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Whole genome amplification and long read sequencing using ONT V.1

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We use this protocol and it's working

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Abstract

A genome reference is a prerequisite for a complete understanding of the biology and evolution of a species. However, the major challenge remains to obtain high-quality DNA and RNA from the majority of organisms. Therefore, there is a need for having a protocol to bypassed the challenging stage of obtaining axenic cultures and limited the amount of DNA material from a limit individual. This protocol build on whole genome sequencing single nematode. With multiple displacement amplification (MDA) allows the genome from a single nematode to be amplified and can sequence with both long- and short-read sequencing. This protocol can be completed within two week including genome amplification and sequencing. Also, combines MDA and Oxford Nanopore sequencing and provides a cost- and labor-effective solution to generate complete assemblies in organisms with as little as 50 picograms of starting material and assemble a draft genome assembly.

Materials

REAGENTS

22 gauge needle

0.2ml PCR tube

DNA LoBind Tubes, Eppendorf, #EP0030108078

⊗ 1X PBS (Phosphate-buffered saline)

⊗ REPLI-g Single Cell Kit **Qiagen Catalog #150345**

⊗ AMPure XP **Bechman Coulter Catalog #A63882**

⊗ T7 Endonuclease I - 1,250 units **New England Biolabs Catalog #M0302L**

⊗ Qubit dsDNA HS Assay Kit **Thermo Fisher Scientific Catalog #Q32854**

⊗ Ligation Sequencing Kit **Oxford Nanopore Technologies Catalog #SQK-LSK109**

⊗ Flow Cell Priming Kit **Oxford Nanopore Technologies Catalog #EXP-FLP002**

⊗ NEBNext Quick Ligation Module - 100 rxns **New England Biolabs Catalog #E6056L**

⊗ NEBNext FFPE DNA Repair Mix - 96 rxns **New England Biolabs Catalog #M6630L**

⊗ NEBNext Ultra II End Repair/dA-Tailing Module - 96 rxns **New England Biolabs Catalog #E7546L**

EQUIPMENT

Magnetic separator

Vortex mixer

Microfuge

Thermal cycler

Rotator mixer

Magnetic separator

Qubit

Gridion

Troubleshooting



[Optional] extraction and denature of genomic DNA

- 1 Prepare DLB Lysis buffer:
 - 33 μL REPLI-g DLB buffer
 - 3 μL 1M DTT
- 2 Add 4 μL PBS buffer in 0.2ml PCR tube.
- 3 Transfer worm to the 0.2ml PCR tube with 4 μL PBS buffer.

Note

[Optional] You may cut the worm with 22 gauge needle. This may release the cells from the cuticles

- 4 Add 3 μL DLB Lysis buffer. Incubate sample on thermocycler at 65 °C for 00:10:00 10m
- 5 Add 3 μL of REPLI-g Stop Solution. Store the sample on ice.

Whole genome amplification

8h 38m 30s

- 6 Prepare REPLI-g polymerase master mix
 - 9 μL Nuclease-free water
 - 29 μL REPLI-g Reaction Buffer
 - 2 μL REPLI-g DNA Polymerase

**Note**

The difference of these kits is the final amount of expected amplified templates from the polymerase master mix (SC 40 µg, midi 40 µg, and mini 10 µg with 10ng DNA). We initially started with SC, but were only able to obtain midi and mini kits during the COVID pandemic. We have inserted this explanation and option in the publicised protocol (**doi here**).

- 7 Add  40 µL REPLI-g polymerase master mix to each reaction.

8h 3m

Note


set heated lid temperature to  70 °C)


Incubate sample at  30 °C for  08:00:00 .

Inactivate reaction at  65 °C for  00:03:00 .

Store amplified DNA at  4 °C .


- 8 Dilute  1 µL amplified DNA to 100X in dH₂O.



Take  2 µL diluted amplified DNA for quantification with Qubit dsDNA High sensitivity assay.

- 9 Warm AMPure XP beads to  Room temperature .



Resuspend the AMPure XP beads by vortexing.

- 10 Transfer the sample to a 1.5 ml Microtubes.

Add  90 µL of resuspended AMPure XP beads to the amplification reaction and thoroughly mixed.


- 11 Incubate the sample on a Tube Revolver for  00:10:00 at  Room temperature .

10m

- 12 Keep the tube on the magnet until eluate is clear and colorless, and pipette off the supernatant. Wash the beads with  200 µL of freshly prepared  70 % (v/v)

30s






ethanol for  00:00:30 and remove the ethanol using a pipette and discard.
Spin down and place the tube back on the magnet. Pipette off any residual ethanol.

13 Allow to dry for  00:05:00


5m


Note


Do not dry the pellet to the point of cracking.

14 Remove the tube from the magnetic rack and resuspend pellet in  30 μL Nuclease-free water. Incubate for  00:20:00 at  Room temperature .

20m

15 Spin down and place the tube back on the magnet until the eluate is clear and colorless. Remove and retain  30 μL of eluate in a clean 1.5 ml Microtube.

16 Dilute  1 μL purified amplified DNA to 100X in dH₂O.

Take  2 μL diluted purified amplified DNA for quantification with Qubit dsDNA High sensitivity assay.

T7 endo I digestion

1h 15m 30s



- 17 For each reaction, mix the reagents in the following order in a clean 0.2 ml PCR tube. Add nuclease-free water until final volume of 25 μL . Mix gently by flicking the tube, and spin down.

A	B
1.5 μg (X μl)	amplified DNA
3 μl	NEBuffer 2
1.5 μl	T7 Endonuclease I
25-X μl	Nuclease-free water

- 18 Incubate at 20 $^{\circ}\text{C}$ for 00:30:00 and 65 $^{\circ}\text{C}$ for 00:30:00 in thermal
cycler.

1h

- 19 Resuspend the AMPure XP beads by vortexing.
Add 60 μL of resuspended AMPure XP beads to the amplification reaction and thoroughly mixed. Incubate the sample on a Tube Revolver for 00:10:00 at
 Room temperature .

10m

- 20 Keep the tube on the magnet until eluate is clear and colorless, and pipette off the supernatant.

- 21 Wash the beads with 200 μL of freshly prepared [M] 70 % (v/v) ethanol for
 00:00:30 and remove the ethanol using a pipette and discard. Spin down and place the tube back on the magnet. Pipette off any residual ethanol.


30s

- 22 Repeat step 21 again.

- 23 Allow to dry for 00:05:00 , but do not dry the pellet to the point of cracking.

5m



- 24 Remove the tube from the magnetic rack and resuspend pellet in  49 μ L Nuclease-free water. Incubate for 20 minutes at RT. Spin down and place the tube back on the magnet until the eluate is clear and colorless.
- 25 Remove and retain 49 μ L of eluate in a clean 1.5 ml microtubes.
- 26 Take 2 μ L purified DNA for quantification with Qubit dsDNA High sensitivity assay.

ONT library prep and ONT sequencing

- 27 ONT library prep were followed ONT Ligation sequencing gDNA (SQK-LSK109) protocol.