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Soil Metagenome ONT

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Continual development of ONT and PB long read extractions

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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....	Ultrall_man.pdf	FFPE_man.pdf	QuickT4_man.pdf	SPRI_man.pdf	FastDNA_SPIN_Kit_Pro...	Zymo_shield_man.pdf
9.3MB	484KB	550KB	449KB	565KB	221KB	628KB

Guidelines

- Fully equilibrate Ampure XP SPRI beads to  Room temperature before use.
- Fully equilibrate Qubit solution to  Room temperature before use.
- Fully equilibrate 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)
- 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 Catalogue no. 41121704
- Qubit tubes  0.2 mL Catalogue 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 100 % (v/v) EtOH before use.
- Prepare 10 mL of fresh 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  100-500 mg/mL .
- 4 Incubate for at least  04:00:00 at room temperature or  4 °C  Overnight  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 lysing 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 $^{\circ}\text{C}$.



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 $^{\circ}\text{C}$ DES elution buffer to the binding matrix and agitate the spin filter until a slurry has formed.

- 36 Incubate the  Sample at  56 $^{\circ}\text{C}$ 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 μ L RNase A at 20 mg/mL . Mix tube by flicking and then incubate at Room temperature for 00:02:00 . 2m
- 39 Add 60 μ L 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 μ L of 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 $^{\circ}\text{C}$ 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 $^{\circ}\text{C}$. Incubate the reaction using the following conditions: 1h   
-  20 $^{\circ}\text{C}$  00:30:00
 -  65 $^{\circ}\text{C}$  00:30:00
 -  4 $^{\circ}\text{C}$ 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.
- 59 Aspirate and discard the supernatant without disturbing the beads.
- 60 Add 200 μL of freshly prepared 80 % (v/v) EtOH across the beads and incubate at Room temperature for > 00:00:30 .
- 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.
- 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 $^{\circ}\text{C}$ for 00:10:00 .
- 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).
- 78 Aspirate and discard the supernatant without disturbing the beads.
- 79 Remove the Sample from the magnetic rack and add 250 μ L 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 .
- 85 Remove the tube from the magnetic rack and add 33 μ L 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.



5m



30s





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 [IM] 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 equilibrate 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 . 
- 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 PromethION 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.