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# High molecular weight gDNA extraction after Mayjonade et al. optimised for eucalyptus for nanopore sequencing V.8

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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, Jérome 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 acides 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 µl |
| | 1 mM EDTA pH 8 | 0.5 M | 20 µl |
| | 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 µl |
| | Milliq Water | | 4.44 ml |

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

*- 0.4% beads final are 40 µL 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 µL in the final 10 mL), there should be no change in size selection, the beads have a binding capacity of 5 µg/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 µl 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 of 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 µl of the previous (step 1) prepared stock solution, mix well, spin down and pipette everyting 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 seperate days if DNA is stored at 4°C.

## Prepare lysis buffer

1 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 µL |
| | 1% (w/v)! Sodium metabisulfite | 190.1 g/mol | 0.1 g |
| | 5 mM Dithiothreitol (DTT) | 1 M | 50 µL |
| | Milliq Water | | 4.3 mL |

Heat lysis buffer to 64 °C for 30 minutes.

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

## 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.

## Grinding

3 Before ginding make sure tissue is completely frozen in liquid nitrogen and do the grinding steps as quickly as possible to avoid freeze thawing. The grinding reck 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)

## Extraction I

4 Add 700 uL 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)

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

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

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

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

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

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

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

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)

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)

15 Remove the supernatant without disturbing the beads

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

17 Remove supernatant without disturbing the beads

18 Repeat the washing steps 15 - 16 once more

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

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

21 Add 80 uL of TE buffer preheted to 50°C and resuspend the beads by flicking the tube (make sure they are not aggregated anymore)

22 Incubate the resuspended beads for 10 minutes at room temperature

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

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

24 Transfer the supernatant (eluted DNA) into new tube

## QC I

25 Meassure 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 (not exceeding 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 pipettet into one tube.

27 Fill up to approximatelly 500 µL with TE-buffer

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

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

30 Transfer the upper phase (DNA) into a new tube and discard the 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)

31 Add 50 µL (0.1 V) 3 M Sodium Acetate (NaAc) and mix by inverting tube

32 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 sample is very clean, which is desireable, this will be seethrough. So only is recognisable by a thickening of the solution and probably airbubbles bound to precipitate (at least that what it looks like to me).

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

(If nothing precipiates 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.

34 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)

35 Add 1 mL 70 % Ethanol to pellet to wash off salt

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

37 Remove supernatant

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

39 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)

40 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 over night at room temperature

## QC II

41 Meassure Qubit and Nanodrop values again (as in step 25)