High Molecular Weight genomic DNA extraction from Gram-negative bacteria for long reads sequencing (Xanthomonas ssp.)

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Abstract

Third generation sequencing aims to sequence long DNA molecules. High-quality and high-molecular-weight DNA is needed to fully benefit from the potential of third-generation sequencers. The cost of sequencing continues to decrease but DNA extraction kits remain expensive. Here is a fast and cheap protocol to extract genomic DNA from Gram-negative bacteria. The original protocol has been fine-tuned for Xanthomonas ssp., which is a tricky bacteria as it secrets a lot of extracellular polysaccharides. We found that bacterial culture can impact the purity of the final DNA solution. In addition, the lysis temperature and mixing by pipetting are critical to get an efficient homogenization as the sample becomes very viscous at some steps (see 'Assessment of various parameters.xlsx' attached below).

High yield Nanopore runs can be achieved with these gDNA samples (see 'Nanopore sequencing report.pdf' attached below).

Acknowledgements:

I would like to thank Harihar Jaishree Subrahmaniam, Rekha Gopalan Nair, and BenjaminSchwessinger for their feedbacks and their corrections on this protocol.

Citation: Baptiste Mayjonade High Molecular Weight genomic DNA extraction from Gram-negative bacteria for long reads sequencing (Xanthomonas ssp.). **protocols.io**

dx.doi.org/10.17504/protocols.io.r9id94e

Published: 02 Aug 2018

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.

https://www.future-science.com/doi/10.2144/000114460

The beads solution used in this protocol is the same as in this protocol:

Ramawatar, Benjamin Schwessinger (1523238626). DNA size selection (>3-4kb) and purification of DNA using an improved homemade SPRI beads solution.. **protocols.io** dx.doi.org/10.17504/protocols.io.n7hdhj6

When citing please make sure to mention the original Mayjonade et al. protocol as described above and the protocol for the beads solution.

pH makes a huge difference in the solubility of DNA and beads. If not set properly, beads tend to clump at DNA elution step that could lose as much 60-70 percent of the DNA. So make sure that your elution buffer (e.g. 10 mM Tris or 0.1 x TE) has a pH of 8.

Store beads solution at 4°C. Allow beads solution to come to room temperature before use (30 minutes).

Mix the beads solution well by vortexing before use.

Always use freshly prepared 70 % Ethanol

Before start

Prepare the following solutions:

- **Lysis buffer:**

Lysis buffer	Stock concentration	Final concentration	Volume for 10ml
H2O	-	-	5.25 ml
NaCl	2.5 M	0.5M	2ml
Tris HCl pH=8	1 M	100mM	1ml
EDTA pH=8 0	0.5 M	50mM	1ml
SDS	20%	1.5%	750µl
Total	-	-	10ml

- **Beads solution:**

Beads solution is the same as in this protocol:

Ramawatar, Benjamin Schwessinger (1523238626). DNA size selection (>3-4kb) and purification of DNA using an improved homemade SPRI beads solution.. **protocols.io** dx.doi.org/10.17504/protocols.io.n7hdhj6

Beads solution	Stock concentration	Final concentration	Volume for 10ml
Tris HCl pH=8	1 M	10 mM	100 µl
EDTA pH=8 0	0.5 M	1 mM	20 µl
NaCl	5 M	1.6 M	3.2 ml
PEG 8000	50% (w/v)	11%	2.2 ml
Tween 20	10 % (v/v)	0.20%	200 µl
Washed Sera-Mag beads (v/v)	100%	0.40%	40 µl
H2O	-	-	up to 10ml
Total	-	-	10 ml

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

2- Vortex Sera-Mag SpeedBeads® Carboxyl Magnetic Beads (GE Healthcare) very well and pipette 40 µl into a 1.5 ml tube, put it on the magnetic rack and wait until the solution has cleared up and all beads have bound to the back of the tube

3- Wash beads by removing supernatant and adding 1 ml Milli-Q 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 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.

10-

- **Potassium acetate 5M:**

Dissolve 4.9 g of potassium acetate in 10 ml of molecular grade water.

Materials

Tryptone Caseine Soy Broth 12186620 by Fisher Scientific

RNAse A 19101 by Qiagen

Polyethylene Glycol 8000 (PEG) 10407773 by Fisher Scientific

Elution buffer 19086 by Qiagen

GE Healthcare Sera-Mag SpeedBeads™ Carboxyl Magnetic Beads, hydrophobic 11819912 by Thermo Fisher Scientific

Protocol

 Bacterial culture

Step 1.

Inoculate a single bacterial colony into 10 mL of 10% TSB (i.e. 3g/l) (Fisherbrand™, Tryptone Caseine Soy Broth Product Code.12186620). Cultivate the bacteria for 48h at 28°C under constant agitation (120 rpm).

NOTES

Note: Culture medium and growth time can impact the purity of the gDNA (especially for bacterial species secreting extracellular polysaccharides like *Xanthomonas ssp.*).

See "Assessment of various parameters.xlsx" file attached in the abstract section.

Bacteria lysis and RNA removal

Step 2.

Heat the lysis buffer for >30 minutes at 65°C

NOTES

The composition of the lysis buffer is given in the Guidelines.

Bacteria lysis and RNA removal

Step 3.

Centrifuge 1.8 mL of bacterial culture in a 2 mL tube at 10 000g for 5 minutes

Bacteria lysis and RNA removal

Step 4.

Discard the supernatant by pouring off the liquid removing all growth media.

Bacteria lysis and RNA removal

Step 5.

Set a P1000 pipette on 900 µl and resuspend the bacterial pellet into 900 µl of lysis buffer (preheated at 65°C) by pipetting up and down vigorously 10 times (with an uncut P1000 tip). This step is critical for the yield and it is very important to pipette the complete liquid up and down vigorously so all the liquid enters the pipette tip during mixing. Apparition of foam is good.

Bacteria lysis and RNA removal

Step 6.

Incubate at 65°C in a dry bath for 20 minutes (invert the tube vigorously 10 times after 10 minutes).

NOTES

A temperature below 60°C can significantly reduce the yield due to incomplete lysis.

See "Assessment of various parameters.xlsx" file attached in the abstract section.

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Bacteria lysis and RNA removal

Step 7.

Spin down the tube for 1 second

Bacteria lysis and RNA removal

Step 8.

Add 4 µl of RNAse A 100 mg/ml (QIAGEN, Cat No./ID: 19101)

Bacteria lysis and RNA removal

Step 9.

Mix by inverting the tube 10 times and spin down for 1 second

Bacteria lysis and RNA removal

Step 10.

Incubate at room temperature (18-23°C) for 10 minutes

Proteins and contaminant precipitation

Step 11.

Add 300 µl of 5M Potassium Acetate solution (or 1/3 of the lysis buffer volume)

Proteins and contaminant precipitation

Step 12.

Mix by pipetting vigorously and completely 10 times with a P1000 set on 1000 µl and using a P1000 tip cut at 0.5 cm from the tip end (to minimize DNA shearing).

NOTES

This step is very important to precipitate contaminants and therefore will impact the purity of the final gDNA solution. The solution gets very viscous after Potassium Acetate addition and mixing by inverting the tube is not efficient enough.

Proteins and contaminant precipitation

Step 13.

Centrifuge the tube at 10 000g for 10 minutes at room temperature

Proteins and contaminant precipitation

Step 14.

Transfer the upper phase into a new 2 mL tube without disturbing the pellet (typically 800µl)

Genomic DNA purification

Step 15.

Add the same volume (800 µl) of beads solution previously prepared (make sure beads are at room temperature for >30 minutes and well homogenized via vortexing for approximately 30 seconds)

Genomic DNA purification
Step 16.

Incubate on a rotator at 10 rpm for at least 15 minutes at room temperature

Genomic DNA purification
Step 17.

Spin down the tube for 1 second

Genomic DNA purification
Step 18.

Place the tube on a magnetic rack and wait for 3 minutes (until beads are stuck to the wall of the tube and solution becomes clear)

Genomic DNA purification
Step 19.

Discard the supernatant by pipetting without taking out any beads

Genomic DNA purification
Step 20.

Add 1 mL of fresh 70% ethanol

Genomic DNA purification
Step 21.

Close the tube and resuspend the beads by inverting the tube 20 times. It is very important that the beads are resuspended in ethanol to ensure an efficient wash. If beads are still stuck to the wall of the tube you can flick the tube to resuspend them

Genomic DNA purification
Step 22.

Spin down the tube for 1 second

Genomic DNA purification
Step 23.

Put the tube back on the magnetic rack. At this step, beads move immediately to the wall of the tube

Genomic DNA purification
Step 24.

Remove the supernatant without disturbing the beads

Genomic DNA purification
Step 25.

Repeat the steps 20 to 24 to wash the beads one more time.

Genomic DNA purification
Step 26.

Spin down the tube to bring all the droplets to the bottom of the tube

Genomic DNA purification
Step 27.

Put the tube back on the magnetic rack and remove all the ethanol with a P200 or P10 pipette. Let beads air drying for a maximum of 30 seconds or else it can affect the DNA integrity and reduce the yield.

Genomic DNA elution
Step 28.

Add 100 µl of Elution Buffer (QIAGEN, Cat No./ID: 19086)

NOTES

The composition of Buffer EB is: 10 mM Tris-Cl, pH 8.5.

Genomic DNA elution
Step 29.

Resuspend the beads by flicking the tube (make sure they are not aggregated anymore).

Genomic DNA elution
Step 30.

Incubate the tube for 15 minutes at 37°C.

Genomic DNA elution
Step 31.

Flick the tube again

Genomic DNA elution
Step 32.

Spin down the tube for 1 second

 Genomic DNA elution

Step 33.

Place the tube on a magnetic rack and wait for the solution to become clear. This can be time consuming when the gDNA solution is very concentrated (>200ng/µl). You can add additional elution buffer or just wait for hours!

 Genomic DNA elution

Step 34.

Transfer the solution into a new tube.

 DNA QC

Step 35.

Check the DNA purity by measuring the gDNA concentration with a Qubit and a Nanodrop. Qubit/Nanodrop ratio should be around 1.2 (Qubit concentration is higher than Nanodrop concentration for Xanthomonas gDNA).

Typically gDNA concentration is between 50 and 200 ng/µl.

A260/A280 ratio should be 1.8 – 2. If this ratio is >2 it is likely that the sample is contaminated by RNA. RNA contamination also leads to a shift between Qubit and Nanodrop concentrations. To get rid of RNA, you can follow the additional purification n°1 below (Additional purification 1: RNA removal starting from step 36).

A260/A230 ratio should be 2 – 2.2. If this ratio is <2 you can follow the purification below (Additional purification 2: A260/A230 improvement starting from step 43)

NOTES

Qubit measurement is the most accurate as it is specific for double stranded DNA. Overestimation of the DNA concentration by the Nanodrop means that there are other compounds which absorb at 260 nm and therefore the gDNA is not pure.

 DNA QC

Step 36.

Check the DNA integrity by running the sample on PFGE or capillary electrophoresis. The sample should range from 10-20kb to 100-150kb.

 Additional purification 1: RNA removal

Step 37.

Add 1µl of RNase A 100 mg/ml for 100µl of gDNA solution

Additional purification 1: RNA removal
Step 38.

Mix by inverting the tube 10 times

Additional purification 1: RNA removal
Step 39.

Incubate for 10 minutes at room temperature (18-23°C)

Additional purification 1: RNA removal
Step 40.

Add 1 volume of beads solution prepared (make sure beads are at room temperature for >30 minutes and well homogenized via vortexing for approximately 30 seconds)

Additional purification 1: RNA removal
Step 41.

- Follow the steps 16 to 34.

Additional purification 1: RNA removal
Step 42.

Check the DNA purity and integrity as in the steps 35-36.

Additional purification 2: A260/A230 ratio improvement
Step 43.

NOTES

Some contaminants can be removed with this purification and lead to an increase of the A260/A230 ratio. But some contaminants coprecipitate with the DNA and therefore it is impossible to get rid of them with this purification.

Additional purification 2: A260/A230 ratio improvement
Step 44.

Add 1 volume of beads solution previously prepared (make sure beads are at room temperature for >30 minutes and well homogenized via vortexing for approximately 30 seconds)

Additional purification 2: A260/A230 ratio improvement
Step 45.

Follow the step 16 to 34.

Additional purification 2: A260/A230 ratio improvement
Step 46.

Check the DNA purity and integrity as in the steps 35-36.