19 Spin down the tube for 1 second and place the tube on the magnetic rack to remove the remaining Ethanol

20 Let the beads air-dry for 30 seconds, but not longer because this would decrease elution efficiency

 00:00:30 Air-drying

21 Add 50 µL of TE buffer preheated to 50°C and resuspend the beads by flicking the tube (make sure they are not aggregated anymore)



🧪 50 µL TE buffer

Note

This volume can be changed to the amount needed (between 20 - 100 µl are most useful to handle)

22 Incubate the resuspended beads for 10 minutes at room temperature

(to make sure the DNA can go back into elution)

🕒 00:10:00 Incubation at room temperature

23 Spin down the tube for 1 second and place the tube in the magnetic rack and incubate for 5 -10 minutes (until solution becomes clear)

🕒 00:10:00 Magnetic rack incubation

24 Transfer the supernatant (eluted DNA) into new tube

QC I

25 Measure DNA concentration with a Qubit and absorbance with a NanoDrop.

Aiming for:

Qubit/Nanodrop: 0.5 - 1.0

260:280: 1.8 - 2.0

260:230: 2 - 2.2

For tissue with less secondary metabolites and oils, the purity of the DNA sample most likely should be good and similar to the values described above.

For recalcitrant tissue though, like Eucalyptus the following clean up step is recommended.

Clean up

26 Transfer the DNA solutions into one tube and add TE-Buffer to make up a total volume of 500 µL

If the extraction is done with multiple tubes at a time (which usually is the case), then after the elution step all the eluted DNA can just be pipetted into one tube.

27 Add 500 µL (1 V) Chloroform:Isoamylalcohol (24:1) and invert tube about 100 times (2 minutes at least)



🧪 500 µL Chloroform:Isoamylalcohol (24:1)

28 Centrifuge tube to separate the phases at 8000g at 4°C for 10 minutes

🕒 00:10:00 Centrifugation at 8000g and 4°C

29 Transfer the upper phase (DNA) into a new tube and discard the lower chloroform phase

(depending on how good the transfer worked out one round is usually enough but if some of the intermediate phase has been transferred, steps 28 -29 can be repeated)

30 Add 50 µL (0.1 V) 3 M Sodium Acetate (NaAc) and mix by inverting tube 10-20 times

🧪 50 µL 3 M Sodium Acetate

31 Add 500 µL (1 V) of 100% Ethanol and mix by inverting tube carefully a few times and then let incubate at 4°C for 5 - 10 minutes.

Depending on DNA concentration (for me at 100ng/µL) DNA starts to precipitate but if the sample is very clean, which is desirable, this will be seethrough. So only is recognisable by a thickening of the solution and probably air bubbles bound to precipitate (at least that what it looks like to me).

🧪 500 µL 100% Ethanol

🕒 00:10:00 Incubation at 4°C

32 Centrifuge at 4°C and 5000g for 2 minutes.

(If nothing precipitates out of solution after that, centrifuge for 10 minutes at 10000g) But the short centrifugation time and lower speed are used to select for longer fragments in the beginning.

🕒 00:02:00 Centrifugation at 4°C and 5000g

🕒 00:10:00 Centrifugation at 10000g




- 33 Pipette supernatant off and make sure to do it on the opposite side than the pellet is supposed to be.


If the the pellet was seethrough I would recommend pipetting the supernatant into a new tube just to make sure that if nothing precipitated out and no pellet is seen the supernatant can be centrifuged again at a higher speed and longer.

Sometimes the "pellet" for me is just a seethrough smear along the tube (due to the low speed I guess)

- 34 Add 1 mL 70 % Ethanol to pellet to wash off salt


 1 mL 70% Ethanol

- 35 Centrifuge at 4°C and 8000 g for 5 minutes (here the higher speed shouldn't matter anymore because DNA is pelleted already)

 00:05:00 Centrifugation at 4°C and 8000 g

- 36 Remove supernatant

- 37 Repeat steps 35 - 37 once more and make sure all Ethanol is pipetted off

 [go to step #35](#) Repeat steps 35-37

- 38 Let air dry (for last removal of Ethanol) for 2 - 5 minutes
(here the drying time is not as critical as in the beads drying step as the DNA pellet should easier dissolve than the beads)

 00:02:00 Air-drying


- 39 Add 50 µL of preheated (to 50°C) 10 mM Tris (pH 8) or TE-Buffer and elute DNA as long as necessary for the whole pellet to dissolve.

If it's a large pellet I let it dissolve overnight at room temperature

 50 µL 10 mM Tris (pH 8) or TE-Buffer

QC II

- 40 Measure Qubit and Nanodrop values again (as in step 25)

 [go to step #25](#) Qubit and Nanodrop values