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WORKS FOR ME 1

Accurate profiling of diazotrophic communities using unique molecular identifiers with Nanopore sequencing

COMMENTS 0

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ABSTRACT

This protocol applies the method by Karst et al. (2021), who used UMI-tagged primers to perform highly accurate long-read amplicon analysis on the full-length ribosomal RNA operons, to the nitrogen fixation (*nif*) gene cluster. A recent study has shown that the functional gene *nifH*, which is frequently used in community analysis of diazotrophs, detects many false positives (Mise et al. 2021). Therefore, in this protocol, I used newly developed primers targeting the *nifD-K* gene (approx. 1.8 kbp) to identify the diazotrophic communities. The present protocol is presented using plant litter and mineral soil as examples, but it could potentially be applied in a variety of environmental samples.

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Oxford Nanopore MinION, Nitrogen fixation, nifD-K, Forest soil, Leaf litter

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MATERIALS TEXT

Materials and Reagents

- 0.2 mL PCR tubes
- 1.5 mL tubes
- DNA extraction kit for soil (e.g., ISOIL for Beads Beating kit, Nippon Gene Co., Ltd., Tokyo, Japan)
- Qubit dsDNA HS assay kit (Thermo Fisher Scientific, Waltham, MA, United States)
- Qubit Assay Tubes (Thermo Fisher Scientific)
- 1st PCR primers (PAGE purification) (Integrated DNA Technologies, Coralville, IA)

1. Forward primer (nifD229f_UMI): 5'-CAAGCAGAAGACGGCATACGAGAT NNNYRNNNYRNNNYRNNN TGGGGNCCVRTCAAGGAYAT-3'
2. Reverse primer (nifK476r_UMI): 5'-AATGATACGGCGACCACCGAGATC NNNYRNNNYRNNNYRNNN CCRATSACYTCBGCCATRCA-3'

- 2nd PCR primers (PAGE purification) (Karst et al. 2021)

1. Forward primer (lu_pcr_i1_fw_v7): 5'-ACGAGACTGATT CAAGCAGAAGACGGCATACGAGAT-3'
2. Reverse primer (lu_pcr_i1_rv_v7): 5'-TACAGCGCATAC AATGATACGGCGACCACCGAGATC-3'

- PrimeSTAR Max Polymerase (Takara Bio Inc., Shiga, Japan)
- Nuclease-free water
- KAPA HyperPure Beads (Kapa Biosystems, Inc., Wilmington, MA, USA)
- 80% ethanol (molecular grade)
- Ligation sequencing kit V14 (SQK-LSK114) (Oxford Nanopore Technologies, Oxford, United Kingdom)
- R10.4.1 flow cell (FLO-MIN114) (Oxford Nanopore Technologies)
- NEBNext Companion Module for Oxford Nanopore Technologies Ligation Sequencing (New England BioLabs, Beverly, MA, USA)
- Bovine Serum Albumin (BSA) (Invitrogen, Carlsbad, CA, USA)

Equipment

- Thermal cycler (e.g., TaKaRa PCR Thermal Cycler Dice Touch, Takara Bio Inc.)
- Micropipette (P1000, P200, P100, P20, P10, and P2)
- Vortex mixer
- Microfuge
- Magnetic stand (e.g., NGS MagnaStand (YS-Model) 8Ch × 0.2 mL PCR tube, Nippon Genetics Co., Ltd., Tokyo, Japan)
- Qubit fluorometer (Qubit 4 fluorometer, Thermo Fisher Scientific)
- Nanopore sequencer (e.g., MinION Mk1C, Oxford Nanopore Technologies)

2h 10m

DNA extraction

2h

1 Extract total genomic DNA (gDNA) from 500 mg of fresh soil and 100 mg of air-dried litter using the ISOIL for Beads Beating kit (Nippon Gene Corporation, Tokyo, Japan), a CTAB-based DNA extraction kit.

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2 Measure the extracted gDNA concentration using a Qubit fluorometer with the Qubit dsDNA HS assay kit (Thermo Fisher Scientific, Waltham, MA, USA). 10m

Tagging *nifD-K* genes with UMIs (1st PCR) 15m

3 Prepare PCR-master mix in a 0.2 mL PCR tube with the following: 5m

Component	Volume (total 50 µL)
Nuclease-free water	X µL
Forward primer (nifDK229f_UMI, 10µM)	2.5 µL
Reverse primer (nifK476r_UMI, 10 µM)	2.5 µL
PrimeSTAR Max Premix	25 µL
Template DNA	Y µL (50 ng)

4 Run the following PCR program: 10m
[98°C 10 sec → 55°C 15 sec → 72°C 30 sec] × 2 → 8°C ∞

Clean-up of 1st PCR products 30m

5 Clean up the 1st PCR products using KAPA HyperPure Beads (Kapa Biosystems, Inc., Wilmington, MA, USA)

5.1 Equilibrate the beads solution at room temperature and allow the beads to resuspend completely.

5.2 Add 25 µL beads solution to the 50 µL PCR products (0.5× bead solution/sample ratio) and mix by pipetting up and down multiple (10 to 20) times.

5.3 Incubate the tube at room temperature for 5 min. 5m

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5.4 Place the tube on a magnetic rack (NGS MagnaStand (YS-Model) 8Ch × 0.2 mL PCR tube, Nippon Genetics Co., Ltd., Tokyo, Japan) to capture the beads. Incubate until the liquid is clear (~3 min).

5.5 Discard the supernatant.

5.6 Wash beads by adding 200 µL fresh 80% ethanol.

5.7 Incubate the tube on the magnetic rack at room temperature for 30 sec. 30s

5.8 Discard the supernatant.

5.9 Repeat the previous washing steps (4.6-4.8).

5.10 Dry the beads at room temperature for 3 min. 3m

5.11 Remove the tube from the magnetic rack.

5.12 Elute the purified DNA by adding 21 µL of nuclease-free water and mix by pipetting.

5.13 Incubate the tube at room temperature for 5 min. 5m

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1m

5.14 Place the tube on the magnetic rack to capture the beads. Incubate until the liquid is clear (1 min).

5.15 Transfer the supernatant to a new PCR tube.

2h 10m

Amplification of UMI tagged sequences (2nd PCR)

10m

6 Prepare PCR-master mix in a 0.2 mL PCR tube with the following:

Component	Total volume (100 µL)
Nuclease-free water	20 µL
Forward primer (lu_pcr_i1_fw_v7)	5 µL (0.5 µM)
Reverse primer (lu_pcr_i1_rv_v7)	5 µL (0.5 µM)
PrimeSTAR Max Premix	50 µL
Template DNA (1st PCR products)	20 µL

2h

7 Run the following PCR program:
[98°C 10 sec → 68°C 5 sec → 72°C 30 sec]×30 → 68°C 5min → 8°C ∞

30m

Clean-up of 2nd PCR products

30m

8 Clean up the 2nd PCR products using KAPA HyperPure Beads (0.5× bead solution/sample ratio) following the same procedure as in Section 5.

10m

Quality control

10m

9 Measure the DNA concentration of the purified 2nd PCR products using a Qubit fluorometer with the Qubit dsDNA HS assay kit.

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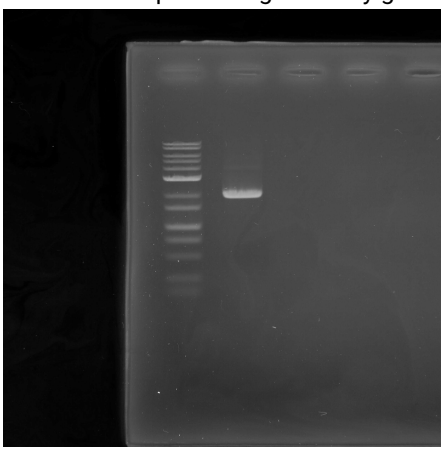
10 OPTIONAL:

Check the amplified fragments by gel electrophoresis.



Lanes:
1. 1kb DNA ladder
2. Purified 2nd PCR products

Nanopore sequencing

11 Conduct "DNA repair and end prep", "Adapter ligation and cleanup", and "Priming and loading of SpotOn flow cell" according to the Ligation Sequence Amplicon V14 protocol (ligation-sequencing-amplicons-sqk-lsk114-ACDE_9163_v114_revE_29Jun2022-minion.pdf).

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