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Soil Metagenome PacBio V2 V.2

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We are still developing and optimizing this protocol

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Abstract

This protocol describes the sample collection to sequence acquisition workflow for PacBio Revio long-read sequencing of a complex soil sample using SMRT Bell library prep kit 2.0 and Revio SMRT cells.

Attachments



[SPRI_man.pdf](#)

565KB



[FastDNA_SPIN_Kit_Pro...](#)

221KB



[Zymo_shield_man.pdf](#)

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

Reagents

-  Zymo DNA/RNA Shield **Fisher Scientific Catalog #50-125-1706**
-  FastDNA™ SPIN Kit for Soil **MPBio Catalog #116560200-CF**
-  Monarch RNase A **New England Biolabs Catalog #T3018L**
-  Ethanol (100%, Molecular Biology Grade) **Fisher Scientific Catalog #BP2818500**
-  Molecular Biology Grade Water **Fisher Scientific Catalog #10154604**
-  Agencourt AMPure XP **Beckman Coulter Catalog #A63880**
-  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**
- PacBio SPK 2.0



- Barcoding overhang kit 8A

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  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
with occasional mixing. 
- 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

1h 38m 15s

- 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  46 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.

PacBio SPK 2.0 library preparation

5h 6m

53 DNA blunting

Prepare the following reaction on ice. Scale the reaction volume by sample number.

	A	B
	Reagent	Volume (μl)
	Enzyme Dilution Buffer	4.0
	DNA Prep Additive	1.0

- 54 Prepare the following reaction in a 0.2 ml thin walled PCR tube for each sample and return to ice. Use DNA Prep Additive from step 53.

	A	B
	Reagent	Volume (μl)
	DNA	45.0
	DNA Prep Buffer	7.0



	A	B
	NAD	1.0
	DNA Prep Additive	1.0
	DNA Prep Enzyme	1.0
	Total	55

55 Mix the sample by flicking and spin down to collect.

56 Incubate the Sample in a thermal cycler at 37 °C for 00:15:00 then place the sample On ice .

15m

57 DNA damage repair (FFPE).

Add 2 µL of DNA damage repair mix v2 to each Sample and mix by flicking then spin down to collect.

58 Incubate at 37 °C for 00:30:00 then place the Sample On ice .

30m

59 End-repair and A-tailing.

Add 3 µL of End Prep Mix to each Sample mix by flicking and spin down to collect.

60 Incubate the Sample at 20 °C for 00:30:00 , then at 65 °C for 00:30:00 then place the sample On ice

1h

61 SMRT bell adapter ligation

Defrost one barcoded overhang SMRT bell adapter per sample, mix by flicking, spin down and place On ice

62 Add 5 µL of a single barcoded overhang SMRT bell adapter to each Sample , mix by flicking, spin down and place On ice .



63 Add 30 μL of Ligation mix, 1 μL of Ligation additive and 1 μL of Ligation Enhancer to each sample, mix by flicking, spin down and return to ice.

64 Incubate the sample at 20 $^{\circ}\text{C}$ for 01:00:00 then at 4 $^{\circ}\text{C}$ for up to 16 h.

1h

65 Incubate the Sample at 65 $^{\circ}\text{C}$ for 00:10:00 then place On ice .

10m

66 Nuclease treatment

Prepare the following reaction and place on ice. Scale the reaction volume by sample number.

	A	B
	Reagent	Volume (μL)
	Enzyme A	4.0
	Enzyme B	1.0
	Enzyme C	1.0
	Enzyme D	2.0

67 Add 8 μL of Enzyme mix to each Sample and mix by flicking then spin down to collect.

68 Incubate at 37 $^{\circ}\text{C}$ for 01:00:00 then place the sample On ice .

1h

69 Spri cleanup.

Add 47.25 μL (0.45x) of SPRI beads at Room temperature and mix by flicking.

70 Incubate on a rotational mixer for 00:10:00 .

10m



71 Briefly spin to collect the  Sample .

72 Place the sample on a magnetic rack and allow the solution to fully clear.

73 Aspirate and discard the supernatant without disturbing the beads.

74 Add  200 μL of [M] 80 % (v/v) EtOH across the beads and allow to sit for  00:00:30 .

30s

75 Aspirate and discard the EtOH.

76 Repeat steps 74 and 75, then continue.

77 Spin down to collect the tube contents and return to the magnetic rack.

78 Promptly remove residual EtOH with a pipette.

79 Add  31 μL of PB 1X Elution buffer to the beads and mix by flicking.

80 Incubate the  Sample at  37 °C for  00:30:00 with occasional mixing.

30m

81 Place sample on a magnetic rack and allow the sample to clear.

82 Aspirate  31 μL of  Sample and transfer to a new 1.5 ml lo-bind tube.



- 83 Add  1 μL of  Sample to  9 μL of water and mix by flicking.
- 84 Quantify  2 μL of diluted sample using a Qubit fluorometer and HS assay kit.
Quantify  1 μL of diluted library on a tape station or similar fragment analyser.
- 85 Dilute SPRI bead clean up
- Add  350 μL of SPRI beads to  650 μL of 1 x PB elution buffer and mix by flicking.
- 86 Dilute the  Sample to < 10 ng/ μL with PB elution buffer.
- 87 Add 3.7 x volume of  Sample of dilute SPRI beads to the  Sample and mix by flicking.
- 88 Place on a rotational mixer for  00:30:00 at  Room temperature
- 89 Briefly spin the sample to collect and place on a magnetic rack, then allow the sample to clear.
- 90 Aspirate and retain the supernatant in a new 1.5 lo-bind tube.
- 91 Add  250 μL of [M] 80 % volume EtOH across the beads and allow to sit for  00:00:30 . 30s
- 92 Remove and discard the EtOH without disturbing the beads.
- 93 Repeat the wash step and briefly spin to collect residual EtOH.
- 94 Remove residual EtOH then add  21 μL of 1x PB elution buffer to the beads and mix by flicking.



- 95 Incubate the  Sample at  37 °C for  00:30:00 with occasional mixing then return to the magnetic rack.
- 96 Dilute  1 µL of final library in  9 µL of molecular grade water and retain for QC.
- 97 Aspirate  20 µL of final library to a new 1.5 ml lo-bind tube and place on ice.
- 98 Store final library at  4 °C for imminent SMRT cell loading or a  -20 °C for longterm storage.

30m