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Soil Metagenome ONT

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Abstract

This protocol describes the sample collection to sequence acquisition workflow for Oxford Nanopore long-read sequencing of a complex soil sample using a ligation sequencing kit kit LSK-114 and R10.4.1 FLO-PRO114M flowcells.

Attachments

genomic-lsk114-prom.... 9.3MB

Ultrall_man.pdf 484KB

FFPE_man.pdf 550KB

QuickT4_man.pdf 449KB

SPRI_man.pdf 565KB

FastDNA_SPIN_Kit_Pro.. 221KB

Zymo_shield_man.pdf 628KB

Guidelines

- Fully equilibriate Ampure XP SPRI beads to Room temperature before use.
- Fully equilibriate Qubit solution to Room temperature before use.
- Fully equilibriate Tape station screen tape and reagents to Room temperature before use.
- Over drying DNA bound to Ampure XP SPRI beads can reduce Sample recovery.
- Additional time must be given for the Ampure XP beads to clear from the adapter ligation reaction due to the high viscosity of the solution. Failure to provide adequate time for the supernatant to clear can result in Sample loss.

Materials

Equipment

- Bench top centrifuge
- Thermal cycler
- Qubit 4 Fluorometer **Thermo Fisher Scientific Catalog #**Q33238
- Capillary electrophoresis instrument (e.g. Agilent Tapestation 4200) **Contributed by users**
- Rotational mixer
- Heat block
- Magnetic rack
- Fridge 4 °C
- Freezer -80 °C
- Ice bucket
- Pipette set (P10, P20, P200, P1000)
- Soil corer
- Soil Sieve
- Soil collection plate
- Plate sealer
- Top pan balance
- Weigh boat
- Spatula
- Measuring cylinder 100 mL
- Conical flask 250 mL
- P2 Solo device and compatible compute

Reagents

- Zymo DNA/RNA Shield **Fisher Scientific Catalog #**50-125-1706
- FastDNA™ SPIN Kit for Soil **MPBio Catalog #**116560200-CF
- Quick T4 DNA Ligase **New England Biolabs Catalog #**E7180S
- Monarch RNase A **New England Biolabs Catalog #**T3018L
- NEBNext Ultra II End Repair/dA-Tailing Module - 24 rxns **New England Biolabs Catalog #**E7546S
- NEBNext FFPE DNA Repair Mix - 24 rxns **New England Biolabs Catalog #**M6630S
- Ligation Sequencing Kit V14 **Oxford Nanopore Technologies Catalog #**SQK-LSK114
- Ethanol (100%, Molecular Biology Grade) **Fisher Scientific Catalog #**BP2818500
- Molecular Biology Grade Water **Fisher Scientific Catalog #**10154604

- Agencourt AMPure XP **Beckman Coulter Catalog #**A63880
- PromethION R10.4.1M flow cell **Oxford Nanopore Technologies Catalog #**FLO-PRO114M
- Qubit 1X dsDNA HS Assay Kit **Thermo Fisher Scientific Catalog #**Q33230
- Genomic DNA Reagents **Agilent Technologies Catalog #**5067-5366
- Genomic DNA ScreenTape **Agilent Technologies Catalog #**5067-5365

Consumables

- Eppendorf lo-bind Falcon tubes 5 mL Catalog no. 0030122348
- Eppendorf lo-bind microfuge tubes 1.5 mL Catalog no. 0030108051
- Qubit™ Assay Tubes **Invitrogen - Thermo Fisher Catalog #**Q32856
- Thin-walled PCR tubes 0.2 mL Catalog no: AB-2000
- Cryovials 2 mL Cataloge no. 41121704
- Qubit tubes 0.2 mL Cataloge no. Q32856
- P1000 Wide bore pipette tips
- P1000 pipette tips
- P200 Pipette tips
- P20 pipette tips
- P10 pipette tips
- Crushed ice

Safety warnings

Safety information

- Binding Matrix contains components that, when in contact with human tissue, may cause irritation. Wear personal protective equipment to prevent contact with the skin or mucous membranes (gloves, lab coat, and eye protection).
- EtOH (100%) is a highly flammable liquid and vapour. It can cause serious eye irritation**.** Keep away from heat, hot surfaces, sparks, open flames and other sources of ignition.

Before start

- Dilute the concentrated SEWS-M solution with 100 mL of [M] 100 % (v/v) EtOH before use.
- Prepare 10 mL of fresh [M] 80 % (v/v) EtOH.

1. Soil sample collection and storage

1 Collect approximately 15 g of soil using a sterile soil corer or similar device and transfer to a sterile soil sieve and collection plate.

2 Homogenise the soil sample by passing it through the soil sieve and collecting the output on the collection plate below the sieve. The use of a sterile plate sealer can be used to facilitate sieve homogenisation.

3 Use a top pan balance, spatular and weigh boat to weigh 10-50 g of homogenised soil Sample and transfer to a 250 mL conical flask. Promptly suspend the soil in 100 mL of Zymo DNA/RNA shield for a final concentration of [M] 100-500 mg/mL .

4 Incubate for at least 04:00:00 at room temperature or 4 °C Overnight with occasional mixing. 16h

5 Gently mix the bulk sample by hand to form a homogenous solution then aspirate 1000 µL of the total sample using a P1000 pipette and wide bore tip. Transfer the Sample into a new and sterile 2 mL cryovial and close the lid securely.

6 Repeat step 5 until the total volume of sample has exhausted or the desired number of aliquots have been achieved.

7 Snap freeze and store Sample at -80 °C for future use.

2. DNA extraction and sample cleanup

8 Defrost the Sample for 00:15:00 On ice prior to commencing DNA extraction. 15m

9 Transfer 1000 µL of the Sample to a new and clean 1.5 ml lo-bind Eppendorf tube using a P1000 pipette and wide bore pipette tip.

10 Centrifuge at 5000 x g, 00:04:00 to pellet the Sample . 4m

11 Aspirate and discard the supernatant without disturbing the pellet.

12 Resuspend the pellet in 978 µL of sodium phosphate buffer, then transfer the Sample to a new and clean lyzing matrix E tube.

13 Add 122 µL of MT buffer to the Sample , mix by inversion and incubate On ice for 00:05:00 . 5m

14 Place the matrix tube in a MPBio FastPrep instrument (or similar) and homogenise for 00:00:10 at 5.0 ms then return the Sample to ice. 10s

15 Incubate the Sample On ice for 00:05:00 . 5m

16 Repeat steps 14 and 15 then continue to step 17.

17 Add 250 µL of protein precipitation solution (PPS) to a new and clean 1.5 mL lo-bind Eppendorf tube and chill On ice .

18 Remove the lysing matrix-E tube from the ice and centrifuge at 14000 x g, 00:04:00 to pellet the debris. 4m

19 Decant the supernatant from the lysing matrix E tube into the pre chilled PPS without disturbing the pellet, then mix the Sample by inversion 10 times and place On ice .

20 Incubate the Sample On ice for 00:10:00 to facilitate protein precipitation. 10m

21 Remove the Sample from the ice and centrifuge at 14000 x g, 00:02:00 to pellet the protein precipitate. 2m

22 Homogenise the binding matrix immediately before use. Add 1000 µL of resuspended binding matrix to a clean 5 mL lo-bind tube.

Safety information

- Binding Matrix contains components that, when in contact with human tissue, may cause
irritation. Wear personal protective equipment to prevent contact with the skin or mucous
membranes (gloves, lab coat, and eye protection).

23 Gently decant the Sample directly into the binding matrix without disturbing the protein pellet, secure the lid and mix the Sample by inversion until a homogeneous solution has formed.

24 Incubate the Sample on a rotational mixer at 30 rpm, Room temperature , 00:10:00 . 10m

25 Add 1000 µL of DES to a new and clean 1.5 mL lo-bind tube and place the tube into an active heat block set to 56 °C .

26 Remove the Sample from the rotational mixer and transfer 750 µL of sample to an MPbio spin filter using a wide bore P 1000 pipette tip. Return the Sample to the rotational mixer.

27 Centrifuge the spin filter at 14000 x g, 00:02:00 and discard flow through. 2m

28 Repeat steps 26 and 27 until all the binding matrix has been processed through the spin filter.

29 Add 500 µL of prepared SEWS-M buffer to the Sample bound binding matrix, close the lid securely and suspend the beads in the SEWS-M solution by flicking the tube.

Safety information

EtOH (100%) is a highly flammable liquid and vapour. It can cause serious eye irritation**.** Keep away from heat, hot surfaces, sparks, open flames and other sources of ignition.

30 Centrifuge the Sample at 14000 x g, 00:02:00 then discard the flow through. 2m

31 Repeat steps 29 and 30 then continue to step 32.

32 Centrifuge the Sample at 14000 x g, 00:02:00 to collect excess EtOH. 2m

33 Remove the spin filter from the catch tube and place it into a new and clean 1.5 mL lo-bind Eppendorf tube.

34 Allow the binding matrix to air dry for 00:02:00 . 2m

35 Add 100 µL of 56 °C DES elution buffer to the binding matrix and agitate the spin filter until a slurry has formed.

36 Incubate the Sample at 56 °C for 00:10:00 with intermittent agitation. 10m

37 Centrifuge the Sample at 14000 x g, 00:02:00 . Discard the spin filter and retain the Sample eluate. 2m

38 Allow the Sample to equilibrate to Room temperature and add 1 µL RNase A at [M] 20 mg/mL . Mix tube by flicking and then incubate at Room temperature for 00:02:00 . 2m

39 Add 60 µL of Room temperature Ampure XP beads to the Sample and mix by flicking the tube until a homogeneous solution has formed.

40 Incubate the Sample on a rotational mixer for 00:10:00 at Room temperature . 10m

41 Briefly spin down the Sample and place on a magnetic rack.

42 Once the Sample has cleared, aspirate and discard the supernatant without disturbing the beads.

43 Gently add 200 µL of [M] 80 % (v/v) EtOH across the beads and incubate on the magnetic rack for at least 00:00:30 . 30s

44 Aspirate and discard the supernatant without disturbing the beads.

45 Repeat steps 43 and 44 then continue to step 46.

46 Remove the Sample from the magnetic rack and briefly centrifuge at < 1000 x g to collect any residual EtOH then return the Sample to the magnetic rack. 5s

47 Promptly remove the excess EtOH with a P10 pipette and tip without disturbing the beads.

48 Allow the beads to air dry for 00:00:30 or until the beads are satin in appearance. Do not over dry the beads, avoid excess EtOH carry over. 30s

49 Remove the Sample from the magnetic rack and add 50 µL of molecular grade water to the beads.

50 Resuspend the beads in the water by flicking the tube until a homogenous solution has formed.

51 Incubate the Sample at 37 °C for 00:10:00 then return the Sample to the magnetic rack. 10m

52 Once the solution has cleared, Quantify 1 µL of Sample using a Qubit 4 fluorometer or similar device.

3. End prep and FFPE repair

53 Transfer 2-2.5 µg of Sample to a new and clean 0.2 mL thin walled PCR tube and adjust the volume to 48 µL using molecular grade water, then place the Sample On ice.

54 Add the following reagents to the Sample in the order listed. Mix the reaction by flicking between the addition of each reagent and return to ice.

3.5 µL NEB Next FFPE repair buffer
3.5 µL NEB Ultra II End prep reaction buffer
2 µL NEB Next FFPE repair enzyme mix
3 µL Ultra II End prep enzyme mix

55 Briefly centrifuge the Sample to collect the contents in the bottom of the tube and place into a thermal cycler with the heated lid set to 105 °C. Incubate the reaction using the following conditions: 1h

20 °C 00:30:00
65 °C 00:30:00
4 °C Hold

56 Transfer 60 µL of Sample to a new and clean 1.5 mL lo-bind Eppendorf tube.

57 Add 60 µL of resuspended Room temperature Ampure XP SPRI beads to the Sample and mix by flicking until a homogeneous solution has formed. Incubate the Sample on an active rotational mixer at 30 rpm, Room temperature , 00:10:00.

58 Briefly centrifuge the tube to collect the Sample (< 1000 x g), then place the Sample on a magnetic rack and allow the solution to clear completely. 5m

59 Aspirate and discard the supernatant without disturbing the beads.

60 Add 200 µL of freshly prepared [M] 80 % (v/v) EtOH across the beads and incubate at Room temperature for > 00:00:30. 30s

61 Aspirate and discard the supernatant without disturbing the beads.

62 Repeat steps 60 and 61 then continue to step 63.

63 Briefly centrifuge the Sample to collect residual EtOH in the bottom of the tube and replace on the magnetic rack.

64 Aspirate and discard the residual EtOH using a P10 pipette and tip.

65 Allow the Sample to air dry until the beads are satin in appearance (00:00:30). Avoid over drying the beads to the point of cracking. Restrict residual EtOH carryover. 30s

66 Remove the Sample from the magnetic rack and add 60 µL of molecular grade water to the beads. Suspend the beads by flicking the tube and place in a heat block at 37 °C for 00:10:00. 10m

67 Remove the Sample from the heat block and place directly into a magnetic rack and allow the solution to clear.

68 Transfer 60 µL of Sample to a new 1.5 mL lo-bind Eppendorf tube and place On ice.

4. Adapter ligation

69 Mix the ONT ligation adapter (LA) and NEB Quick T4 ligase by flicking then briefly spin down to collect the contents in the bottom of the tube and place them On ice .

70 Thaw the ONT ligation buffer (LNB) at room temperature, mix by pipetting, then place On ice .

71 Thaw ONT Elution buffer (EB) and ONT long fragment buffer (LFB) at room temperature, mix by flicking, spin to collect and place On ice .

72 Mix the following reagents in order in a 1.5 mL lo-bind Eppendorf tube. Mix the reaction by flicking between the addition of each reagent and place On ice .

- 60 µL Sample from previous step
- 25 µL ONT Ligation Buffer (LNB)
- 10 µL NEBNext Quick T4 ligase
- 5 µL ONT Ligation Adapter (LA)

73 Thoroughly mix the ligation reaction by flicking until a homogenous solution is achieved. Incomplete mixing can result in a reduced ligation efficiency.

74 Incubate the Sample for 00:20:00 at Room temperature . 20m

75 Add 40 µL of resuspended Room temperature Ampure XP SPRI beads to the Sample and mix by flicking until a homogenous solution has formed.

76 Place the Sample on an active rotational mixer and incubate at 30 rpm, Room temperature , 00:10:00 .

77 Briefly centrifuge the Sample to collect the contents at the bottom of the tube, then place the Sample on a magnetic rack and allow the solution to clear. The Sample is viscous and will require additional time to fully clear (~ 00:05:00). 5m

78 Aspirate and discard the supernatant without disturbing the beads.

79 Remove the Sample from the magnetic rack and add 250 µL of ONT long fragment buffer (LFB) across the beads then suspend the beads in the LFB by flicking the tube.

80 Briefly centrifuge the Sample to collect the solution at the bottom of the tube and return the Sample to the magnetic rack and allow the solution to fully clear.

81 Repeat steps 78 - 80 then continue to step 82.

82 Sample Aspirate and discard the supernatant, then briefly centrifuge the Sample to collect residual LFB at the bottom of the tube then return the Sample to the magnetic rack.

83 Aspirate and discard residual LFB using a P 10 pipette and tip.

84 Allow the Ampure XP SPRI beads to air dry for 00:00:30 .

30s

85 Remove the tube from the magnetic rack and add 33 µL of ONT Elution buffer (EB) across the SPRI beads. Resuspend the beads by flicking then briefly spin to collect a homogenous solution at the bottom of the tube.

86 Transfer the Sample to a heat block at 37 °C and incubate for 00:10:00 .

10m

87 Replace the Sample on the magnetic rack and allow the solution to clear.

88 Quantify the [DNA] and fragment size distribution of a [M] 10 % (v/v) dilution of the Sample in molecular grade water using a Qubit 4 fluorometer, 1x HS assay kit and Tape station Genomic screen tape.

89 Aspirate 32 µL of Sample and transfer to a new 1.5 mL lo-bind Eppendorf tube. Store the final library On ice until ready to load. Proceed directly to the next step in this protocol.

5. PromethION Flow cell priming, loading and sequence acquisition

55m

90 Remove PromethION flow cell(s) from the fridge and allow to equilibriate to Room temperature for approximately 00:20:00 .

20m

91 Thaw the Sequencing Buffer (SB), library beads (LIB), Flow Cell Tether (FCT), and Flow Cell Flush (FCF) at Room temperature .

92 Briefly mix all solutions by flicking then spin down the tubes to collect the contents and return to ice.

93 Add the following reagents to a 1.5 mL lo-bind Eppendorf tube, mix by pipetting up and down then store On ice .

1170 µL Flow Cell Flush (FCF)
30 µL Flow Cell Tether (FCT)

94 Locate the PromethION flow cell securely in the sequencing device by.

95 Rotate the sample port valve clockwise to expose the sample port.

96 Remove ~ 20 µL of storage buffer from the sample loading port using a P1000 pipette and tip. Draw back the buffer by placing the end of the pipette tip into the sample port and manually increasing the set pipette fill volume.

97 Load 500 µL of the prepared Flow Cell Flush Buffer through the exposed sample port of the PromethION flow cell. Care must be taken not to introduce air to the loading channel and flow cell during this step.

98 Proceed with the protocol while incubating the flush buffer on the flow cell for 00:05:00 at Room temperature . 5m

99 Resuspend the library loading beads (LIB) by pipetting then transfer 68 µL of LIB to a new 1.5 mL lo-bind tube and place on ice.

100 Mix the Sequencing buffer (SB) by flicking then transfer 100 µL of SB to the 68 µL of LIB and return to ice.

101 Complete the flow cell flush by adding an additional 500 µL of the previously prepared Flow Cell Flush buffer to the PromethION flow cell through the sample port.

102 Set a P1000 pipette and tip to 200 µL and mix the Sequencing Buffer (SB) and library loading beads (LIB) mixture by pipetting up and down.

103 Aspirate 168 µL of the SB and LIB mixture and dispense directly into the Sample with enough force to mix all reagents. Proceed directly to the next step.

104 Aspirate 200 µL of Sample using the P 1000 and pipette then load into the PromerthION flow cell through the exposed sample port. Ensure no air bubbles are present in the end of the pipette and loading channel prior to loading the final library. Load the entire library by manually reducing the set pipette volume.

105 Close the sample port valve by rotating it anti-clockwise and ensure the light shield is located securely.

106 Incubate the flow cell for 00:30:00 at Room temperature . 30m

107 Complete the requested sequencing parameters on the MinKNOW GUI and commence sequence acquisition.