High molecular weight gDNA extraction.

Protocol for:

Extraction of high-molecular-weight genomic DNA for the long-read sequencing of single molecules.

Baptiste Mayjonade[1], Jérôme Gouzy[1], Cécile Donnadieu[2], Nicolas Pouilly[1], William Marande[3], Caroline Callot[4], Nicolas Langlade[1] and Stéphane Muños[1]

1. LIPM, Université de Toulouse, INRA, CNRS, Castanet-Tolosan, France

2. Get-PLAGE, Université de Toulouse, INRA, CNRS, Castanet Tolosan, France.

3. CNRGV, Université de Toulouse, INRA, CNRS, Castanet Tolosan, France.

4. CRCT, INSERM, Université de Toulouse, CNRS, Toulouse, France.

LEGEND

⇨ **Warning**

REAGENTS

Sodium Chloride Biotechnology Grade (VWR, Radnor, PA, USA).
1M TRIS, pH 8.0 biotechnology grade (Amresco, Solon, OH, USA).
0.5M EDTA, sterile solution biotechnology grade (Amresco, Solon, OH, USA).
Sodium Dodecyl Sulfate (SDS), 20% Solution Biotechnology Grade (Amresco, Solon, OH, USA).
Polyvinylpyrrolidone average mol wt 40,000 (Sigma, St. Louis, MO, USA).
Sodium metabisulfite ReagentPlus®, ≥99% (Sigma, St. Louis, MO, USA).
Potassium acetate for molecular biology, ≥99.0% (Sigma, St. Louis, MO, USA).
Polyethylene Glycol 8000 (PEG) (Fisher Scientific, Pittsburgh, PA, USA).
Buffer EB (QIAGEN, Germantown, MD, USA).
Sera-Mag SpeedBead magnetic carboxylate modified particles, DSMG-CM, 1 um, 5% solids ((Fisher Scientific, Pittsburgh, PA, USA).
RNase A 100 mg/ml (QIAGEN, Germantown, MD, USA).
Ethanol absolute AnalaR NORMAPUR® ACS, Reag. Ph. Eur. analytical reagent (VWR, Radnor, PA, USA).

PROCEDURE

Before starting:

- Warm the lysis buffer at 65°C for at least 30 minutes.
- Preheat a water bath or a heating block to 50°C.
- Cool down the centrifuge and the rotor (2ml tubes) at 4°C.
- Incubate the elution buffer at 50°C.
- Allow Serapure to come to room temperature prior to use.
-

A. CELLS LYSIS

1. From tissues: tissues must be immediately frozen in liquid nitrogen after harvest and stored at -80°C. The tissues are grinded in fine powder with liquid nitrogen in a pre-cooled mortar or using an automated grinder (like Ultra Centrifugal Mil ZM 200 with a 0.5-mm sieve, Retsch). The finest the powder is, the better the yield, the purity and the integrity will be.

   Add 600 µl of preheated buffer and 4 µl of RNAse A (100 mg/ml QIAGEN) in a 2 ml tube. Add 100 mg of tissue powder (note: adjust the quantity of materials if needed) and immediately homogenate by inverting the tube 20 times. It is very important to quickly homogenate the lysate to neutralize DNAse. Homogeneity of the lysate is also important for the overall yield. Short vortexing can be performed but could lead to DNA shearing.

   From liquid cultures: pellet bacteria or cells from an appropriate volume of culture by centrifugation at 5000g for 10 minutes at room temperature. Add 600 µl of preheated buffer and 4 µl of RNAse A (100 mg/ml QIAGEN) and immediately homogenate by pipetting up and down 10 times with a wide bore tip. It is very important to quickly homogenate the lysate to neutralize DNAse. Homogeneity of the lysate is also important for the overall yield. Short vortexing can be performed but could lead to DNA shearing.

2. Incubate the tube at 50°C during 30 minutes and mix by inverting the tube 20 times every 10 minutes. A rotator, placed in an oven at 50°c, can be alternatively used to keep the lysate homogenous.

⇨ The lysate must be homogenous. Aggregates of material can decrease the lysis and that will therefore reduce the yield of the extraction.

B. CONTAMINENTS PRECIPITATION WITH POTASSIUM ACETATE

3. Add 200µl (or 1/3 of the lysis buffer volume) of 5M Potassium Acetate and mix by inverting the tube 20 times in order to obtain a homogenous solution to fully precipitate the proteins and the polysaccharides that will complex with SDS. It is important to incubate at 4°C after the addition of Potassium Acetate.

4. Centrifuge at 5000 g for 10 minutes at 4°C.

C. GENOMIC DNA PURIFICATION

5. Transfer the supernatant to a new 2 ml tube without disturbing the pellet.

6. Add one volume of binding buffer and 1:18 (v:v) of Serapure beads previously prepared (vortex the beads solution for 20 seconds before use to ensure that the beads are completely resuspended).

7. Mix by inverting the tube 20 times

8. Incubate with a gentle agitation (with a rotator or a rocker platform) for 10 minutes at room temperature.

9. Spin down the tube for 1 second.

10. Place the tube in a magnetic rack for 3 minutes (until the solution becomes clear). The actual time required to collect beads may vary according to samples.

11. Remove the supernatant without disturbing the beads pellet.

12. Add 1 ml of wash solution, remove the tube from the magnetic rack and mix by inverting the tube 20 times.

13. Spin down the tube for 1 second.

14. Place the tube in the magnetic rack and wait for 30 seconds (until the solution becomes clear).

15. Remove the supernatant without disturbing the beads pellet.

16. Repeat steps 10 to 12.

17. Spin down the tube for 1 second and place the tube on the magnetic rack to remove the remaining washing solution.

18. Let the beads air-dry for 1 minute with the cap open. Do not let the beads dry more than 1 minute as this will significantly decrease elution efficiency.

19. Add 80 µl of the elution buffer EB preheated to 50°C.

20. Resuspend the beads by flicking the tube. It is important that the beads are not aggregated.

Aggregates of the beads can significantly reduce the elution efficiency.

21. Spin down the tube for1 second and place the tube in the magnetic rack. Let the solution to become clear. If DNA solution is highly concentrated, it can take a long time. In this case, it is recommended to let the tube in the magnetic rack overnight or to add more elution buffer.

22. Transfer 75 µl of the eluted gDNA solution in a new tube.

23. Measure the absorbance of the gDNA solution at 230, 260 and 280nm with a spectrophotometer (NanoDrop 2000; ThermoFisher; Waltham; MA; USA). $A_{260/280}$ ratio must be between 1.8 and 2. $A_{260/230}$ ratio must be between 2 and 2.2. $A_{260/280}$ ratio higher than 2 or $A_{260/230}$ ratio higher than 2.2 could be indicative of a contamination by RNA.

24. Determine the concentration of the double-strand DNA with a fluorimetric-based method (Qubit 3.0 Fluorometer; ThermoFisher; Waltham; MA; USA).

25. Evaluate the integrity of the gDNA on pulsed field electrophoresis or on a system allowing the separation of high molecular weight DNA molecules with a sufficient resolution.

D. OPTIONAL PURIFICATION

The following additional purification steps can improve the $A_{260/280}$ and $A_{260/230}$ ratios.

Before starting:

- Allow Serapure to come to room temperature prior to use.
- Mix the beads by vortexing for 20 seconds before use

26. Add one volume of Serapure beads to the DNA solution.

27. Follow steps 7 to 25.

    Steps 12 to 14 are replaced by the following washing step: add 2 ml of wash solution by keeping the tube on the magnetic rack and wait for 30 seconds. Repeat it as indicated in step 16.

SOLUTIONS PREPARATION

- Lysis buffer (use **fresh solution for optimal results):**

Component	Quantity	[final]
Polyvinylpyrrolidone 40	0.1 g	1%
Sodium metabisulfite	0.1 g	1%
Sodium Chloride	0.29 g	0.5 M
TRIS, 1M, pH 8.0	1 ml	100 mM
EDTA, 0.5M	1 ml	50 mM
SODIUM DODECYL SULFATE (SDS), 20%	625 µl	1.25 %
Water molecular biology grade	Adjust to 10 ml	-

⇨ Mix and incubate at 65°C during at least 30 minutes. The solution need to be clear before use.

- Serapure beads solution (adapted from Nadin Rohland and David Reich, 2012):

2% Sera-Mag SpeedBead magnetic carboxylate modified particles (wash 4 times with water to remove sodium azide).
18% PEG 8000
1 M NaCl
10 mM Tris-HCl pH 8.0
1 mM EDTA pH 8.0
Optional: 0.05% Tween 20
Note: AMPURE XP (Beckman Coulter, Brea, CA, USA) can be used but they must be washed 4 times with water and resuspended in their initial buffer.

- 5M Potassium Acetate solution:

Dissolve 4.9 g of potassium acetate in a final volume of 10 ml

- Binding buffer solution:

2 g of PEG8000
1.75 g of sodium chloride
Adjust to 10 ml with molecular biology grade water
Mix until the solution becomes clear.

⇨ If PEG8000 is not dissolved, it can lead to a poor yield because PEG8000 makes gDNA to bind to the beads.

- Washing solution:

Mix 35 ml of absolute ethanol and 15 ml of molecular biology grade water.

Reference:

Rohland, N., and D. Reich. 2012. Cost-effective, high-throughput DNA sequencing libraries for multiplexed target capture. Genome Res. **22**:939-46.

TROUBLESHOOTING

Problem	Suggested solution
Low purity ($A_{260/280}$ and $A_{260/230}$ ratio)	▪ Add an incubation step at 4°C for 10 minutes after step 3. ▪ Repeat step 16 twice. ▪ Perform the optional purification procedure (steps 26-27). ▪ Resuspend the beads in the washing solution during the additional purification procedure. ▪ Repeat step 16 twice during the optional purification. ▪ Repeat the optional purification procedure.
Low yield	▪ Improve the grinding to obtain a finer powder. ▪ Check if PEG8000 is dissolved to obtain a clear binding buffer solution. ▪ Check if the beads are completely resuspended during step 17 (the beads should not be aggregated). ▪ Increase the temperature up to 65°C during the lysis (step 2). The temperature cannot exceed 65°C for 1 hour (it could lead to DNA damages). ▪ Extend the lysis to 1 hour or more (step 2). ▪ Increase the SDS concentration to 2% in the lysis buffer solution.