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Bacterial and fungal DNA extraction protocol for long-read whole genome sequencing

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DNA extraction protocol HoloE2Plant

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Disclaimer

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Abstract

In the context of the ERC Starting Grant HoloE^{2}Plant N° 101039541 “Exploring the Holobiont concept through a Plant Evolutionary Experiment study”, long read bacterial and fungal reference genomes were performed with PacBio Sequel II technology. The purpose of these genomes is to help in the Synthetic Microbial Communities (SynComs) construction. As DNA extraction is a crucial step prior to obtain high-quality genomes, we developed a two-days DNA extraction protocol efficient for the extraction of High-Molecular-Weight (HMW) genomic DNA on both gram-positive/negative bacteria and fungi. To our knowledge, this is the first protocol enabling to extract HMW DNA from both bacteria and fungi. Our protocol was adapted from the four protocols provided in the references.

Attachments

PDF
Precautions for safe...
220KB

PDF
Product references.p...
219KB

Materials

Equipments

- Agitator system for Eppendorf tubes
- 50 ml Eppendorf tubes
- Dry bath and water bath
- Eppendorf centrifuge
- Eppendorf tubes (1.5 ml and 2 ml)
- Freeze-dryer (Heto PowerDry LL3000 freeze dryer of ThermoScientific)
- Fume hood
- Ice
- Magnetic rack
- 15 ml culture tubes or Petri dishes
- Membrane Millipore Express ® PLUS 0.22 µm (hydrophilic polyethersulfone)
- Rotator system for the bacterial culture incubation (only for liquid medium growth)
- Small glass beads (0.5 mm diameter)
- TissueLyser (or another sample grinder)
- Vortex

Solution and buffer preparation

- Nutrient Broth medium with the addition of $CaCl_2$ for bacterial growth

	A	B
	Beef extract	3.0 g
	Peptone	5.0 g
	Glucose	1.0 g
	Yeast extract	0.5 g
	Agar (only if growing strains on solid medium)	15 g
	Osmotic water	1L

Autoclave the medium at 121°C for 20min, after autoclaving filter 40 g/L of $CaCl_2$ with 0.22µm Millipore filters. Add 1ml of the filtered $CaCl_2$ in 1L of medium.

- Inhibitory Mold Agar medium for fungal growth

	A	B
	Pancreatic Digest of Casein	3.0 g
	Sodium Phosphate (Na_3PO_4)s	2.0 g

	A	B
	Peptic Digest of Animal Tissue	2.0 g
	Magnesium Sulfate (MgSO4)	0.8 g
	Bacto Yeast Extract	5.0 g
	Ferrous Sulfate (FeSO4)	0.04 g
	Dextrose (Glucose $C_6H_{12}O_6$)	5.0 g
	Sodium Chloride (NaCl)	0.04 g
	Starch	2.0 g
	Manganese Sulfate ($MnSO_4$)	0.16 g
	Dextrin from corn	1.0 g
	Bacto Agar	15.0 g
	Osmotic water	1L

Check pH and adjust around 6.0

- Lysis buffer

	A	B	C	D
		Stock concentration	Final concentration	Volume (x48 samples)
	Milli-Q water	-	-	30 ml
	NaCl	2.5 M	0.5 M	10 ml
	Tris HCl pH=8.0	1 M	100 mM	5 ml
	EDTA pH=8.0	0.5 M	50 mM	5 ml

As we had 2 magnetic racks for 24 samples each, volumes were calculated for 48 samples.

- SDS 20%

- Lysozyme (100 mg/ml)

Note : it is necessary to filtered lysozyme with 0.22 µm Millipore filters prior the extraction. The filtered solution can be stored up to 3 months at -20°C.

- Proteinase K (20 mg/ml)

- 5M Potassium acetate

- Chloroform:Isoamyl alcohol (24:1)

- Isopropanol

- 3M Sodium acetate
- 70% ethanol
- Elution buffer: 5 mM Tris-HCl, pH 8.5
- RNAse A (100 mg/ml)
- Beads buffer

	A	B	C	D
		Stock concentration	Final concentration	Volume (x48 samples)
	Tris HCl pH=8.0	1 M	10 mM	500 µl
	EDTA pH=8.0	0.5 M	1 mM	100 µl
	NaCl	5 M	2.5 M	25 ml
	PEG 8000	50% (w/v)	18%	9 g
	Sera-Mag Bead	100%	2%	1 ml
	Milli-Q water	-	-	Up to 50 ml

Sera-Mag™ SpeedBead Carboxylate-Modified [E7] Magnetic Particles:
The stock needs to be stored at 4°C. Before use, beads must be placed at to room temperature for 30 min and well mixed to obtain a homogenized solution.

Beads preparation

1. Vortex Sera-Mag beads stock vigorously and pipette 1 ml of them into a 1.5 ml Eppendorf tube.
2. Place on a magnetic rack the 1.5 ml tube containing the beads and wait until all beads have bound to the back of the tube.
3. Wash the beads by removing the supernatant without touching the beads.

Add 1 ml of sterilized Milli-Q water for elution, take the tube off from the magnet, mix well, spin down and put back the beads on the magnet and wait that the beads are assembled.

4. Discard supernatant without touching the beads.
5. Repeat the washing step (steps 2- 5) 3 times.
6. At the last washing step, take off the tube from the magnet and add 1 ml Milli-Q water. Washed beads can be store at 4°C for one month.

Beads buffer preparation

1. Add 9 g of PEG-8000 to a 50 ml Eppendorf tube.
2. Add 25 ml of 5 M NaCl to the PEG-8000.
3. Add 500 µl of 1 M Tris-HCl and 100 µl 0.5 M EDTA.
4. Adjust the volume at 49 ml by adding Milli-Q sterilized water.

5. Mix for 3-5 min until the PEG is dissolved and heat the tube at 65°C until the solution become clear.
6. Filter the solution with Membrane Millipore Express ® PLUS 0.22 µm filters.
7. Mix 1 ml of washed Sera-Mag beads and transfer into the prepared solution.
8. Gently mix until the solution is homogeneous but do not vortex.
9. Pace the bead solution in a dark container and store at 4°C for no more than a week.

Bacterial and fungal growth

1 Bacterial growth

Bacteria growth can be realized on Petri dishes (solid medium) or in a liquid medium (into 15ml tubes). Both techniques give high quality HMW bacterial DNA.

To grow a bacterium into Petri dishes, streak the bacterial strain from the collection tube (normally placed at -80°C) onto Nutrient Agar plates with the addition of $CaCl_2$. Incubate the bacteria at the appropriate
temperature.

To grow a bacterium in liquid conditions, inoculate a single bacterial colony (previously obtained from a Nutrient Agar plate) into 10 mL of Nutrient Broth. Incubate the bacterial strain at appropriate temperature under constant agitation (120 rpm) until the stationary phase is reached.

2 Fungal growth

Place a fungal stab (obtained from the culture collection tube stored at 4°C) on four Inhibitory Mold Agar plates. Collect the mycelium from the four plates into a 5ml Eppendorf tube and incubate it at -80°C for 24h. Lyophilize the mycelium for 48h at -55°C. In this study we utilized the Heto PowerDry LL3000 freeze
dryer of ThermoScientific.

Bacterial and fungal lysis

3 Before to start:
i) Fill 2mL Eppendorf tubes with 0.5 mL of 1 mm glass beads
ii) Heat the lysis buffer for ≥ 30 min at 65°C before use

4 Bacterial lysis

If growing bacteria in Nutrient Broth, centrifuge the bacterial culture at 5000g for 10 min, discard the supernatant and resuspend the bacterial pellet in preheated 800 µL of lysis buffer by pipetting vigorously.

If growing the bacteria on Nutrient Agar plates, pick few isolated bacterial colonies (take the equivalent of two toothpick tips) and mix them into the preheated 800 µl of lysis buffer.

For both methods, transfer the obtained suspension into the 2ml Eppendorf tube containing the

glass-beads. Mix the solution and incubate at 65°C for 30 min.

5 Fungal lysis

Weigh 30 mg of dried mycelium and place it into the 2ml Eppendorf tube containing the glass beads. Add 800 µl of lysis buffer preheated at 65°C.
Note: for some fungal strains, it is necessary to independenlty extract the DNA in several tubes. In this case, pool the independent DNA extractions in order to obtain a suitable amount of DNA.

Mix the sample by using a Tissue Lyser at speed 6 m/s for 1 min and incubate at 65°C for 30 min.

6 Common steps to both bacteria and fungi
- Add 60 µL of 20% SDS stock.
- Homogenize the samples by using a Tissue Lyser at speed 6 m/s for 1 min.
- Add 20 µl of lysozyme 100 mg/ml and incubate 30 min at 37°C.
- Add 8 µl of proteinase K and incubate overnight at 56°C.

Protein precipitation (common to both bacteria and fungi)

7 Centrifuge 1 min at 10,000 x g.

8 Transfer the supernatant to a new tube (~ 650 µL) containing 200 µL of 5M potassium acetate.

9 Mix and incubate on ice for 5 min.

10 Centrifuge at maximum speed for 2 min. Transfer the supernatant to a new tube (~ 500 µl).

Genomic DNA purification (common to both bacteria and fungi)

11 Add 350 µL of chloroform:Isoamyl alcohol (24:1).

12 Invert the tubes several times until the solution is homogenous and centrifuge 5 min at maximum speed.

13 Take upper phase into a new tube (~ 300 µl) without disturb the inner phase.
Note: if the upper phase contains traces of the microbial suspension, repeat the "chloroform:isoamyl alcohol" step twice.

14 Mix the cleaned aqueous phase with an equal volume of isopropanol and a 1/8 volume of 3M sodium acetate.
Note: DNA will precipitate and form "strings".

15 Incubate the samples on ice for 10 min.

16 Centrifuge at maximum speed for 5 min to obtain a DNA pellet.

17 Discard the supernatant by reversing the tubes and let the samples dry for 5 min under the hood.

18 Wash the pellet with 70% of ethanol and add 600 µl of 70% ethanol, centrifuge 5 min at maximum speed, discard the supernatant by reversing tubes and let the samples dry for 5 min under the hood.

19 Resuspend the pellet in 200 µl of Elution Buffer and add 900 µl of beads buffer.
Note: This will purify DNA and remove small DNA fragments.

20 Incubate on a rotator at 10 rpm for 45 min at room temperature.

21 Spin down the tubes for 1 second.

22 Place the tubes on a magnetic rack and wait for 5 min (until solution becomes clear).

23 Discard the supernatant by pipetting without disturbing the beads.

24 Add 70% ethanol to the tube.
Note: Beads must be completely immersed in ethanol.

25 Flick the tubes to resuspend beads, spin down and put the tubes back on the magnetic rack. Remove the ethanol without disturbing the beads.

26 Repeat the steps 24 to 25 and remove the ethanol with a P200 / P10 pipette.

27 Let the samples air drying on the magnetic rack for 5 min.
Note: Beads must not dry too much otherwise resuspension can be difficult.

Genomic DNA elution (common to both bacteria and fungi)

28 Add 100 µl of Elution Buffer.

29 Resuspend the beads by gentling pipetting and spin down the tube for 1 second.
Note: Make sure the beads are not aggregated.

30 Incubate the tubes for 15 min at 37°C.

31 Spin down the tubes for 1 second.

32 Place the tubes on the magnetic rack and wait until the beads are well anchored and the solution is clear.

33 Transfer the solution, consisting in the HMW DNA, into a 1.5ml Eppendorf tube.

RNase treatment (common to both bacteria and fungi)

34 Add 5 µl of 100 mg/ml RNase A.

35 Incubate at 50°C for 15 min.

DNA quality control (common to both bacteria and fungi)

36 Determine the DNA concentration with NanoDrop™ or Qubit® Fluorometer.
$A_{260/280}$ ratio should ideally be between 1.8 and 2.
$A_{260/230}$ ratio should ideally be between 2 and 2.2.

37 To check that DNA is not degraded or contaminated with RNA, migrate the DNA samples on 1.5% agarose gel.

Example of bacterial and fungal DNA quality control

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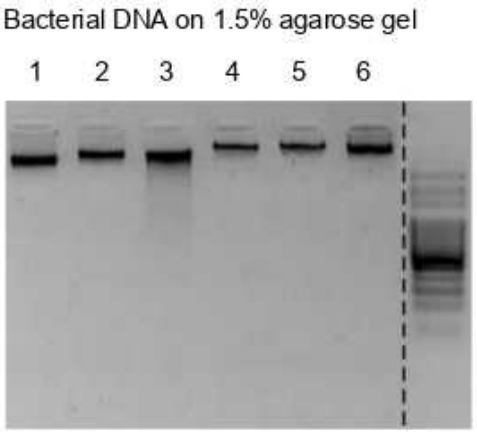

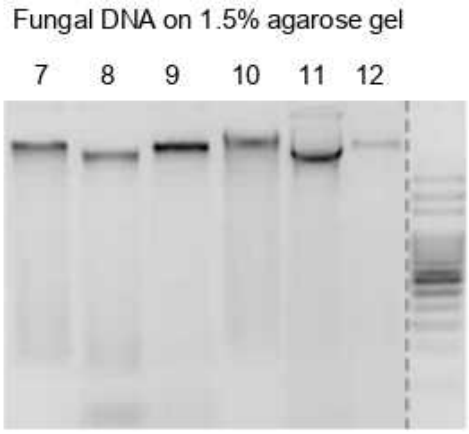

Ladder : 100 pb, higher fragment = 1 kb

	A	B	C	D	E	F
	Gram-staining	Bacterial species	Number in the agarose gel	DNA concentration ng/µl	Quality OD260/280	Quality OD260/230
	negative	*Sphingomonas sp*	1	110	2.10	2.00
	positive	*Actinobacteria sp*	2	45	2.20	2.30
	positive	*Arthrobacter sp*	3	79	2.10	2.00
	negative	*Pseudomonas moraviensis*	4	173	2.20	2.00
	positive	*Actinobacteria sp*	5	35	2.00	1.70
	negative	*Pseudomonas viridiflava*	6	91	2.00	1.87

	A	B	C	D	E
	Fungal species	Number in the agarose gel	DNA concentration ng/µl	Quality OD260/280	Quality OD260/230
	Acremonium furcatum	7	178	1.76	0.89
	Neopyrenochaeta inflorescentiae	8	738	2.02	1.55
	Aureobasidium pullulans	9	1174	2.11	1.91

	A	B	C	D	E
	Metapochonia suchlasporia	10	362	2.05	1.91
	uncultured fungus	11	862	2.04	1.96
	Ascochyta rabiei	12	79	2.04	1.54

Nucleic acid quantifications was performed with Nanodrop^{TM}.

All DNAs have been successfully sequenced with the PacBio Sequel II HiFi technology.

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